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Research Article

Development and Validation of a Rapid Chemometrics Assisted RP-HPLC with PDA Detection Method for the Simultaneous Estimation of Pyridoxine HCl and Doxylamine Succinate in Bulk and Pharmaceutical Dosage Form

P. Giriraj and T. Sivakkumar

Department of Pharmacy, Faculty of Engineering and Technology, Annamalai University, Tamil Nadu 608002, India

Correspondence should be addressed to T. Sivakkumar; sivat27@rediffmail.com

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Simple, rapid, precise, and accurate RP-HPLC method was developed and optimized with the help of chemometric tool for the simultaneous estimation of pyridoxine HCl and doxylamine succinate in bulk and pharmaceutical dosage form. Optimization was done by central composite design in response surface methodology. Based on the trial and error, percentage of organic phase (methanol) in mobile phase, flow rate, and molarity of the buffer were selected as factors. Resolution and retention time were used for the estimation of system response during the optimization procedure. The optimized condition was used and the separation was carried out on phenomenex C_{18} column (150 × 4.6 mm; i.d, 5 μ particle size) using the mobile phase containing 49.37% of methanol and 50.63% of phosphate buffer (45.14 mM) at a flow rate of 1 mL/min. Retention time was found to be 1.884 minutes for pyridoxine HCl and 3.959 minutes for doxylamine succinate. The calibration curves were found to be linear from 10 to 70 μg/mL and 10 to 90 μg/mL for pyridoxine HCl and doxylamine succinate with their correlation coefficient values 0.9995 and 0.9997. LOD and LOQ were found to be 23.5 ng/mL and 71.1 ng/mL for pyridoxine HCl and 99.9 ng/mL and 302.6 ng/mL for doxylamine succinate.

1. Introduction

Pyridoxine hydrochloride (PYH) is chemically 3,4-pyridinediacetonitrile, 5-hydroxy-6-methyl, hydrochloride (Figure 1). It is a water-soluble vitamin, involved principally in amino acid, carbohydrate, and fat metabolism [1, 2].

Doxylamine succinate (DOX) is chemically N,N-dimethyl-2-[8-methyl-8-(2-pyridyl) benzyloxy] ethylamine hydrogen succinate (Figure 2). It is an antihistaminic with antimuscarinic and pronounced sedative effect [1–3].

Literature survey exposed that there are few UV [1, 2, 4–7] and HPLC [2, 8–19] methods that were reported for these drugs individually and combined with other drugs. There is no article available in the literature regarding chemometrics approach used in the RP-HPLC method development for the simultaneous estimation of PYH and DOX.

Developing and optimizing isocratic HPLC techniques [20] could be a sophisticated practice that needs synchronized fortitude of several factors. HPLC methods were optimized by time-consuming trial-and-error approach for the last several years, resulting only in an obvious optimum and information regarding the sensitivity of the factors on analytes separation and interaction between factors is not available. Hence, any one of the chemometric methods which includes the overlapping resolution maps, factorial design [21], and response surface methodology [22–25] can be useful.

This paper describes the development and validation of a fast, easy, and sturdy RP-HPLC method for the simultaneous estimation of PYH and DOX using design of experiment (DOE) approach. Optimization of the developed method by using central composite design (CCD) in response surface

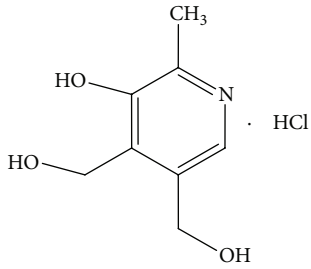


Figure 1: Structure of PYH.

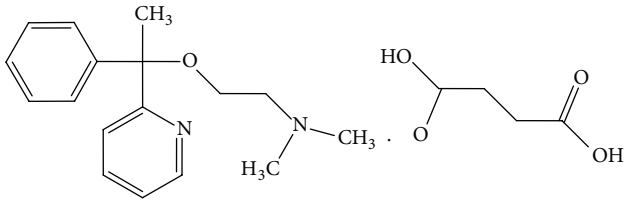


Figure 2: Structure of DOX.

methodology (RSM). Applying the chemometrics loom allows a relatively controlled range of experiments to outline the factors that have an effect on the chromatographic behavior of investigated substances and to get conditions for the analysis.

2. Materials and Methods

2.1. Chemicals and Reagents. PYH and DOX working standards were received as gift samples from Softgel Healthcare Pvt. Ltd., Pondicherry, India. Disodium potassium hydrogen orthophosphate (analytical grade) and methanol (HPLC grade) (S.D Fine Chemical Pvt. Ltd., Mumbai, India) were used throughout these experiments. HPLC grade water was collected from the Milli-Q system. The marketed tablets (Doxinate) used containing 10 mg of PYH and DOX per tablet were manufactured by Maneesh Pharmaceutical Pvt. Ltd., Solan (H.P), India.

2.2. Instrumentation and Chromatographic Conditions. A Shimadzu HPLC system consists of a LC-20AD solvent delivery system (pump), SPD-M20A photodiode array detector, Rheodyne injector with 20 μL loop volume, and LC-Solution assisted for data collections and processing. Phenomenex luna C_{18} column (150 × 4.6 mm; i.d, 5 μ particle size) was used as a stationary phase with the use of 49.37% v/v of methanol and 50.63% v/v of phosphate buffer (45.14 mM; pH 4.5) delivered at a flow rate of 1 mL/min. Detection was carried out by PDA detector at 261 nm. Mobile phase was filtered through 0.45 μ membrane filter before using.

2.3. Preparation of Phosphate Buffer Solution. 6.4080 gm of disodium hydrogen orthophosphate (45.14 mM) was dissolved in sufficient water (HPLC grade) with aid of sonicator. Then 10 mL of triethylamine was added and the volume was made up to 1000 mL with water. Finally pH was adjusted to 4.5 with orthophosphoric acid.

2.4. Preparation of Standard Solution. 10 mg of PYH and DOX was accurately weighed and transferred into a separate 25 mL volumetric flask and sufficient mobile phase was added to dissolve it. Then the solution was sonicated for 10 min. Final volume was adjusted with the mobile phase. 1 mL was pipetted out from the above solution and transferred into a 10 mL volumetric flask and diluted up to the mark with mobile phase to get standard solution with the concentration of 40 μg/mL of DOX and PYH. Resulting solution was then filtered with 0.45 μ membrane filter.

2.5. Preparation of Sample Solution. Twenty tablets were accurately weighed and finely powdered. A quantity of powder weight equivalent to 10 mg of PYH and DOX was weighed and transferred to a 25 mL volumetric flask and sufficient mobile phase was added to dissolve it. Then the solution was sonicated for 20 min. Final volume was adjusted with the mobile phase and filtered with 0.45 μ membrane filter. 1 mL was pipetted out from the above filtrate and transferred into a 10 mL volumetric flask and diluted up to the mark with mobile phase to get sample solution with the concentration of 40 μg/mL of DOX and PYH.

2.6. Software. Experimental design, data analysis, and desirability function calculations were performed by using Design-Expert trial version 7.0.0. (Stat-Ease Inc., Minneapolis).

2.7. Experimental Design. Trial experiments indicated that the factors, such as percentage of methanol in the mobile phase, flow rate, and buffer molarity, were the main factors that affected the retention time and resolution among the chromatographic conditions. Therefore a CCD-RSM was used to methodically examine the influence of these three critical factors on retention time (tR_2) and resolution (Rs_2). The details of the design are listed in Table 1. For each factor, the experimental range was selected on the basis of the results of trial experiments. The value range of the variables was the percentage of methanol in the mobile phase (A) of 40–50% V/V, flow rate (B) of 0.6–1 mL/min, and buffer molarity (C) of 40–50 mM. A total of 20 tests were conducted in randomized order.

3. Results and Discussion

3.1. Optimization of CCD. The CCD-RSM constitutes a different approach because it offers the possibility of investigating a high number of variables at different levels with only a limited number of experiments. The variables in Table 1 were chosen taking into account our preliminary experiments and showed the experimental results concerning the tested variables on retention time and resolution. The two response values ranged from 3.108 to 15.545 and 10.473 to 28.476. A mathematical relationship between factors and responses

Table 1: CCD consists of experiments for the study of three experimental factors with the results.

Std.	Run	Type	Factor 1 *A*: methanol (%)	Factor 2 *B*: flow rate (mL/min)	Factor 3 *C*: buffer molarity (mM)	Response 1 Retention time (tR_2)	Response 2 Resolution (Rs_2)
1	17	Fact	40.00	0.60	40.00	12.794	25.821
2	4	Fact	50.00	0.60	40.00	6.679	16.234
3	18	Fact	40.00	1.00	40.00	8.434	20.939
4	10	Fact	50.00	1.00	40.00	3.108	11.356
5	16	Fact	40.00	0.60	50.00	14.492	24.313
6	20	Fact	50.00	0.60	50.00	6.482	14.586
7	8	Fact	40.00	1.00	50.00	7.904	21.299
8	2	Fact	50.00	1.00	50.00	4.959	10.473
9	1	Axial	36.59	0.80	45.00	15.545	28.476
10	9	Axial	53.41	0.80	45.00	4.853	10.984
11	11	Axial	45.00	0.46	45.00	9.584	22.248
12	19	Axial	45.00	1.14	45.00	4.169	13.81
13	12	Axial	45.00	0.80	36.59	6.155	17.356
14	6	Axial	45.00	0.80	53.41	5.509	13.238
15	14	Center	45.00	0.80	45.00	5.985	14.124
16	13	Center	45.00	0.80	45.00	5.768	14.167
17	5	Center	45.00	0.80	45.00	5.986	14.256
18	15	Center	45.00	0.80	45.00	5.955	17.174
19	7	Center	45.00	0.80	45.00	5.973	14.141
20	3	Center	45.00	0.80	45.00	5.979	14.12

Table 2: Reduced response models and statistical parameters obtained from ANOVA (after backward elimination).

Response	Regression model	Adjusted R^2	Model P value	% C.V.	Adequate precision
tR_2	$+5.96 - 2.96 * A - 1.84 * B + 0.73 * AB + 1.58 * A^2 + 0.40 * B^2$	0.9576	<0.0001	9.40	23.952
Rs_2	$+14.88 - 5.06 * A - 2.28 * B - 0.78 * C + 1.82 * A^2 + 1.22 * B^2$	0.9603	<0.0001	6.07	25.378

was generated by response surface regression analysis using Design-Expert software.

It is momentous to scrutinize the curvature term utilizing CCD with center points before starting optimization procedure. ANOVA generated for central composite design exhibited that curvature is significant for both responses (tR_2 and Rs_2). Since P value is less than 0.05, quadratic model should be advised. The quadratic mathematical model of the three independent factors is given in

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2. \quad (1)$$

Statistical parameters obtained from ANOVA for the reduced model are granted in Table 2. In order to get more realistic model insignificant terms with corresponding P value > 0.05 were eliminated through backward elimination process. Since R^2 habitually decreases when a regressor variable is eliminated from regression model, in statistical modeling, the adjusted R^2, which takes the number of regressor variables into account, is generally chosen [26].

In the present study the adjusted R^2 values were well within the agreeable bounds of $R^2 > 0.88$ [27] which revealed that the experimental data is in good fit with the second order polynomial equations. All the reduced models P value < 0.05 implies that the reduced models are significant. In this study, the signal (response) to noise (deviation) ratio was in the range of 23–25, which showed an adequate signal (ratio greater than 4 is desirable [28]) and therefore the model is significant for the separation process. The coefficient of variation (% C.V.) for all the models that were found in less than 10% (model can be advised sensibly reproducible if it is less than 10%) revealed that the models were reproducible.

As can be glimpsed in Table 2 and Figures 3 and 4, the positive interaction of A and B is statistically important for tR_2 and Rs_2. Altering the factors A and B, low to high levels powerfully affect (decreasing order) the tR_2 followed by C. Altering the factor A, low to high levels powerfully affect (decreasing order) the Rs_2 followed by B and then C. So this study discloses that expanding factors A, B, and C will reduce the tR_2 and Rs_2. So, high level of factors A, B, and C will give a shorter run time.

The abovementioned interaction graphs (Figure 5) appear with two nonparallel lines indicating the positive interaction between factor A and factor B on the retention time and resolution.

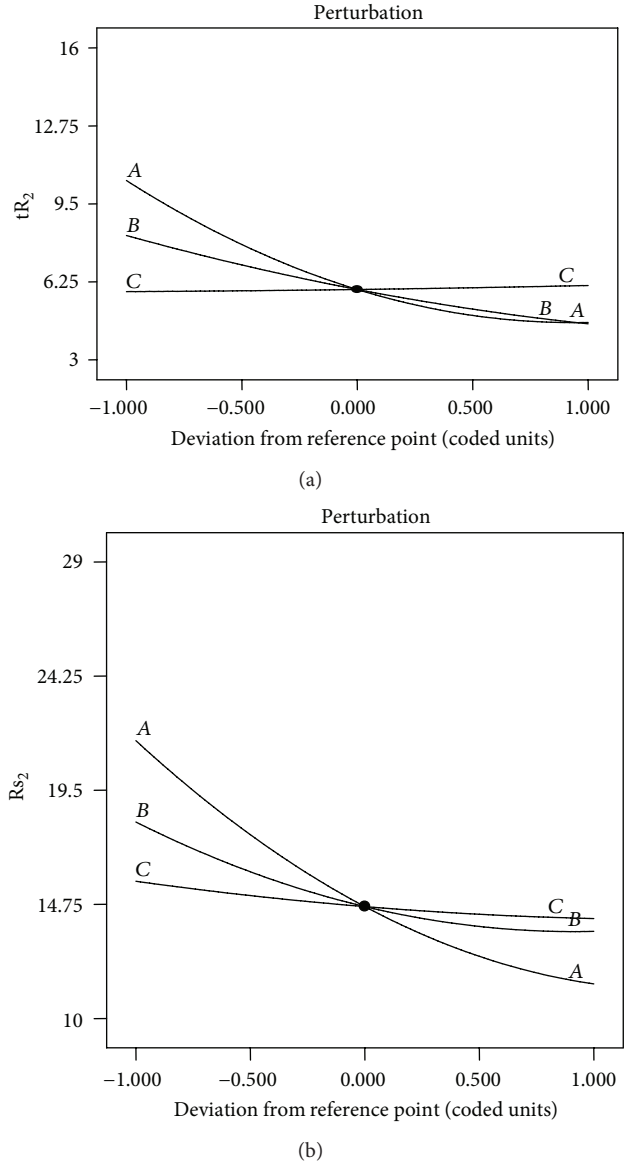


Figure 3: Perturbation plots showing the effect of each of the independent variables on retention time and resolution: (a) tR_2 and (b) Rs_2.

3.2. Global Optimization. In the present study, the recognized criteria for the optimization were resolution between the critical peaks and elution time. Derringer's desirability function was used to optimize two responses with distinct goals [29]. The Derringer desirability function, D, is characterized as the geometric mean, weighted, or otherwise, of the individual desirability functions. The sign that characterizes the Derringer desirability function is

$$D = \left[d_1^{p1} \times d_2^{p2} \times d_3^{p3} \times \cdots \times d_n^{pn}\right]^{1/n}, \quad (2)$$

where pi is the heaviness of the response, n is the number of responses, and di is the individual desirability function of each response. Desirability function (D) can take values from 0 to 1. Weights can vary from 0.1 to 10. Weights lower than 1 give less importance to the goal, whereas weights greater than 1 give more importance to the goal. In the present study, pi values were set at 1 for all the three responses. A value of D close to 1 indicates that the combination of the different criteria is matched in a global optimum [22]. The criteria for the optimization of each individual response are shown in Table 3. The criteria I have been proposed for selecting an optimum experimental condition for analyzing routine quality control samples. As can be seen under the criteria I, the responses tR_2 and Rs_2 minimized in order to shorten the analysis time. The response surface obtained from the global desirability function is presented in Figure 6. From the figure it can be concluded that there was a set of coordinates, producing high desirability value (D = 0.9732), with the methanol concentration (A) of 49.37% and flow rate of 1 mL/min (B) and buffer molarity 45.14 mM (C). The predicted response values corresponding to the latter value of D were $tR_2 = 3.7657$ and $Rs_2 = 10.473$. The prediction efficiency of the model was confirmed by performing the experiment under the optimal condition and the corresponding chromatogram is shown in Figure 7.

In order to acquire the predictability of the suggested model, the affirmation between experimental and predicted responses for the predicted optimums I is shown in Table 4. The percentage of prediction error was calculated by (3). The mean errors for tR_4 and Rs_2 are well within the acceptable range:

$$\text{predicted error} = \frac{\text{experimental value} - \text{predicted value}}{\text{predicted value}} \times 100. \quad (3)$$

3.3. Validation. The developed and optimized method was validated as per ICH guidelines [30]. The method was validated in terms of specificity, system suitability, linearity, precision, accuracy, robustness, LOD, and LOQ. The specificity of the method was established by injecting the blank and placebo (synthetic mixtures). It was observed that there is no interference of the placebo and blank with principal peaks; hence, the method was specific for these two drugs. System performance was developed by system suitability parameters such as retention time, theoretical plates, asymmetric factor and resolution were calculated and percentage RSD was found to be less than 2%, its indicating good performance of the system. The calibration curves were found to be linear from 10 to 70 μg/mL and 10 to 90 μg/mL for pyridoxine HCl and doxylamine succinate with their correlation coefficient values (R^2) 0.9995 and 0.9997, indication of good correlation between concentration and responses. LOD and LOQ were found to be 23.5 ng/mL and 71.1 ng/mL for pyridoxine HCl and 99.9 ng/mL and 302.6 ng/mL for doxylamine succinate. Recovery studies were carried out at the levels of 50%, 75%, 100%, 125%, and 150% of known amounts of PYH and DOX that were added to the placebo (synthetic mixture). The mean percentage recovery of PYH and DOX was found to be 98.75–102.25% and 98.47–101.37%, respectively, showing

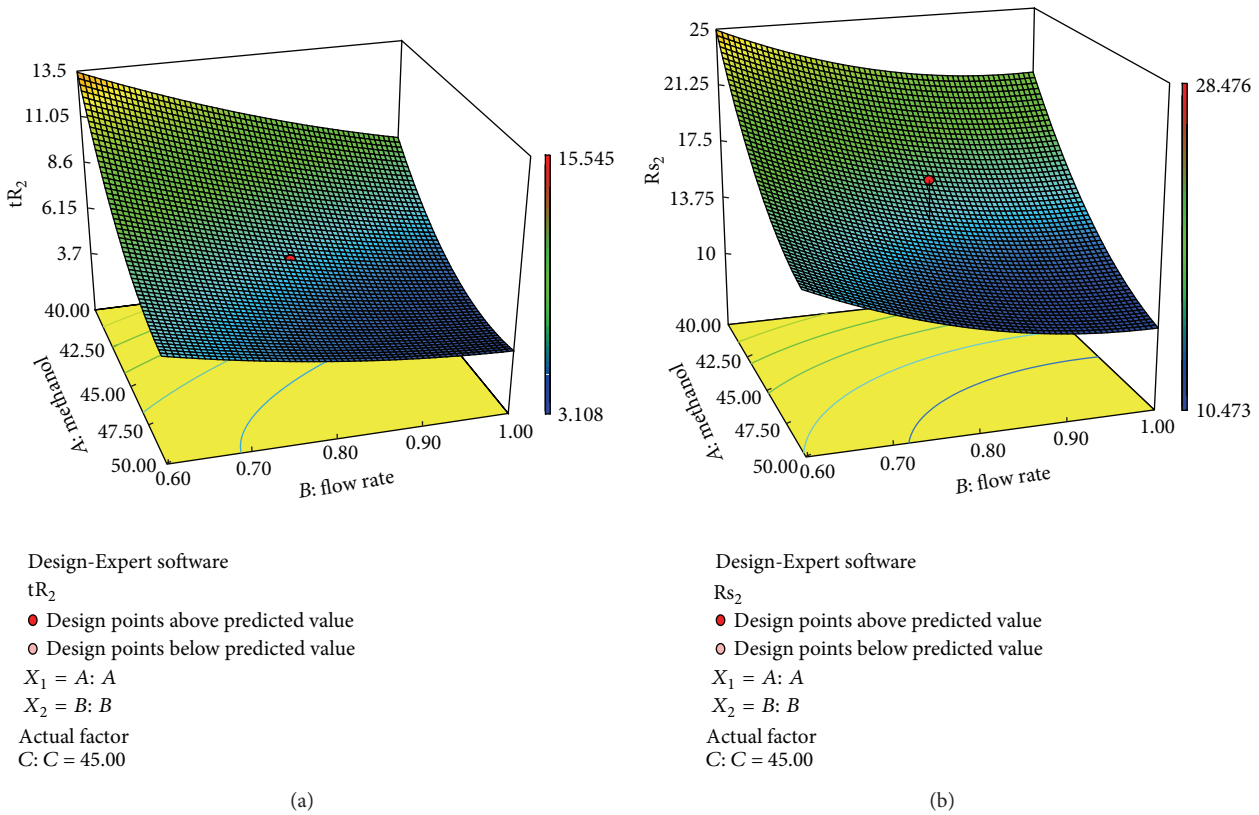


Figure 4: Response surface related to percentage of acetonitrile and flow rate: (a) tR_2 and (b) Rs_2.

Table 3: Criteria for optimization of individual responses.

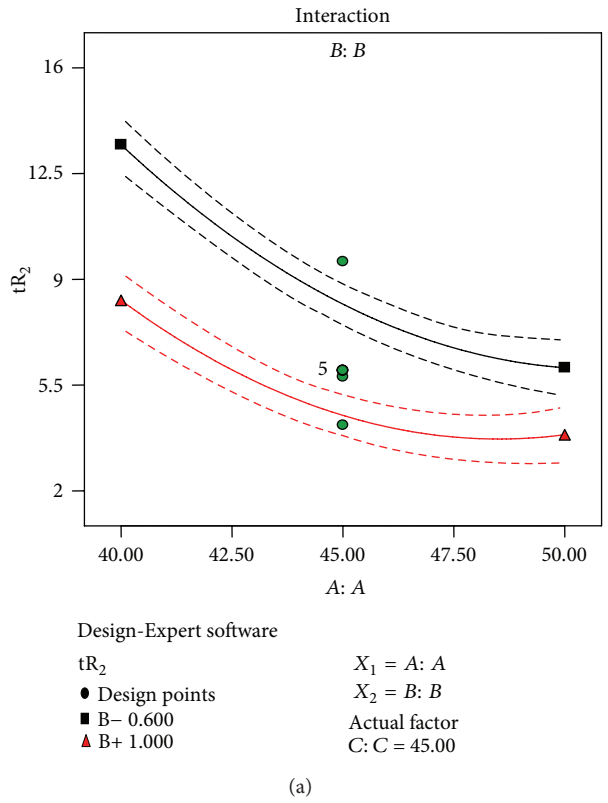
Responses	Lower limit	Upper limit	Criteria I	
			Goal	Importance
tR_2	3.108	15.545	Minimize	3
Rs_2	10.473	28.476	Minimize	3

Table 4: The comparison of observed and predicted values under optimum condition.

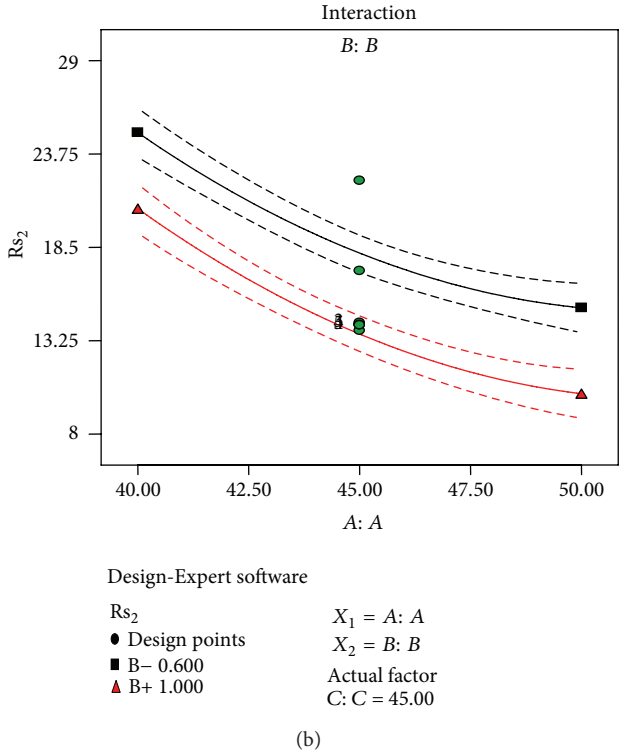
Optimum condition		Response	Experimental value	Predicted value	% error
Factor	Condition				
Methanol	49.37%				
Flow rate	1 mL	tR_2	3.959	3.7657	5.14
Buffer molarity	45.14 mM	Rs_2	10.921	10.473	4.10

the accuracy of the proposed method (Table 5). Precision study was performed by injecting the sample solution, at least 3 times at 0 hrs, 8th hrs, 16th hrs, and 6 times at day-1, day-2, day-3, different analyst and different instrument. The amount of PYH and DOX present in sample solution was calculated and found to be 97–102% and 98–102%, respectively. % RSD was found to be less than 2% (Table 5). Robustness of the method was checked by small deliberate changes made in the method parameters such as wavelength (±2 nm), flow rate (±0.1 mL), mobile phase ratio (±2%), and pH (±0.05), but these changes did not affect the method results (Table 5). Standard and sample solution stabilities were checked up to 3 days at room temperature and we measured the responses on one time on each day. No degradation of PYH and DOX was observed during this period.

All the validation parameter results (Table 5) were indicating that the developed and optimized method was suitable, linear, precise, accurate, and robust for the simultaneous



(a)



(b)

Figure 5: Interaction graph showing the effect of the positive interaction between factor A and factor B on the retention time and resolution.

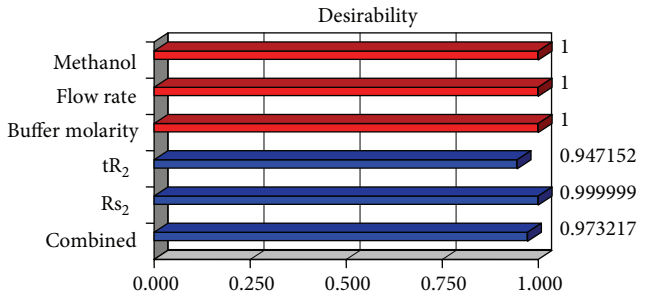


Figure 6: Response surface bar graph for the global desirability function.

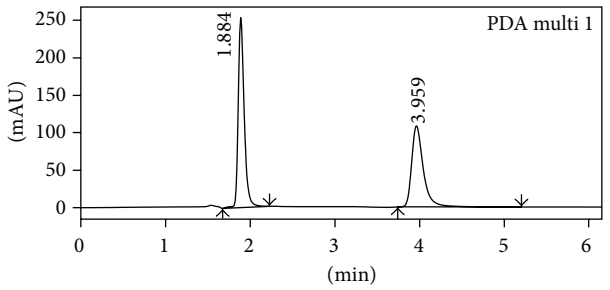


Figure 7: Chromatogram of PYH and DOX under optimal condition.

estimation of PYH and DOX in bulk and pharmaceutical dosage forms.

3.4. Method Applied to the Marketed Dosage Forms. The assay was performed on marketing dosage forms as per above described procedure (Section 2.5); then six replicate injections were given into HPLC without changing the proposed method conditions. The amount of PYH and DOX present in each tablet was found to be 100.24 mg and 101.39%, respectively (Table 5).

4. Conclusion

The proposed method represents an efficient and easily accomplishable approach to resolving the problem of searching for optimum RP-HPLC conditions. The quadratic model obtained demonstrates a strong influence of the organic phase variations on retention time and resolution and smaller but significant influence of buffer molarity. The investigation also showed that chromatographic techniques coupled with chemometric tools provide useful information of separation and elution time, making this combined technique a powerful analytical tool. From the results obtained, it can be concluded that the developed and optimized method was specific, suitable, linear, accurate, precise, and robust for the estimation of PYH and DOX. Therefore, this RP-HPLC method can be

Table 5: Validation results of developed and optimized reverse phase chromatographic method.

Parameter	PYH (avg. %)	DOX (avg. %)	
Specificity	No interference		
	Accuracy		
50%	102.23	100.10	
75%	100.60	98.47	
100%	98.72	100.61	
125%	100.50	99.87	
150%	100.61	100.37	
	Precision—Repeatability		
0 hrs	99.26	99.34	
8 hrs	99.18	99.34	
16 hrs	99.20	99.33	
	Precision—Intermediate		
Day-1	99.29	100.42	
Day-2	99.42	99.89	
Day-3	98.90	98.37	
Instrument-1	99.41	99.34	
Instrument-2	101.24	100.25	
Analyst-1	100.20	99.35	
Analyst-2	100.26	99.34	
Lab-1	99.42	99.31	
Lab-2	99.96	99.33	
	Robustness		
Flow rate	0.9 mL/min	99.28	99.69
	1.1 mL/min	99.25	100.24
Wavelength	+2 nm	98.20	99.31
	−2 nm	101.68	99.40
Mobile phase	+2%	99.73	100.11
	−2%	100.21	100.26
pH	+0.05	100.23	99.96
	−0.05	99.42	100.28
Assay		100.24	101.39

used for routine quality control analysis in pharmaceutical environment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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