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A RAPID - CHEMO METRICS ASSISTED RP-HPLC METHOD WITH PDA DETECTION FOR THE SIMULTANEOUS ESTIMATION OF OFLOXACIN AND NIMORAZOLE IN PURE AND PHARMACEUTICAL FORMULATION

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ABSTRACT

A simple, rapid, sensitive, cost effective and reproducible chemo metrics assisted RP-HPLC method was developed, optimized and validated for the simultaneous estimation of the Ofloxacin and Nimorazole in pure and pharmaceutical formulation. The developed method was optimized by using Central composite design (CCD) in resoponse surface methodology (RSM). Based on the trial and error, percentage of tetrahydrofuran in mobile phase, flow rate and buffer molarity were selected as factors. Resolution (Rs_2) and retention time (tR_2) were used for the estimation of system response during the optimization procedure. Derringer's desirability function was used to concurrently optimize the selected two responses. Separation of ofloxacin and nimorazole was achieved on phenomenex C_{18} column (150 X 4.6 mm i.d, 5µ particle size) with a mobile phase consisting of 25% of tetrahydrofuran and 75% of Phosphate buffer (25mM) was delivered at a flow rate of 1.2mL/min and photodiode array detection at 235nm. The method was found to be linear over the concentration range of 20 – 60µg/mL for ofloxacin and 50 – 150µg/mL for nimorazole with their correlation coefficient values of 0.9995 and 0.9997 respectively. LOD and LOQ were found to be 20.62ng/mL and 62.49ng/mL for ofloxacin, 188.65ng/mL and 571.68ng/mL for nimorazole. The % RSD value of accuracy and precision study was found to be less than 2%. The proposed method is simple, rapid, sensitive, cost effective and reproducible. Hence

this method can be used for routine quality control analysis of ofloxacin and nimorazole in pure and pharmaceutical dosage form.

KEYWORDS

Response surface methodology, Central composite design, Derringer's Desirability function, RP-HPLC, Oflaxacin, Nimorazole.

INTRODUCTION

Ofloxacin (OFX) is a fluoroquinolone derivative with potent activity against a broad spectrum of bacteria. Chemically, it is (±)-9-fluoro-2, 3-dihydro-3-methyl-10- (4-methyl-1-piperazinyl)-7-oxo-7H-pyrido-[1,2,3-de]-1,4-benzoxazine -6-carboxylic acid [1] (Figure 1). It is mainly used as an antibacterial for the treatment of urinary tract infection and sexually transmitted diseases.

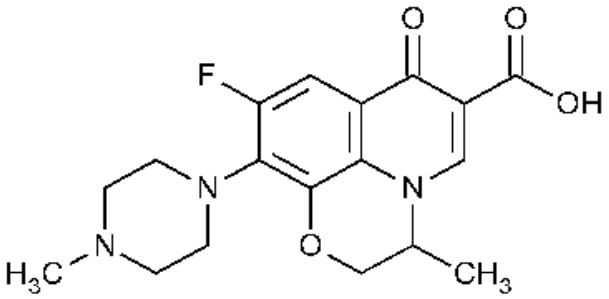


FIGURE 1: Structure of OFX

Nimorazole (NIM) is a 5-nitroimidazole, which is closely related to Metronidazole in structure and activity. Nimorazole is used as a hypoxic sensitizer concomitantly with radiotherapy for head and neck cancers and could from the similarities with Metronidazole theoretically lead to increased effect of anticoagulant therapy. Nimorazole chemically known as 4-[2-(5-nitro-1*H*-imidazole-1-yl)ethyl] morpholine (Figure 2) [2].

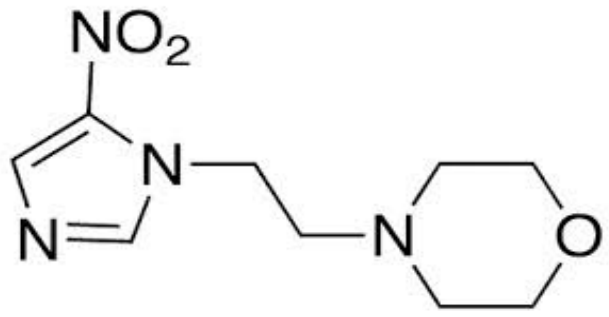


FIGURE 2: Structure of NIM

Literature survey stated that, few analytical methods such as, HPLC [3-7], LC –MS [8,9] HPTLC [10,11] and UV [12-18] were reported for the estimation of OFX and NIM either individually or combined with other drugs. However , no method is reported in the literature concerning chemometrics approach used for the method development for the simultaneous estimation of OFX and NIM.

Developing and optimizing isocratic HPLC methods are a difficult procedure that requires instantaneous determination of several factors. In order to optimize more than one response at a time, the chemometric methods which includes factorial design [19] and response surface methodology [20 – 24] were applied.

EXPERIMENTAL

Samples

Working standards of OFX (99.35 %) and NIM (99.68%) were kindly supplied by AN therapeutics, (Pondicherry, India). Tetrahydrofuran HPLC Grade (S.D fine chemical Ltd., Mumbai, India) and HPLC grade water, arranged from Milli-Q-Academic system, Millipore, Bangalore, India, were used throughout the experiments. The pharmaceutical formulation used in this study was NIMORAZ O tablets (Lupin Ltd, Mumbai, India) procured from the local market and labelled to contain 200mg OFX and 500mg NIM per tablet.

Instrumentation and Chromatographic conditions

A shimadzu HPLC system consist of LC-10AT-vp Solvent develivery system (pump), SPDM – 10AVP photodiode array detector, Rheodyne injector with 20µL loop volume, LC-Solution assisted for data collections and processing. The mobile phase consisted of 25 % of THF and 75 % of Phosphate buffer (25mM) was delivered at a flow rate of 1.2 mL/min. Separation was achieved using a 150mm X 4.6 mm (i.d.) Phenomenex luna C_{18} column with an average particle size of 5µ and the column was kept at an ambient temperature. The column effluent was monitered at 294 nm by PDA detection. The mobile phase was filtered through 0.45µ filter before using.

Preparation of Phosphate Buffer Solution

3.4023 gm of potassium di - hydrogen orthophosphate (25 mM) was dissolved in sufficient water (HPLC grade) with aid of sonicator. Then 5 mL of tri ethanol amine was added and the volume was made up to 1000ml with water. Finally pH was adjusted to 5 with ortho phosphoric acid.

Standard stock solution

Standard stock solutions of 500 µg/ml of OFX and NIM were prepared separately in methanol. From the stock solutions, the mixed standard solutions were prepared to contain 40µg/ml of OFX and 100 µg/ml NIM.

Sample solution

Twenty tablets were accurately weighed and finely powdered. A quantity of powder weight equvalent to 20mg of OFX and 50mg of NIM were weighed and transferred to a 100 ml volumetric flask. Sufficient amount mobile phase was added and the resulting solution was sonicated for 20 minutes. Then the final volume was adjusted with mobile phase and filtered by vaccum filtration. From the filtrate 10mL was taken and transferred to a 50 mL volumetric flask, final volume was adjusted to 50mL with mobile phase so as to get working concentration of 40µg/ml of OFX and 100 µg/ml NIM.

Software

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

Experimental design

In RSM the most popular design CCD was selected for this experiment. Three factors at two levels was used to optimize the chormatographic conditions. Percentage of THF in mobile phase (A), flow rate (B) and buffer molarity (C) were selected in the variation of levels of 15 - 25 % v/v, 0.8ml/min - 1.2 ml/min and buffer molarity 15 - 25 mM respectively. Two responses (Retention time (tR_2) and resolution (Rs_2)) were measured in each run, of total 20 runs and conducted in randomized order.

RESULTS AND DISCUSSION

Optimization of design

Factorial design and RSM are usually included for the optimization of isocratic HPLC conditions in chemometric methods. Choice of key factors examined for optimization were based on initial experiments and from the literature. The three factors (A B and C) and two responses (tR_2 and Rs_2) were selected for the optimization process. Totally 20 runs were generated by the software and all the experiments were performed in randomized order to decrease the effects of uncontrolled variables that may bring in unfairness on the measurements. The design and measured responses are represented in Table 1.

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

Std	Run	Type	Factor 1	Factor 2	Factor 3	Response 1	Response 2
			A: Methanol (%)	B: Flow rate mL/Min	C: Buffer molarity (mM)	Retention time (tR_2)	Resolution (Rs_2)
1	11	Fact	15.00	0.80	15.00	6.465	5.235
2	5	Fact	25.00	0.80	15.00	4.3	7.175
3	19	Fact	15.00	1.20	15.00	4.318	4.603
4	17	Fact	25.00	1.20	15.00	2.871	5.91
5	14	Fact	15.00	0.80	25.00	6.189	5.098
6	4	Fact	25.00	0.80	25.00	4.29	5.873
7	10	Fact	15.00	1.20	25.00	4.136	4.894
8	9	Fact	25.00	1.20	25.00	2.878	5.295
9	3	Axial	11.59	1.00	20.00	6.622	4.013
10	12	Axial	28.41	1.00	20.00	3.281	5.559
11	8	Axial	20.00	0.66	20.00	6.502	6.609
12	13	Axial	20.00	1.34	20.00	3.234	5.843
13	6	Axial	20.00	1.00	11.59	4.521	7.154
14	18	Axial	20.00	1.00	28.41	4.217	5.564
15	1	Center	20.00	1.00	20.00	4.306	6.493
16	16	Center	20.00	1.00	20.00	4.308	6.491
17	15	Center	20.00	1.00	20.00	4.305	6.495
18	20	Center	20.00	1.00	20.00	4.309	6.449
19	2	Center	20.00	1.00	20.00	4.311	6.497
20	7	Center	20.00	1.00	20.00	4.301	6.493

Before initialising an optimization procedure, it is obligatory to investigate the curvature term using CCD with center points. ANOVA generated for CCD publicized that, curvature is important for both the responses (tR_2 and Rs_2). Since p-value is less than 0.05, quadratic model was considered. The quadratic mathematical model for three independent factors was given in Equation (1).

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2 \quad (1)$$

Statistical parameters obtained from ANOVA for the reduced model were given in Table 2. In order to get more realistic model, unimportant terms with corresponding p value > 0.05 were removed during backward elimination process. Since R^2 always decreases when a regressor variable is eliminated from regression model, the adjusted R^2 which takes the number of regressor variables in to account is generally selected in statistical modeling [25].

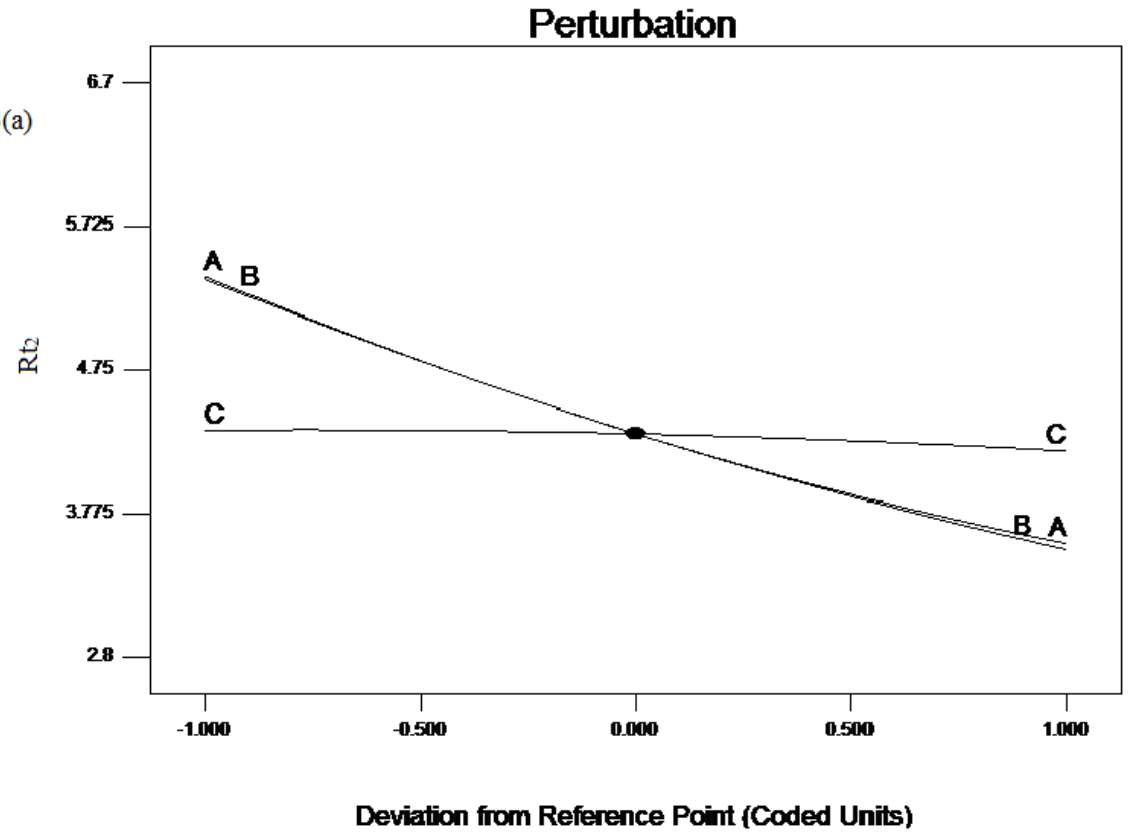
TABLE 2: Response models with statistical parameters obtained from ANOVA

Response	tR_2	Rs_2
Regression model	+ 4.32 - 0.91*A - 0.92*B - 0.071*C + 0.17*A* B + 0.057*A*C + 0.014*B*C + 0.16* A^2 + 0.13* B^2 - 0.046* C^2	+ 6.49 + 0.51*A - 0.29*B - 0.32*C- 0.13*A*B - 0.26*A*C+0.14* B*C - 0.65*A^2-0.14* B^2-0.095* C^2
Adjusted R^2	0.9654	0.9350
Model p value	<0.0001	<0.0001
% C.V.	4.68	3.68
Adequate precision	25.579	22.688

The adjusted R^2 values were well with in the satisfactory limits of $R^2 > 0.88$ [26], publicized that the experimental datas are in good fit with the second order polynomial eauations. Since p-value is < 0.05, reveals that, all the reduced models are significant. In this study, the signal (response) to noise (deviation) ratio was found to be in the range of 22 - 26 (ratio greater than 4 is desirable [27], suggestive of an adequate signal to noise ratio and therefore the model is significant for the separation process. The % C.V. of all the models were found to less than 10% revealed that all the models were reproducible, (model can be considered reasonably reproducible if % C.V is less than 10%).

From the Table 2, the interaction term with the largest absolute coefficients among the fitted models is AB (+0.17), AC (+0.057) and BC (+0.014) of tR_2 model. The positive interaction between AB, AC and BC is stastically significant (< 0.0001) for tR_2 and BC (+ 0.14) for RS_2. Changing the factor B, A, and C from low level to high level strongly affect (decreasing order) the tR_2 and also changing the factor B, C and A from low level to high level strongly affect (decreasing order) the Rs_2. So this study indicated that increasing the factors A, B and C will reduce the tR_2 and Rs_2. A high level of factor A, B and C will give a shorter run time.

3(a)


Perturbation
6.7
5.725
4.75
3.775
2.8
Rt2
A
B
C
C
B A
-1.000
-0.500
0.000
0.500
1.000
Deviation from Reference Point (Coded Units)

3(b)

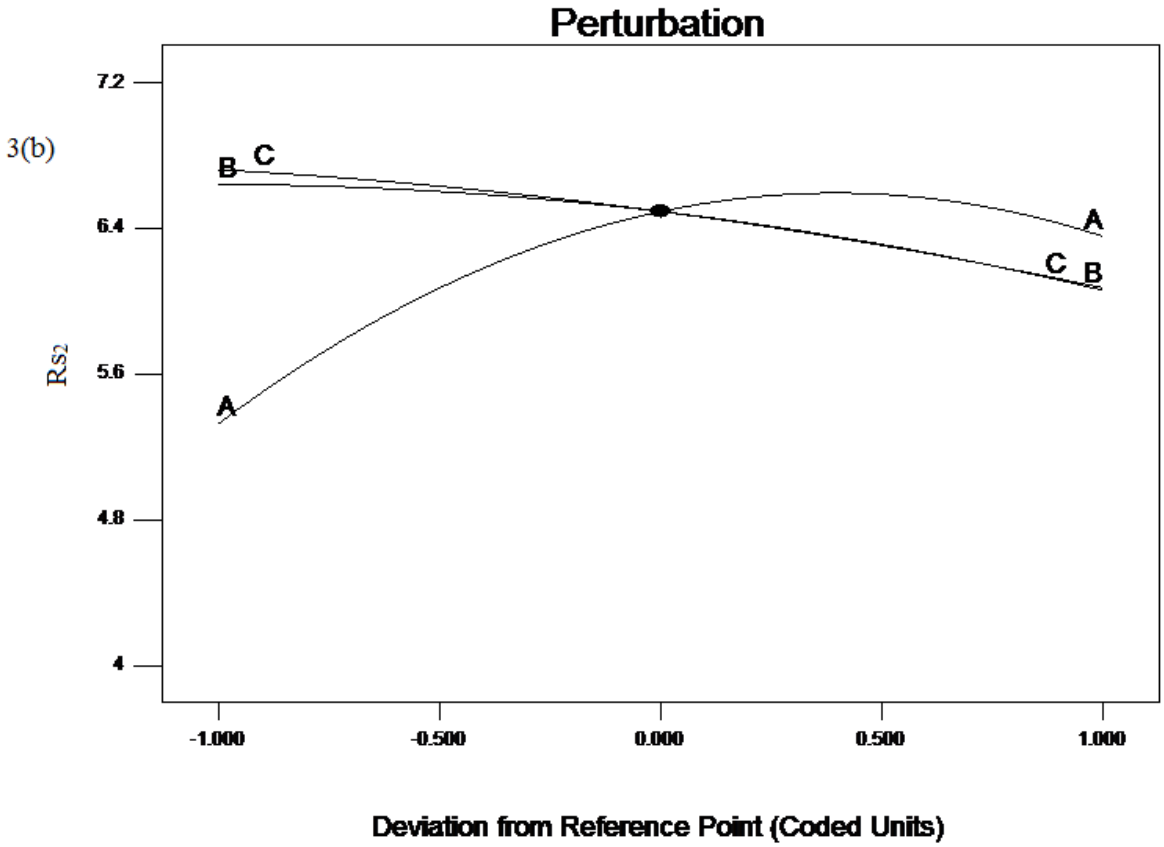
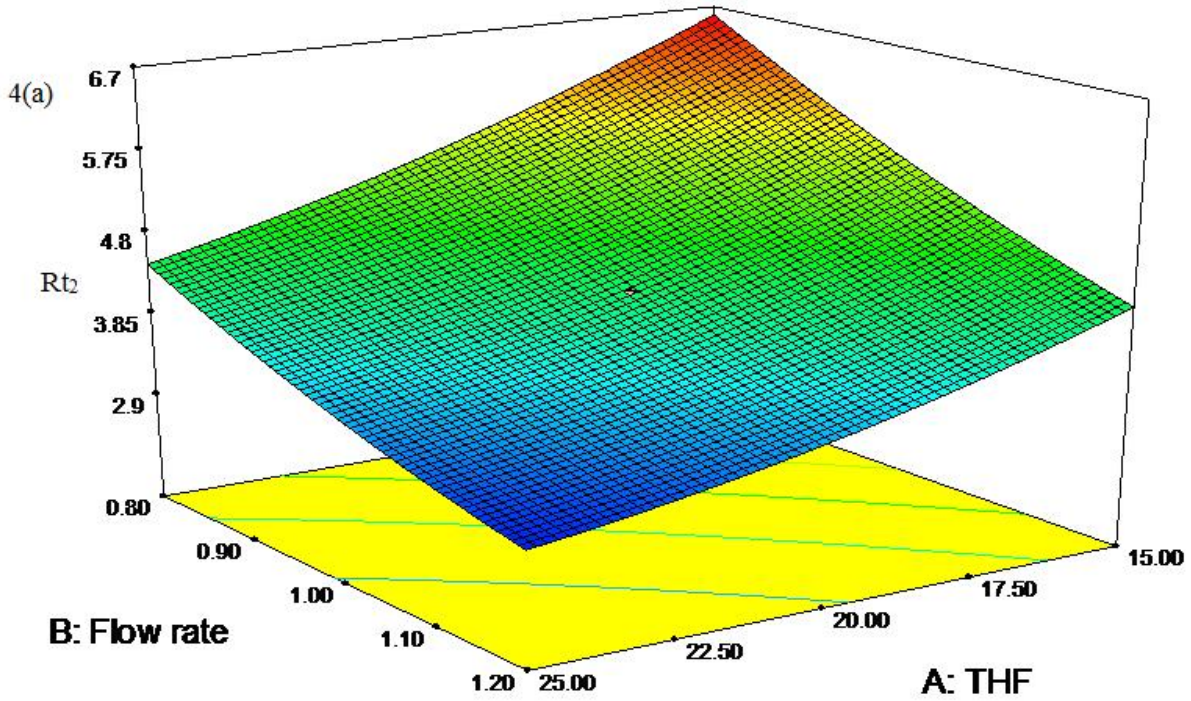

Perturbation
7.2
6.4
5.6
4.8
4
Rs2
B C
A
C B
A
-1.000
-0.500
0.000
0.500
1.000
Deviation from Reference Point (Coded Units)

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



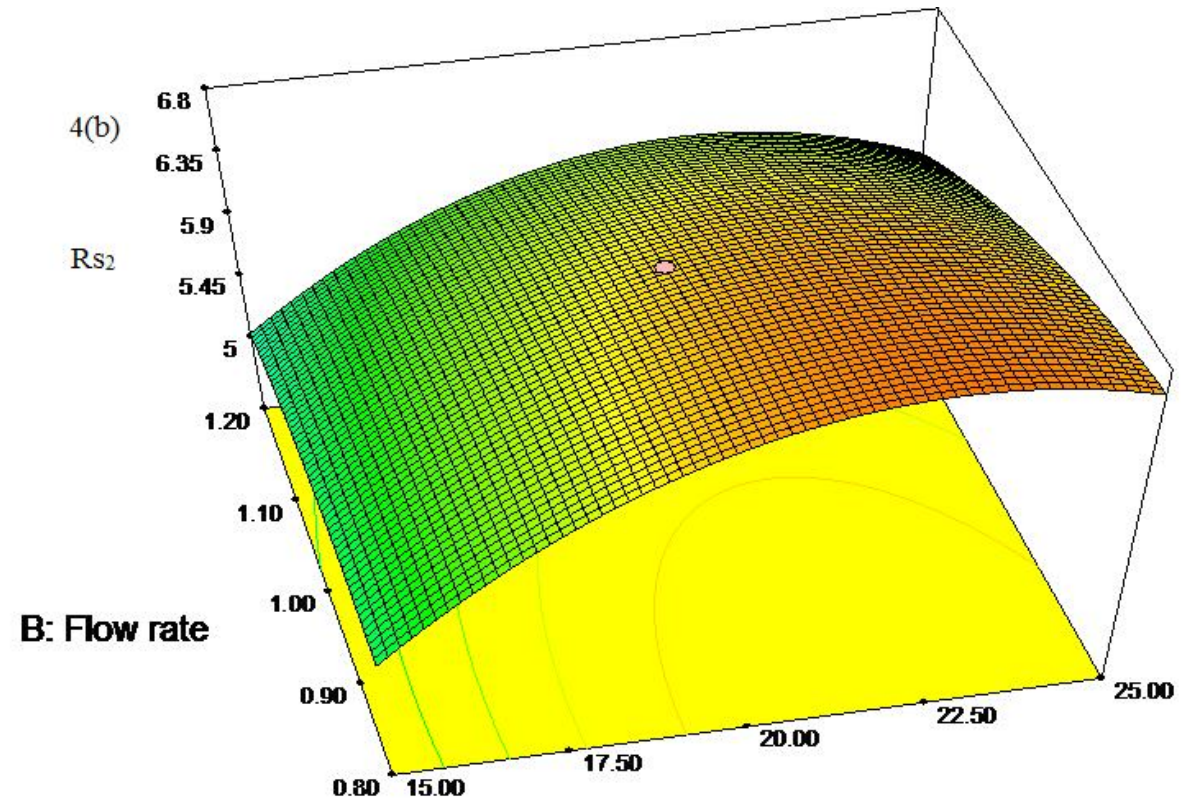


FIGURE 4: Response surface related to percentage of THFl and flow rate (4a) tR_2 and (4b) Rs_2

Petrubation plots and response surface plots were offered (Figure 3 & 4) for predicted models in order to given a improved understanding of the investigated method. This type of plots represented the effect of an independent factor on a specific response with all other factors assumed constant at a reference point [20]. A steepest slope or curvature represents the sensitiveness of the response to specific factor. Figure 3(a) showed that Flow rate (B) percentage of THF(A), and Buffer molarity (C) is the most important effect on tR_2. Figure 3(b) showed that Flow rate (B), Buffer molarity (C) and percentage of THF(A) is the most important effect on Rs_2. Factor c have slightly less effect on tR_2 but strongly affect the Rs_2. But all these three factors are vital for the shorter run time.

Global optimization

In the present study, the identified criteria for the optimization were; resolution between the critical peaks, capacity factor, and elution time. Derringer's desirability function was used to optimize the two responses with same target [28]. The Derringer's desirability function(*D)*, is defined as the geometric mean, weights, or otherwise, of the individual desirability functions. The expression that defines the Derringer's desirability function is:

$$D = [d_1^{p1} \text{ X } d_2^{p2} \text{ X } d_3^{p3} \text{ X } \ldots \text{ X } d_n^{p}{}_n]^{1/n} \qquad (2)$$

Where *n* is the number of responses and p_n is the weight of the responses.Weight of the response is the relative importance of each of the individual functions d_i. The relative importance p_i is a comparative scale for weighting each of the resulting d_i in the overall desirability product and it varies from the least important (p_i = 0.1) to the most important (p_i = 10). Desirability function (*D*) can take values from 0 to 1. Weights can range 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, p_i 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 [20]. The criteria for the optimization of each individual response are shown in Table 3. Criteria I have been proposed for the selecting an optimum experimental condition for analysing routine quality control samples. As can be seen under criteria I, the reponses tR_2 and Rs_2 were minimized in order to shorten the analysis time. Following the conditions and restrictions as mentioned above, the optimization procedure was carried out. The response surface obtained for the global desirability function is presented in Figure 5. From the Figure 5 it can be concluded that there was a set of coordinates producing high desirability value (D = 0.748) were THF concentration of 25%,

flow rate of 1.20 ml/min and buffer molarity 25mM. The predicted response values corresponding to the later value of *D* were; $tR_2 = 2.90467$ and $Rs_2 = 5.25887$. The prediction efficiency of the model was confirmed by performing the experiment under the optimal condition and the corresponding chromatogram was shown in Figure 6.

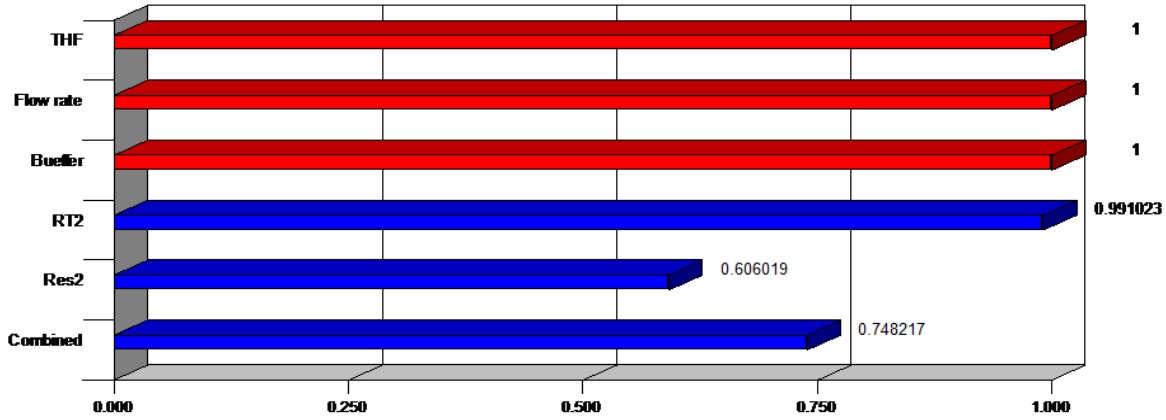


FIGURE 5: Response surface obtained for the global desirability function

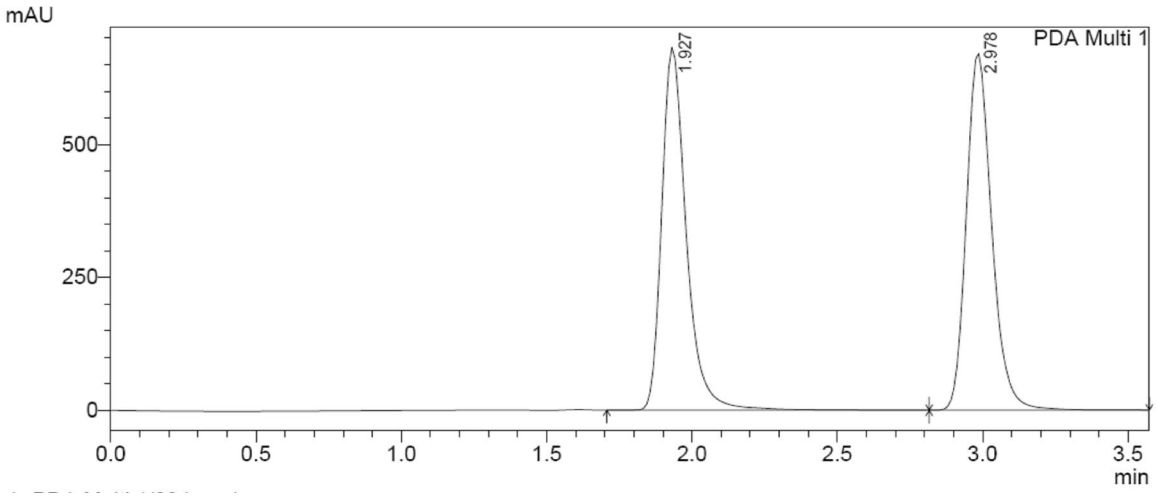


FIGURE 6: Chromotagram of OFX and NIM under optimal condition

TABLE 3: Criteria for optimization of individual responses

Responses	Lower limit	Upper limit	Criteria I	
			Goal	Importance
tR_2	2.871	6.622	Minimize	3
Rs_2	4.013	7.175	Minimize	4

In order to investigate the predictability of the proposed model, the agreement between experimental and predicted responses for the predicted optimums I are shown in Table 4. The Percentage of prediction error was calculated by Equation (3) [29].

Predicted Error = Experimental – Predicted / Predicted x 100 (3)

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

Optimum condition		Response	Experimental valvue	Predicted value	% Error
Factor	condition				
THF	25%	tR_2	2.978	2.90467	2.52
Flow rate	1.2 ml	Res_2	6.109	5.25877	4.98
Buffer molarity	25mM				

Validation

The developed and optimized method was validated as per ICH Q2(R_1) guidelines [30]. Specificity was performed by comparing the peaks observed in sample solution, blank and placebo (synthetic mixtures). No interference was observed. Hence observed peaks in sample solution was the actual peak of OFX and NIM shown that, the method was specific. System performance was developed by system suitability parameters such as retention time, theoratical plates, asymetric factor and resolution were calculated and percentage RSD was found to be less than 2 % indicating the good performance of the system. The method was found to be linear over the concentration range of 20 – 60 µg/mL for ofloxacin and 50 – 150 µg/mL for nimorazole with their correlation coefficient values (R^2) of 0.9997 and 0.9995 respectively, indicating that good correlation existing between concentration and responses (Figure 7 & 8).

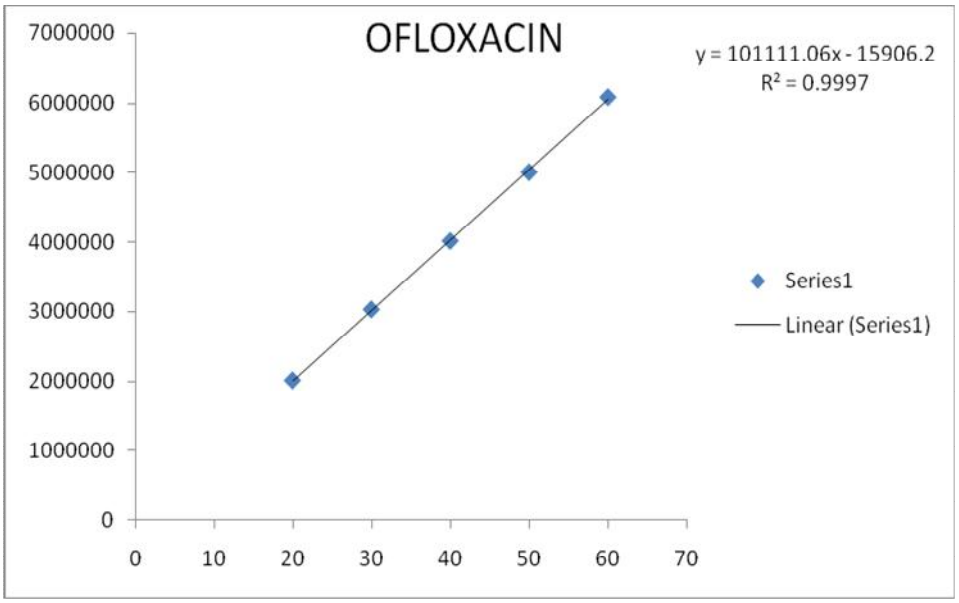


FIGURE 7: Calibration curve for OFX

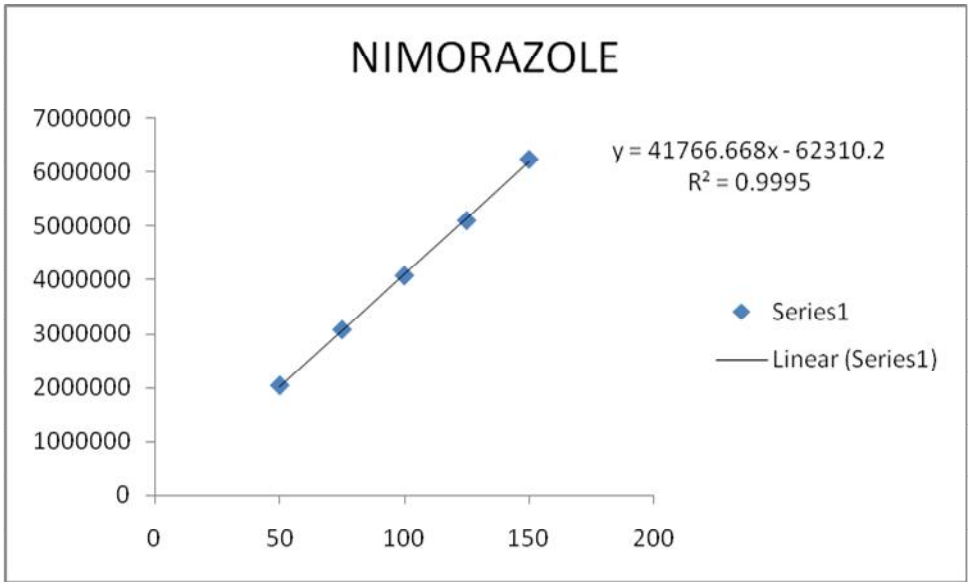


FIGURE 8: Calibration curve for NIM

Accuracy was performed at various levels of 50% 75%, 100%, 125% and 150% of lablel claim. The amount of OFX and NIM recovered in all the levels were found to be close to 100 %, indicative of good accuracy of the proposed method (table 5). Precision study was performed by injecting the sample solution 3 times at 0hrs, 8hrs,16th hrs and 6 times at day-1, day-2,day-3, by different analysts and in different instruments. The amount of OFX and NIM present in sample solution was found to 99.20 -102.15 % and 98.24 – 101.25 % (table 5). % RSD was found to less than 2%. Robustness of the method was determined by small delibrate changes were made in the method parametres such as wavelength (±2nm), flow rate (±0.1ml), mobile phase ratio (±2%) and pH (± 0.05). But these changes, not affected the method results indicated that the method was robust (table 5). Standard and sample Solutions stability were checked up to 3 days at room temperature and the reponses were measured at one time on each day. Results revealed that there was no degradation of OFX and NIM.

All the validation parameters results (Table 5) were indicating that the developed and optimized method was specific, suitable, linear, precise, accurate and robust for the simultaneous estimation of OFX and NIM in pure and pharmaceutical dosage form.

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

Parameter	OFX (avg %)		NIM (avg %)
Specificity	**No interference**		
Accuracy			
50 %	100.04		99.85
75%	100.11		100.36
100%	99.54		100.14
125%	99.71		99.93
150%	100.56		101.25
Precision - Repeatability			
0 hrs	100.17		100.56
8 hrs	99.89		99.32
16 hrs	99.25		98.24
Precision - Intermediate			
Day -1	100.78		99.80
Day - 2	99.60		99.62
Day - 3	99.20		99.48
Instrument -1	99.22		99.45
Instrument - 2	101.14		100.75
Analyst -1	99.89		99.69
Analyst -II	99.23		99.52
Column -1	99.24		99.67
Column -II	100.56		100.89
Robustness			
Flow rate	1.1 ml/min	99.23	99.54
	1.3 ml/min	99.78	99.69
wavelength	+ 2 nm	101.89	98.80
	- 2 nm	98.59	100.92
Mobile phase	+ 2 %	99.30	99.15
	- 2 %	102.15	99.56

pH	+ 0.05	99.51	99.62
	- 0.05	99.16	99.69
Assay		99.20	99.32

Method apllication to the marketed formulation

Sample solution of the marketed formulation was prepared as per the above procedure as described in the preparation of sample solution. Six replicate injections were given in to HPLC without changing the proposed method procedure. The amount of OFX and NIM present in each tablet was calculated and found to be 198.40 mg and 496.61 mg respectively (Table 5).

CONCLUSION

This developed method is considered as the first method for the simultaneous estimation of OFX and NIM using Chemo metrics assisted RP-HPLC with Photodiode array detection. The various validation characteristics were applied and determined, to assure the suitability of the method.This investigation also proved that, the chromatographic techniques coupled with chemometric tools provide a complete profile of separation process, making this combined technique a powerful analytical tool. Therefore, this validated RP-HPLC-PDA method can be readily adapted for the simultaneous estimation of OFX and NIM in pure and pharmaceutical dosage form as a routine quality control analysis.

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