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Article

Optimization of HPLC method using central composite design for estimation of Torsemide and Eplerenone in tablet dosage form

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A simple, precise, accurate and robust high performance liquid chromatographic method has been developed for simultaneous estimation of Torsemide and Eplerenone in tablet dosage form. Design of experiment was applied for multivariate optimization of the experimental conditions of RP-HPLC method. A Central composite design was used to study the response surface methodology and to analyse in detail the effects of these independent factors on responses. Total eleven experiments along with 3 center points were performed. Two factors were selected to design the matrix, one factor is variation in ratio of Acetonitrile and the second factor is flow rate (mL/min). Optimization in chromatographic conditions was achieved by applying Central composite design. The optimized and predicted data from contour diagram comprised mobile phase (acetonitrile, water and methanol in the ratio of 50: 30: 20 v/v/v respectively), at a flow rate of 1.0 ml/min and at ambient column temperature. Using these optimum conditions baseline separation of both drugs with good resolution and run time of less than 5 minutes were achieved. The optimized assay conditions were validated as per the ICH guidelines (2005). Hence, the results showed that the Quality by design approach could successfully optimize RP-HPLC method for simultaneous estimation of Torsemide and Eplerenone.

Keywords: Torsemide. Eplerenone. Quality by Design. RP-HPLC method. Tablet.

INTRODUCTION

Torsemide (TOR) is a loop diuretic drug, chemically it is a 3-Pyridine sulfonamide, N- [[(1-methyl ethyl) amino] carbonyl]-4- [(3-methyl phenyl) amino]-1-Isopropyl- 3- [(4-m-toluidino-3-pyridyl) sulfonyl] urea (Figure 1). It is useful in the treatment of hypertension or edema associated with congestive heart failure, renal disease and hepatic disease (Rang *et al*., 2007). Eplerenone (EPL) is designated Chemically as methyl (1'R,2R,2'S,9'R,10'R,11'S,15'S,17'R)- 2',15'-dimethyl-5,5'-dioxo-18'-oxaspiro[oxolane-2,14'pentacyclo [8.8.0.0^{1},^{17}.0^{2},^{7}.0^{11},^{15}] octadecan] -6'-ene-9'- carboxylate (Figure 2) is a compound of class Steroid Lactones and used to treat Edema associated with CHF. Eplerenone is an aldosterone antagonist used as an adjunct in the management of chronic heart failure. It is clinically used as antihypertensive and diuretic (Rang *et al*., 2007). Torsemide is official in the United States Pharmacopoeia 2007 and Eplerenone is official in Indian Pharmacopoeia 2014 and are analysed by Liquid chromatographic method.

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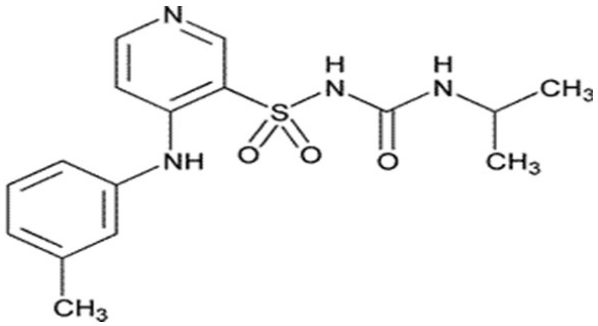

FIGURE 1 - Chemical structure of Torsemide.

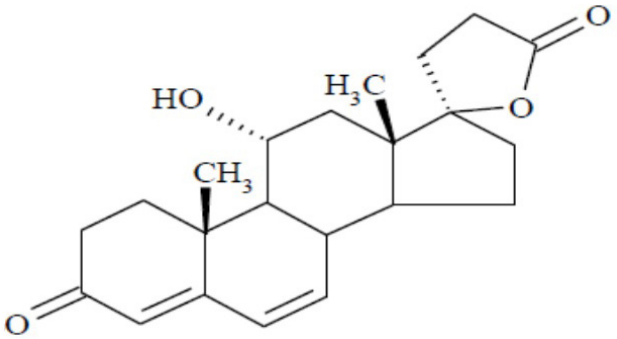

FIGURE 2 - Chemical structure of Eplerenone.

After referring different articles and extensive literature survey it has been revealed that there are a few reverse phase high performance liquid chromatographic (RP HPLC) method available for simultaneous estimation of TOR and EPL in bulk and pharmaceutical dosage form. But there is no method available by using an experimental design approach. A few analytical methods were reported in the literature for the determination of TOR alone. Capillary electrophoresis method (Akesolo *et al,*. 2017), Voltametric method (Fernández *et al.,* 1994), Spectrophotometric methods (Bagade,Patil, 2010; Krishna, Sankar, 2008; Golher, Kapse, Singh, 2010), RP HPLC (Zaazaa *et al,*.2016; Jovic *et al.,* 2012; Ghodke, Poul, Sorde, 2014; Khan, Loya, Saraf, 2008; Shukla e*t al.,* 2012), RP-LCMS (Zhang *et al.,* 2016; Jovic *et al.,* 2012), Thermal method (Rollinger *et al.,* 2003) and HPTLC (Kakde *et al.,* 2011) method for determination of TOR in pharmaceutical dosages were developed. TOR was estimated, with other antihypertensive drugs Spironolactone, Moxifloxacin, Levofloxacin, Gemifloxacin and Pseudoephidrine using spectrophotometric methods from bulk and pharmaceutical dosage forms (Reddy, Sayanna, Venkateshwarlu.,2014; Bhadja *et al.,* 2014a**;** Golher,Kapse,Singh, 2010; Sharma *et al.,* 2010b; Sasikala, Sayanna, Venkateshwarlu, 2015) and also with Spironolactone and Amiloride using the RP -HPLC method in binary mixtures (Deshpande *et al.,* 2012; Bhalodiya, Modiya, Faldu, 2014; Bihola, Prajapati, Agrawal, 2018; Dubey *et al.,* 2012; Laxman *et al.,* 2010), The stability indicating RP HPLC method for the determination of TOR with Spironolactone in bulk and pharmaceutical product (Karbhari, Bhoir,Joshi, 2013). HPTLC method is used for simultaneous estimation of TOR with spironolactone and amiloride (Sharma *et al.,* 2010b; Bhadja *et al.,* 2014b; Gaikwad *et al.,* 2010). TOR with spironolactone estimated by HPLC-PDA method (Subramanian, Nagappan, Mannemala, 2015). Few analytical methods have been reported for quantitative determination of EPL are UV spectrophotometry (Shaniya *et al.,* 2016; Shailaja *et al.,* 2015), RP HPLC method in bulk and tablet dosage forms (Rane *et al.,* 2009) and in human plasma (Bukwanik, Filist, Rudzk., 2016), HPLC-ESI-MS (Wen-Juan *et al.,* 2009), one TLC and HPTLC method (Mahajan, Kekar, Shah, 2011). Literature has depicted that the three methods have been reported for simultaneous estimation of TOR and EPL in bulk and tablet form namely, UV spectrophotometric method (Hinge,Patel,Patel, 2019), RP HPLC method (Patel *et al.,* 2016; Kranthi,Jhansi,Ganesh, 2018) and stability indicating HPLC method (Patel *et al.,* 2017a). These methods did not describe the design space as per recent FDA guidelines. Using 'Quality by Design' (QbD) or 'Design of Experiments' (DoE) is recommended to achieve robustness during analytical method validation by statistical quality control monitoring.

The conventional methods comprise trial and error and by varying one factor at a time (Patel *et al.,* 2016; Kranthi *et al.,* 2018).This approach often encounters difficulties in optimizing robust chromatographic conditions because of various factors viz; limited availability of the chromatographic column, solvents, chemicals, and critical physicochemical properties of the analyte. Recently the FDA has approved a few new drug applications (NDA) that have applied the QbD approach to analytical techniques, HPLC and UV Spectrophotometry, in which regulatory flexibility has been granted for movement within the defined method

operable design region (MODR) (Peraman *et al.*, 2015). Since its implementation by the FDA, QbD has been an integral part of the pharmaceutical product development process, impacting its robustness (Awotwe-Otoo *et al.*, 2012). A modern QbD approach is useful for selection of optimized conditions as mobile phase, flow rate etc, and for the robustness study of the HPLC method which requires the assessment of all factors which most strongly influence the results of the method. The experimental verification of many factors simultaneously is impractical and associated with difficulties and more expense. To overcome the challenge and reduce the experimental workload, a thorough understanding of the response of the system quality to system parameters that leads ultimately to the establishment of design space for the method is important (Awotwe-Otoo *et al.*, 2012; Monks *et al.*, 2012).

Hence, in present work, a simple, rapid, precise and robust RPHPLC method was developed for analysis of TOR and EPL which is assisted with DoE, Central composite design (CCD) was used for evaluation of robustness of developed method, followed by graphical interpretation of data by response surface methodology (RSM). Statistical approaches such as Response Surface Methodology can be employed to obtain a method for separation by optimization of operational factors. In contrast to conventional methods, the interaction among process variables can be determined by statistical techniques (Nooshin, Hamid, 2017). CCD (RSM) is a gathering of mathematical and statistical techniques, which provides important information concerning the optimum level of each process variable along with its interactions with other variables and their effects on the response of the process, to acquire optimum chromatographic conditions. With the use of CCD (RSM), the number of experiments can be minimizing without neglecting the interactions between the parameters of the process. This multivariate also improves statistical explanation possibilities and evaluates the comparative significance of several contributing factors even in the incidence of complex interactions. For instance, in a study, a central composite design was used to investigate the effect of critical parameters. The retention factor and resolution were selected as the response variables (Mohammad *et al.,* 2019).

MATERIAL AND METHODS

Material

Torsemide was got as a gift sample from Sun Pharmaceuticals. Eplerenone was supplied as gift sample by Lupin Laboratories. The analytical grade methanol was purchased from Rankem Pvt. Ltd. (India). Tablets (Planep-T) were purchased from a local pharmacy. The distilled water was used for analytical work and rinsing of clean glass wares. Acetonitrile, water and methanol were of HPLC grade of Rankem brand supplied by Avantor Ltd., Thane.

Instrumentation

A Shimadzu LC-2010 CHT equipped with auto sampler and low pressure gradient pump was used. The system has UV detector. The detection wavelength selected was 223 nm. Data was gained and processed by using LC solution software. Chromatographic separation was performed using Shim - pack solar C18 (250 mm × 4.6 mm, 5 µm) column.

Software

Experimental design (CCD), desirability function, and data analysis calculations were performed by using Design-Expert version 10.0.0.1.

METHODS

Diluent

Water: Acetonitrile in the ratio of 50:50 used as diluent.

Preparation of standard stock solution

Standard stock solutions of TOR and EPL were prepared separately by adding 100 mg of the drug to diluent taken in 100ml volumetric flasks and then sonicated for five minutes and the volume was made up with diluent. The resulting solutions contain 1mg/mL of the drug. The stock solutions of TOR and EPL were

further diluted with diluent to get the concentration of 4 µg/mL and 10 µg/mL of TOR and EPL, respectively.

Sample preparation

Twenty tablets were weighed and crushed. From that, a powder equivalent to 25 mg of EPL was weighed accurately and transferred into a 10 mL clean dry volumetric flask. The contents of the flask were dissolved in diluent, sonicated for 30 minutes, and made up to the final volume with diluent and labelled as Sample stock solution.The Sample stock solution was filtered by Whatmann 0.45 µm filters. From this solution, dilutions were made to get the solution containing 25 µg/mL of EPL and 10 µg/mL of TOR. The analysis procedure was repeated three times for the tablet formulation.

Chromatographic procedure

Chromatographic separations were carried out on a Shim - pack solar C18 column (250 mm x 4.6 mm, 5 µm). A mobile phase used was acetonitrile, water and methanol (50:30:20 v/v/v). Wavelength of 223 nm was used for detection, at which both drugs showed a good response. Quantitation was performed by using low pressure gradient at 1.0 mL/min flow rate and column temperature was maintained at ambient temperature. An Injection volume used was 10 µL.

Software aided method optimization

Literature revealed that some design methodologies were employed to study the robustness of HPLC method. They applied in optimizing separation techniques during screening, testing of robustness and also in optimizing formulation, products, or method. Here in the present work, the important chromatographic factors were selected, based on preliminary experiments and after referring the literature. Various factors were considered for method development, including a volume of organic solvents in the mobile phase and flow rate, (Dawud *et al.,* 2014). Thus, the CCD was used to test the effects of two independent chromatographic parameters on the three defined key response variables. The design comprised 11 experimental runs and helped in screening of factors by evaluating their main effect to get outcomes of the study. For a Central composite design there three levels and two factors were selected. The three levels were low (-1), medium (0) and high (+1), whereas factors were (X_1) proportion of organic solvent (Acetonitrile) used in mobile phase (45%, 50% and 55%) and (X_2) flow rate of mobile phase (0.9, 1.0 and 1.1 mL/min). The retention time of TOR (Y_1), retention time of EPL (Y_2), and resolution (Y_3) were used as responses in experimental design and are shown in Table I. The resulting data was fitted into Design-Expert version 10.0.1. Response surface quadratic method was a suitable method and was used to explore, to investigate behaviour of the response around optimized values of the factors and to attain the best system performance (Ficarra *et al*., 2002). The Analysis of variance (ANOVA) was applied to examine the significance of the model. From this optimized method conditions were selected and subjected to verification for the method performance. Accuracy and precision were performed and results were found to be less than 2% RSD, and results of robustness were within a limit (Peraman *et al*., 2015; Patel *et al.,* 2017b). Eleven experiments were constructed using the conditions and observed responses and described in Table II.

TABLE I - Experimental plan of CCD showing factors with levels

Factors	Range levels			
	Code	Low (-1)	Medium (0)	High (+1)
Proportion of organic solvent (Acetonitrile) used in mobile phase	X_1	45	50	55
Flow rate of mobile phase (mL/min)	X_2	0.9	1.0	1.1
Responses				
Retention time of TOR	Y_1	-	-	-
Retention time of EPL	Y_2	-	-	-
Resolution	Y_3	-	-	-

TABLE II - Coded values for factor level and observed responses in CCD for 11 analytical trials

Experiment (Run)	Factor 1 (% Organic Phase) X_1	Factor 2 (Flow rate) ml/min X_2	Retention time of TOR (min) Y_1	Retention time of EPL (min) Y_2	Resolution Y_3
1	45	0.9	2.661	5.641	13.663
2	45	1.0	2.475	5.074	14.76
3	55	0.9	2.395	4.459	8.47
4	55	1.1	1.956	3.66	7.857
5	55	1.0	2.154	4.022	8.139
6	50	1.0	2.233	4.405	9.121
7	50	1.0	2.218	4.403	8.558
8	45	1.1	2.161	4.608	8.849
9	50	1.1	2.041	4.018	8.639
10	50	0.9	2.492	4.913	9.548
11	50	1.0	2.232	4.390	9.258

Method validation

The RPHPLC method was validated according to the International Council for Harmonization (ICH) Q2R (1) (2005) guidelines for system suitability, linearity, detection limit, quantitation limit, precision, accuracy, specificity, and robustness.

System suitability test

According to the ICH guidelines (2005), system suitability tests are an intrinsic component of liquid chromatographic methods. System suitability parameters, viz; number of theoretical plates, resolution, and tailing factor were tested by injecting six replicates of standard solutions containing 4 µg/mL of TOR and 10 µg/mL of EPL before the sample analysis. The results were calculated and percent relative standard deviation found to be < 2.0% that are in acceptable limits. (Ganorkar,Dhumal, Shirkhedkar, 2017).The system suitability parameters are given in Table III.

TABLE III - System suitability parameters

Drugs	Parameters	Mean ± S.D. (n=6)	% R.S.D
TOR	Retention Time	2.233 ± 0.00711	0.3202
	Theoretical Plate	11466.9 ± 34.622	0.3021
	Tailing Factor	1.005 ± 0.00804	0.7954
EPL	Retention Time	4.405 ± 0.0161	0.3660
	Theoretical Plate	30392.04 ± 30.7375	0.1011
	Tailing Factor	1.284 ± 0.0044	0.3497
	Resolution	9.121 ± 0.0407	0.4474

Linearity

Linearity of the developed method was estimated at five different concentrations levels over the range of 4-12 µg/mL for TOR and 10-30 µg/mL for EPL. Each solution of respective sample concentrations was injected

in triplicate. The calibration curve was constructed by plotting the peak area against the concentration, using linear regression analysis.

Accuracy and precision

Accuracy was carried out by spiking a known amount of standard to the tablet solution for each drug at 80, 100 and 120% levels in triplicate and samples were analyzed by the optimized method. Percentage recovery was then calculated for both the drugs. The mean recovery was found to be about 100 %. Precision of the optimized method was determined by studying the intermediate precision and repeatability. Six mixtures of TOR and EPL were assayed to assess the method repeatability and the three different concentration were selected to perform intraday and interday precision (Ganorkar *et al.,* 2017).

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ of TOR and EPL were tested using the standard deviation method. LOD was defined as 3.3 x σ/S and LOQ as 10 x σ/S based on standard deviation of the response (σ) and slope of the calibration curve (S).

Robustness

The robustness of the method refers to its ability to remain unaffected by small and deliberate variations in method parameters. The robustness of the optimized method was investigated by injecting the system suitability solution with minute deliberate changes in the chromatographic parameters, flow rate (0.8-1.2 mL/min), proportion of solvent in mobile phase (48:32:20 and 52:28:20). It was measured based on percent relative standard deviation.

Assay of tablet sample.

Twenty tablets were accurately weighed and powdered separately. A portion of powder equivalent to 25 mg of EPL and 10 TOR was transferred to volumetric flask (100 mL) and dissolved in about 50 ml of mobile phase. The sample solution was sonicated for 30 min using ultrasonic bath and diluted to the mark with mobile phase. The solution was filtered using 0.45 µm membrane filter. The working solutions containing concentrations of 25 µg/mL EPL and 10 µg/mL TOR were prepared by dilution using mobile phase, and 10 µL of filtered solution was injected in HPLC system.

RESULTS AND DISCUSSION

Preliminary studies and factor selection

In literature some methods are reported based on implementation of QbD in analytical method development (Peraman *et al*., 2015).The study was aimed to develop a simple, robust, and cost effective RP-HPLC method for estimation of TOR and EPL in tablet formulation. In this method, the important chromatographic factors were selected, based on preliminary experiments and by referring different articles from the literature. Such investigations to select the factor levels for screening and optimization studies revealed that mobile phase conditions needed to be optimized so that both TOR and EPL would be separated in a short run time. Mobile phase composition of acetonitrile, water and methanol was found to be more suitable for the simultaneous estimation of both the drugs and change in the volume of acetonitrile resulted in a large change in retention time. Hence, it is one of the critical parameters for method development.

After analysing the Ishikawa (Fish bone) diagram and Pareto ranking analysis, preliminary experiments were conducted and the critical parameters selected for further study and the components selected viz; composition of mobile phase and flow rate were found to have the most influential effect on system suitability parameters (Awotwe-Otoo *et al*., 2012; Ficarra *et al.*,2002).

QbD assisted method development

CCD design was used in the present analytical method optimization study. It is an efficient and

comprehensive experimental design based on systematic search of key components for the optimization of RP-HPLC method, two factors; volume of organic phase and flow rate were depicted.

For the RP-HPLC method, a multivariate approach DoE with CCD applied to study the simultaneous variations of the factors on considered responses, such as retention time of TOR (Y1), retention time of EPL (Y2), and resolution (Y3) to test method robustness. Based on the effects of two factors on responses and evaluation of these results, it was practicable to intricate mathematical models that had been attempted to find out the interrelation between the factors and the responses of interest studied. We observed that the best fitted model for CCD was the response surface quadratic model. The model was also validated by ANOVA using Design Expert software. The predicted R-Squared values of retention time of TOR (Y_1) and EPL (Y_2) were in reasonable agreement with adjusted R-Squared values i.e., the difference is less than 0.2, as reported by other authors (Awotwe-Otoo *et al*., 2012; Gundala *et al.,* 2019). A negative predicted R-Squared value for resolution (Y_3) implies that the overall mean may be a better predictor of the response than the current model. Adequate precision, measures the signal-to-noise ratio. A ratio of greater than 4 is desirable, and the responses for the Y_1, Y_2, and Y_3 were 31.232, 234.56, and 7.418, respectively, which shows an adequate precision. This quadratic model can be used to navigate the design space. Model F-value of responses for retention time of TOR (Y_1), retention time of EPL (Y_2), and resolution (Y_3) were 87.81, 4658.25, and 5.82, which implies the model is significant. There is only a 0.01% chance, with Y1 and Y2, while a 3.80% chance for resolution (Y3) than an F-value, showing that this could occur because of noise. Hence, the values of significant responses showed p value < 0.05, suggesting that the model terms are significant. The low standard deviation and high adjusted R-square value shows a good relationship between experimental data and those of fitted models.The ANOVA data is given in Table IV.

TABLE IV - ANOVA regression analysis for models and responses

Response	Mean	SD[a]	%CV[b]	Press value	R^{2c}	Adjusted R^2	Predicted R^2	Adequate precision	SS[d]	D[e]	MS[f]	F[g]	P
Retention time of TOR(Y1)	2.27	0.032	1.39	0.043	0.9887	0.9775	0.9034	31.232	0.44	5	0.087	87.81	0.0001
Retention time of EPL (Y2)	4.51	0.011	0.25	0.004	0.9998	0.9996	0.9985	234.56	3.01	5	0.60	4658.25	0.0001
Resolution (Y3)	9.71	1.24	12.76	70.63	0.8533	0.7066	-0.3490	7.418	44.68	5	8.94	5.82	0.0380

[a]Standard deviation,
[b]Coefficient of variations,
[c]Coefficient of Regression,
[d]Sum of squares,
[e]Degrees of freedom,
[f]Mean sum of squares,
[g]Fischer's ratio

The equations in terms of coded factors can make predictions about the response for given levels of each factor. This equation is useful for identifying the relative impact of the factors by comparing the factor coefficient. Final equations for Y_1, Y_2, and Y_3 are:

TOR:$Y1 = +4.40-0.53X1-0.45X2+0.059X1X2+0.14X1^2+0.055X2^2$;

EPL:$Y2 = +2.24-0.13X1-0.23X2+0.015X1X2+0.055X1^2+0.007211X2^2$;

Resolution:$Y3 = +9.37-2.13X1-1.06X2+1.05X1X2+1.49X1^2+0.86X2^2$

As per the values of coefficient from the above equations and their signs, factors, such as mobile phase composition (X_1) had a negative effect on retention time of TOR and EPL, Y_1 and Y_2 and on resolution, Y_3. Flow rate (X_2) had negative effects on retention time of TOR and EPL, Y_1 and Y_2 and on resolution, Y_3. Interactions of X_1 and X_2 had a positive effect on Y_1, Y_2 and Y_3. The squares of factors, X_1^2 and X_2^2, had positive effects on all chromatographic responses.

Response surface and contour plots were analyzed to see the effect of the factors and their interactions on the responses (Awotwe-Otoo *et al.*, 2012). The contour plots showed curvature, displaying a nonlinear effect of factors on responses. Figures 3 and 4 showed 2D (A) and 3D (B) contour plots displaying the effect of mobile phase ratio (X_1) and flow rate (X_2) on retention time of TOR (Y_1) and EPL (Y_2). A curvilinear increasing trend was observed for the mobile phase ratio (X_1) and flow rate (X_2), which showed higher resolution time of TOR (Y_1) and EPL (Y_2) at lower levels. Therefore, lower levels of X_1 and X_2 were recommended to achieve high retention time of TOR (Y_1) and EPL (Y_2). The study of 3D and 2D contour plots presented in Figure 5 showed curvature effects of the mobile phase ratio (X_1) and flow rate (X_2) on resolution. An increasing curvature trend was observed for both X_1 and X_2, which showed higher resolution at higher levels. Therefore, optimized levels of X_1 and X_2 were recommended to achieve resolution.

A composite desirability applied to get an optimum set of conditions based on the specified goals and boundaries for each response. The desirability function "R", equal to unity, showed the achievement of desired goals in the constraints set and the whole experimental area was explored for the compositions (Awotwe-Otoo *et al.*, 2012), where in constraints set were met to the maximum i.e., unity, as shown in Figure 6. The optimum values of chromatographic conditions of RP-HPLC were selected as mobile phase (X_1) ratio of acetonitrile: water: methanol 50:30:20 and flow rate 1.0 mL/min (Figure 7) which resulted in retention time of TOR (Y_1) 2.233 ± 0.0071, retention time of EPL (Y_2) 4.405 ± 0.016, and resolution (Y1) 9.121 ± 0.040 min, respectively, as shown in Figure 8.

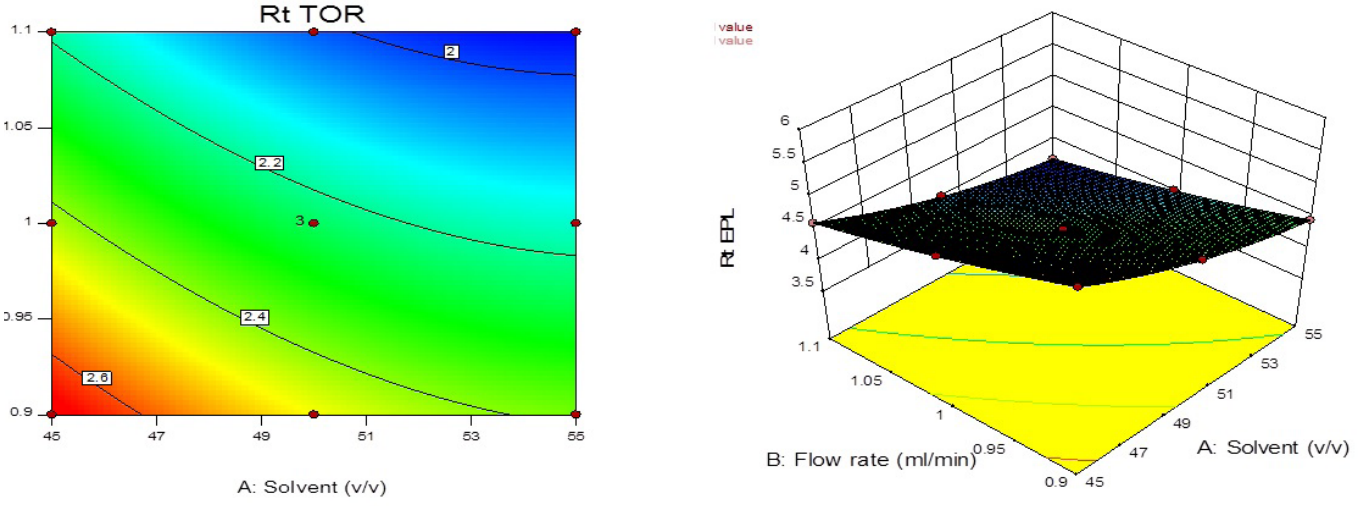

FIGURE 3 - 2D (A) and 3D (B) contour plots showing the effect of mobile phase ratio (X_1) and flow rate (X_2) on retention time of TOR (Y_1).

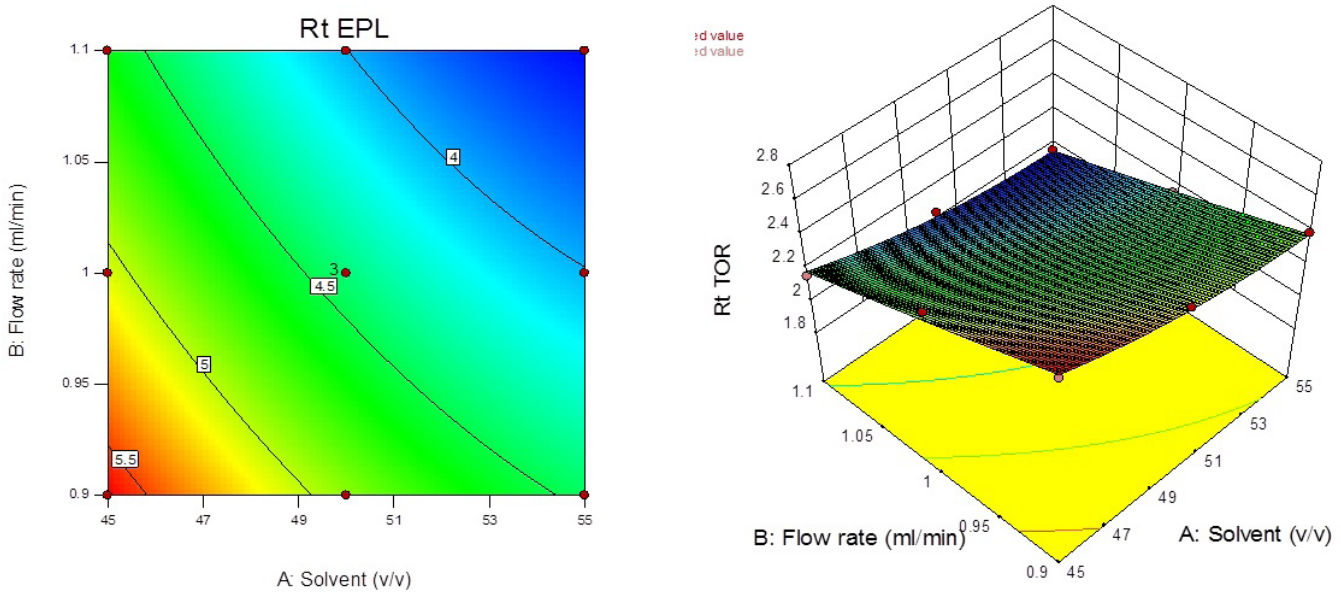

FIGURE 4 - 2D (A) and 3D (B) contour plots showing the effect of mobile phase ratio (X_1) and flow rate (X_2) on retention time of EPL (Y_2).

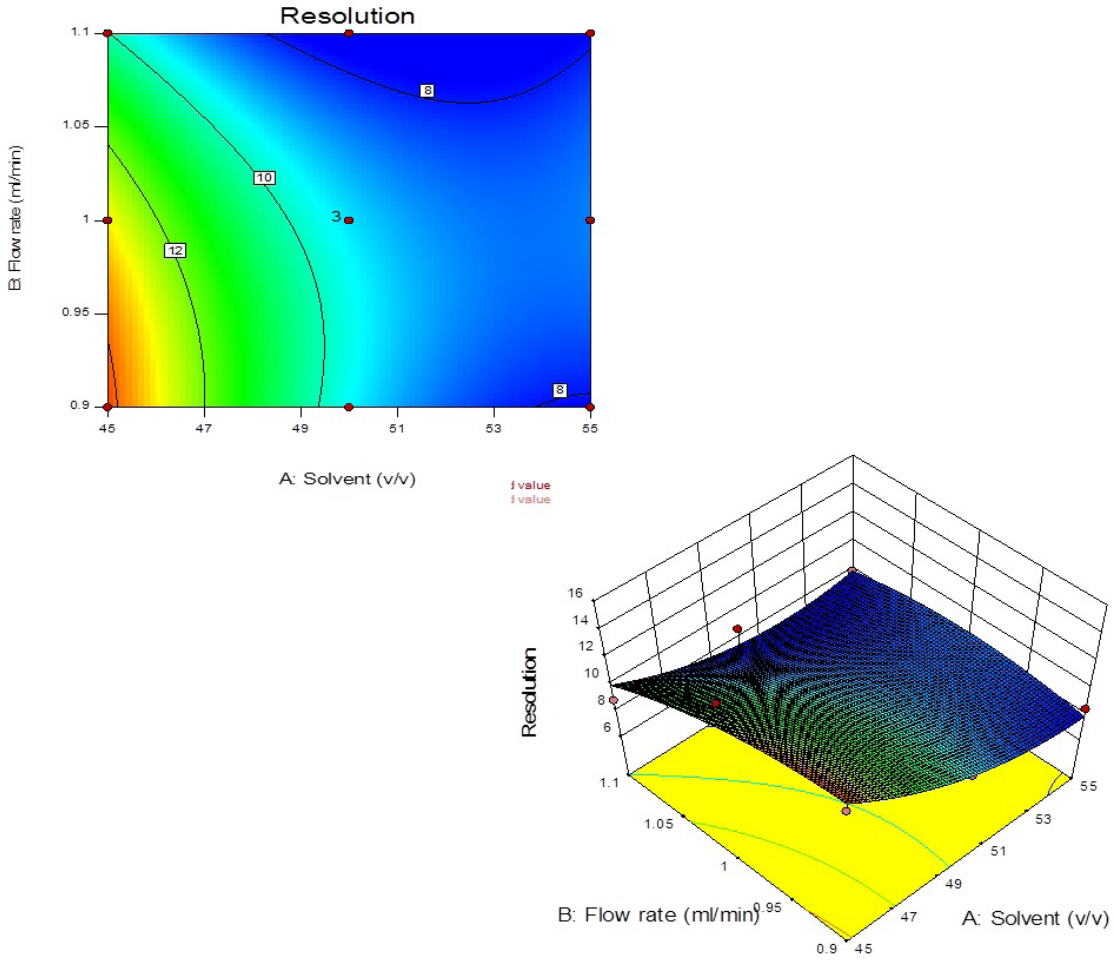

FIGURE 5 - 2D (A) and 3D (B) contour plots showing the effect of mobile phase ratio (X_1) and flow rate (X_2) on resolution (Y_3).

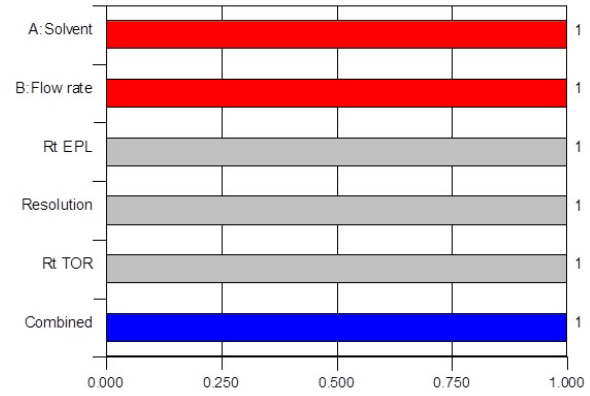

FIGURE 6 - Desirability function representation basis unity=1.

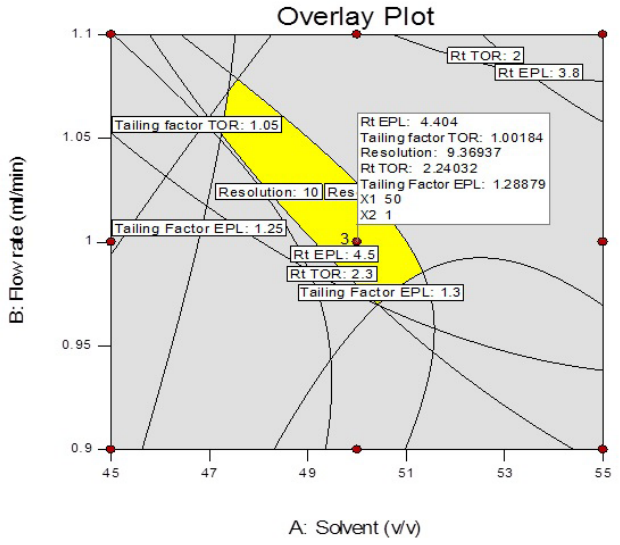

FIGURE 7 - Optimized chromatographic conditions.

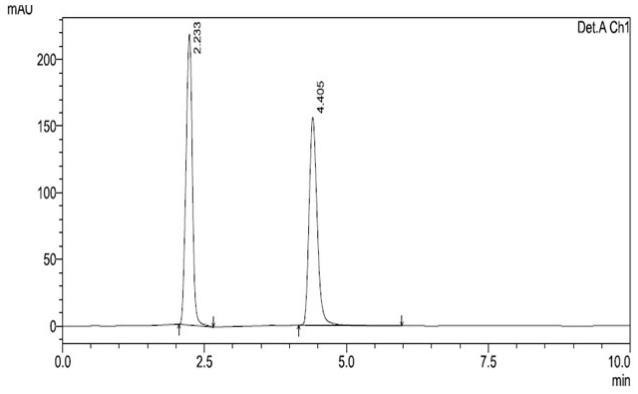

FIGURE 8 - Optimized RP-HPLC chromatogram for TOR and EPL at 223 nm.

Method validation

As per ICH guidelines (2005), system suitability tests were performed in liquid chromatographic method. The column efficiency, as determined from a number of theoretical plates for both the drugs, was found to be over 2000, resolution was 9.121, and tailing was found to be less than 2. The percent relative standard deviation for six replicate injections was found to be 0.6895 in the concentration of 10 µg/mL for TOR and 0.2307 in the concentration of 25 µg/mL for EPL, respectively. As % RSD was found to be less than 2%, it has shown good injection repeatability. Linearity of the developed method was confirmed by plotting the linearity curve over concentrations ranging from 4 to 12 µg/mL for TOR and 10-30 µg/mL for EPL, with a correlation coefficient (r^2=0.9995 and 0.9998) for TOR and EPL respectively, shown in Table V. The obtained correlation coefficient (r^2=0.999) shows an excellent correlation between peak area and concentration. For the recovery study, different concentrations of samples (80, 100, and 120%) of standard concentrations for both drugs were prepared and showed recovery of 99.41–100.53 % and 99.43–100.06 % for TOR and EPL, respectively. Data is shown in Table IV, showing that the developed method has a high level of accuracy with % RSD 0.049 and 0.119 for TOR and EPL, respectively. Intermediate precision and repeatability were carried out and the resultant data are given in Table IV. The precision values for both drugs were less than 2%., showing precision of the method. The LOD and LOQ were found to be 0.12 and 0.38 µg/mL, respectively for TOR, and 0.20 and 0.61 µg/mL, respectively for EPL. The insensitivity of the RP-HPLC method to minor changes in the optimized experimental changes was showed by its robustness to such slight changes. The mobile phase composition and flow rate caused significant effects in the retention time of TOR and EPL, and resolution.

TABLE V - Validation results for TOR and EPL

PARAMETERS		TOR	EPL
System suitability parameters			
No. of theoretical plates	Mean ± SD*	11466.9 ± 34.622	30392.04 ± 30.7375
	% RSD	0.3202	0.3660
Resolution	Mean ± SD	-	9.121 ± 0.0407
	% RSD	-	0.4474
Tailing factor	Mean ± SD*	1.005 ± 0.00804	1.284 ± 0.0044
	% RSD	0.7954	0.3497
Linearity			
Range(µg/ml)		4-12	10-30
Slope		104444	38576
Intercept		208048	192281
Correlation coefficient		0.9997	0.9998
Accuracy			
Recovery studies		99.41 – 100.53	99.43 – 100.06
%RSD**		0.42-0.88	0.27-1.19
Precision			
Repeatability	% RSD	0.5185	0.6538
Intermediate precision	% RSD	0.6356 – 1.121	0.8139 – 0.9822
LOD (µg/mL)		0.1266	0.2035
LOQ (µg/mL)		0.3836	0.6167
Assay % ± S.D.		99.42 ± 0.0732	99.80 ±0.1789

*Mean of six determinations,
**Set of three determinations

CONCLUSION

The present study entails systematic QbD, based development of a simple, rapid, precise, and cost effective RP-HPLC method for simultaneous estimation of TOR and EPL. The experimental design describes the scouting of key components, including mobile phase composition and flow rate. The modelling software facilitated better understanding of the factors influencing optimization of the method and separation of TOR and EPL. CCD applied to optimize the resolution as response between TOR and EPL in a relatively short time (5 min). In the optimized model, the acetonitrile, water and methanol in the ratio of 50:30:20 v/v/v shows the suitability for estimation of TOR and EPL. The flow rate of the mobile phase was optimized at 1.00 ml/min. The validation study supported the selection of the best conditions by confirming that the method was accurate, linear, precise, and robust. Therefore utilization of the response surface technique provides

a better insight for method development and robustness testing. This developed method satisfies the design space concept and is suitable for regulatory submission under regulatory flexibility.

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