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Development of RP-HPLC Method for Simultaneous Determination of Brimonidine Tartrate and Brinzolamide by QbD Approach and Its Validation

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A simple, rapid, accurate and precise reversed phase high performance liquid chromatographic method has been developed for the simultaneous determination of brimonidine tartrate (BRT) and brinzolamide (BRZ). A 3^2 factorial design was utilized to aid in method development and optimization. Effective chromatographic separation was achieved using C18 column (250 × 4.6 mm, 5µm) as a stationary phase and mobile phase consisted of methanol: 0.01 M ammonium acetate buffer (49.5: 50.5, v/v), pH adjusted to 3.8 with acetic acid at a flow rate of 1.1 mL/min at a detection wavelength of 260 nm. The injection volume was 20 µL. Quality by design approach was applied to evaluate the effect of two factors i.e. mobile phase composition and flow rate on the various chromatographic responses (area, number of theoretical plates, resolution, retention time and tailing factor). The retention time of BRT and BRZ were found to be 3.96 and 8.34 min; respectively. Calibration curves were found to be linear over the concentration range of 0.2-1.4 µg/mL for BRT and 1-7 µg/mL for BRZ. The limit of detection and limit of quantitation for BRT were found to be 0.03 µg/mL and 0.09 µg/mL whereas those for BRZ were found to be 0.018 µg/mL and 0.051 µg/mL; respectively. The % recovery of the drugs by developed method was found in the range of 99.04 to 101.67 %. The proposed method was found to be precise as well as robust. The method was successfully applied for quantitative determination of BRT and BRZ in in-house dosage form i.e. suspension.

Keywords: brimonidine tartrate, brinzolamide, RP-HPLC, method validation, quality by design

INTRODUCTION

Glaucoma is an eye disease, wherein the intraocular pressure within the eye is

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enough so as to cause damage to the optic nerve [1]. Brimonidine tartrate (BRT), chemically 5-bromo-6 (2-imidazolidinylideneamino) quinoxaline L- tartrate is a α_2-adrenoreceptor agonistused for the treatment of open-angle glaucoma [2]. The ocular hypotensive effect of this molecule is because of its ability to decrease aqueous humor production [3]. Brinzolamide (BRZ), chemically (R) – 4- (ethyl amino)-3,4-dihydro-2-(3-methoxy propyl)-2H thienol[3,2-e]- 1,2-thiazine-6 sulphonamide 1,1-dioxide, a non-competitive reversible carbonic anhydrase inhibitor is indicated for the treatment of elevated intraocular pressure in patients with glaucoma [4]. Simbrinza ophthalmic suspension is available as a fixed dose combination of BRT (0.2% w/w) and BRZ (1% w/w), which is indicated for the treatment of glaucoma.

Quality by Design (QbD) is a systematic approach that focuses on understanding and control of processes to provide continuous improvement in method development with the desired critical quality attributes [5]. QbD-based analytical method development helps to recognize and reduce sources of variability that may lead to poor method performance. It also ensures that the method meets its proposed performance requirements throughout the product and method life cycle [6-8]. Quality is built into the development of the method itself, resulting in improved separations. USFDA also proposed QbD as important criteria for method development.

Several analytical methods such as UV [9,10], RP-HPLC [11-13], HPTLC [14], UPLC [15], spectrofluorimetric [16], HILIC [17], GC-MS [18], LC/MS/MS [19] and capillary electrophoresis methods [20] are reported for the determination of BRT alone. Few UV [21-24], RP-HPLC [25-28] and HPTLC [29] methods have been reported for estimation of BRT and Timolol (TM). BRZ is official in IP [30] and USP[31]. Methods such as UV spectrophotometry [32], HPLC and HPTLC [33] are reported for simultaneous estimation of BRZ and TM. Few UV derivative spectrophotometric methods [34, 35] have been reported for the determination of BRT and BRZ. To the best of our knowledge, till now no reversed phase high performance liquid chromatographic method has been reported for simultaneous determination of BRT and BRZ utilizing experimental design. Thus, the aim of the present study was to develop, optimize and validate a simple and rapid RP-HPLC method for the simultaneous determination of BRT and BRZ using QbD approach.

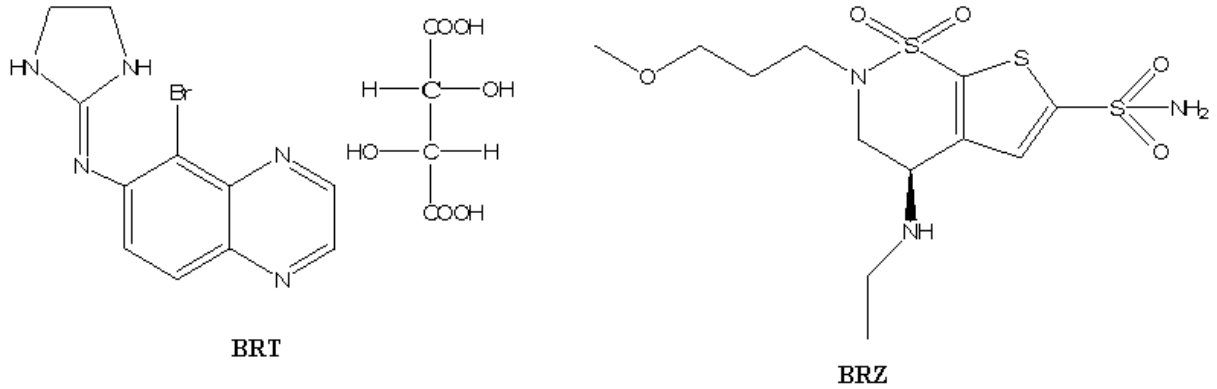

Figure 1. Chemical structure of brimonidine tartrate (BRT) and brinzolamide (BRZ).

MATERIALS AND METHODS

Chemicals and reagents

Reference standards of BRT (purity 98 % w/w) and BRZ (purity 98 % w/w) were obtained from Sun Pharmaceutical Pvt. Ltd, Halol, Gujarat, India. HPLC grade

methanol and water were purchased from Fisher scientific India Pvt. Ltd, Powai, Mumbai. HPLC grade ammonium acetate was purchased from Rankem (RFCL), Haryana, India.

Instruments

Analysis was performed on Cyber lab LC 100HPLC system equipped with binary LC P-100 pump, high pressure gradient mixer (1500 µL) and a UV detector. Data acquisition and processing was done using WS- Workstation software. Equitron digital ultrasonic cleaner was used for mixing the solutions. Precisa digital weighing balance was used for weighing. Equiptronics digital pH meter was used for all pH measurements.

Selection of wavelength

For both drugs, standard solutions of 10 µg/mL were prepared in methanol individually and were scanned in the wavelength range of 200-400nm and the overlain spectrum was obtained. From the overlain spectrum, isoabsorptive point was found to be at 260 nm (Figure 2). Thus, 260 nm was selected as detection wavelength for the simultaneous estimation of both the drugs.

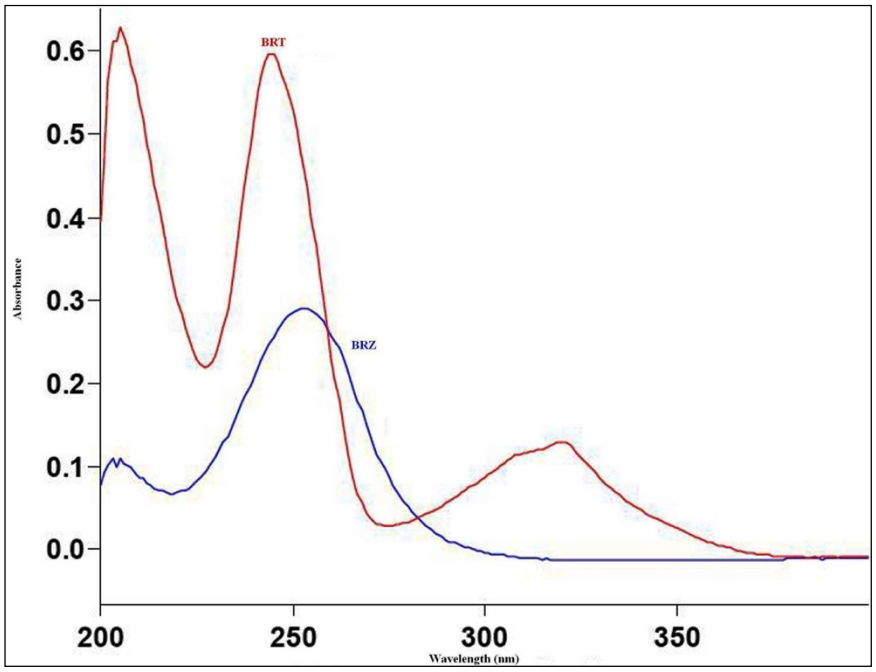

Figure 2. Overlain spectrum of BRT and BRZ.

Method optimization

Initially various mobile phases such as methanol: water (80: 20, v/v); acetonitrile: water (80: 20, v/v); methanol: 0.01 M phosphate buffer (pH adjusted to 3.14 with ortho phosphoric acid) (40: 60, v/v); acetonitrile: methanol: 0.01M phosphate buffer (pH 3.14) (10: 40: 50, v/v/v); methanol: 0.4 % TEA in water (pH adjusted to 3.0 with o-phosphoric acid) (25: 75, v/v) etc. were tried at different flow rates but they didn't produced satisfactory results. After evaluating all the factors like resolution, peak symmetry, number of theoretical plates, time required for analysis; the mobile phase consisting of methanol: 0.01 M ammonium acetate buffer

(pH adjusted to 3.8 with acetic acid) (45: 55, v/v) at a flow rate of 1.0 mL/min was selected for further optimization by QbD.

Software aided method optimization

A 3^2 factorial experimental design was separately applied for both drugs to optimize the chromatographic conditions. A 3^2 factorial design indicates that there are three levels and two factors involved in it. The three levels were low (-1), medium (0) and high (+1) whereas the factors were A (mobile phase ratio) and B (flow rate). The chromatographic responses involved in the trial were area (Y_1, Y_6), number of theoretical plates (Y_2, Y_7), resolution (Y_3, Y_8), retention time (Y_4, Y_9) and tailing factor (Y_4, Y_{10}). This design was specifically selected since it required fewer runs (13) as compared to the others. It was suitable for exploring response surface and creating different models with Design Expert ® (Version 9.0.4, Trial version). The levels selected for both the drugs are described in Table 1. About 13 experimental runs were carried out for both drugs using the different chromatographic conditions and responses were observed as described in Table 2 and Table 3.

Table 1: Experimental factors and levels used in factorial design.

Factor	**Level used**		
Independent variable	Low (-1)	Medium (0)	High (+1)
A= Mobile phase ratio (v/v)	50 : 50	45 : 55	40 : 60
B = Flow rate (mL min^{-1})	0.5	1.0	1.5
Dependent variable			
Y_1,Y_6 = Area of BRT and BRZ; respectively			
Y_2, Y_7 = No. of theoretical plates of BRT and BRZ; respectively			
Y_3, Y_8 = Resolution of BRT and BRZ; respectively			
Y_4, Y_9 = Retention time of BRT and BRZ; respectively			
Y_5, Y_{10} = Tailing factor of BRT and BRZ; respectively			

Analysis of variance (ANOVA) was applied to the response variables to examine the significance of the model. Lack of fit test, which indicated insignificant lack of fit value corresponding to a higher p-value as compared to the model F-value, was also used to examine the applied model.

Chromatographic conditions

Chromatographic separation was achieved on Sun chrome C-18 column (250 × 4.6 mm, 5 µm) and the software aided optimized mobile phase was methanol: 0.01 M ammonium acetate buffer (pH adjusted to 3.8 with acetic acid) (49.5: 50.5, v/v). The flow rate of mobile phase was 1.1 mL min^{-1}. The detection was carried out at 260 nm. The injection volume was 20 µL. The chromatographic run time was 10 min. The mobile phase was filtered before use through a 0.2 µ membrane filter (Sartorius Stedium Biotech, Germany) and degassed for about 15 min.

Preparation of mobile phase

Accurately weighed 0.3854 g of ammonium acetate was dissolved in 500 mL of HPLC grade water. The solution was adjusted to pH 3.8 with 1M acetic acid. The resulting buffer was filtered through a 0.2 µ membrane filter. Required volume of the mobile phase was prepared by mixing methanol and ammonium acetate buffer (49.5: 50.5, v/v). Then the mixture was sonicated for about 15 min to ensure proper mixing and then filtered through a 0.2 µ membrane filter.

Table 2: Observed responses of 13 experimental runs for BRT.

Run	Level	Factor		Response				
		Mobile phase (v/v)	Flow rate (mL/min)	Retention time (min)	Area (mAU)	Resolution	No. of theoretical plates	Tailing factor
1	1, 1	50:50	1.5	3.188	6418.3	15.30	5673.18	1.68
2	0, 0	45:55	1.0	3.580	6891.2	19.18	5918.94	1.54
3	0, 0	45:55	1.0	3.580	6891.2	19.18	5918.94	1.54
4	-1,+1	40:60	1.5	3.912	7011.9	24.97	6165.57	1.73
5	-1,-1	40:60	0.5	6.612	11911.0	26.97	6848.36	2.15
6	0,0	45:55	1.0	3.580	6891.2	19.18	5918.94	1.54
7	-1,0	40:60	1.0	4.144	7326.9	24.76	6316.25	2.15
8	0,0	45:55	1.0	3.580	6891.2	19.18	5918.94	1.54
9	0,-1	45:55	0.5	5.975	11544.8	21.62	6584.08	1.90
10	+1,0	50:50	1.0	3.440	6853.7	15.22	5736.26	1.58
11	+1,-1	50:50	0.5	5.636	11342.2	17.04	6299.36	1.66
12	0,+1	45:55	1.5	3.373	6484.0	19.47	5922.00	1.66
13	0,0	45:55	1.0	3.580	6891.2	19.18	5918.94	1.54

Table 3: Observed responses of 13 experimental runs for BRZ.

Run	Level	Factor		Response				
		Mobile phase (v/v)	Flow rate (mL/min)	Retention time (min)	Area (mAU)	Resolution	No. of theoretical plates	Tailing factor
1	+1,+1	50:50	1.5	6.570	3818.5	15.30	9299.64	1.22
2	0, 0	45:55	1.0	8.802	4452.4	19.18	9651.61	1.18
3	0,-1	45:55	0.5	14.741	7167.5	21.62	13043.5	1.18
4	0,0	45:55	1.0	8.802	4452.4	19.18	9651.61	1.18
5	0,0	45:55	1.0	8.802	4452.4	19.18	9651.61	1.18
6	+1,-1	50:50	0.5	11.629	6833.4	17.04	12292.7	1.22
7	0,0	45:55	1.0	8.802	4452.4	19.18	9651.61	1.18
8	-1,-1	40:60	0.5	19.918	7417.3	26.97	14261.1	1.18
9	-1,0	40:60	1.0	12.425	4455.0	24.76	11647.3	1.16
10	0,0	45:55	1.0	8.802	4452.4	19.18	9651.61	1.18
11	-1,+1	40:60	1.5	11.678	4087.8	24.97	12212.8	1.09
12	+1,0	50:50	1.0	7.108	4316.4	15.22	8951.51	1.06
13	0,+1	45:55	1.5	8.298	3961.5	19.47	10084.2	1.18

Preparation of solutions

Preparation of standard stock solutions

Standard stock solutions of BRT and BRZ were prepared by dissolving 10 mg of each drug separately in separate 100 mL volumetric flasks using methanol as a solvent up to 50 mL, then sonicated for 15 minutes and the final volume was made up to 100 mL with methanol to get the standard stock solutions containing 100 µg/mL of each of BRT and BRZ.

Preparation of working standard solutions

Working standard solution of BRT (10 µg/mL) was prepared by transferring about 1 mL of stock solution of BRT into 10 mL volumetric flask and the volume was made up to the mark by using mobile phase as diluent. Working standard solution of BRZ (10 µg/mL) was prepared by transferring about 5 mL of stock solution of BRZ into 50 mL volumetric flask and diluting it up to mark with mobile phase.

Preparation of mixed standard solution

Required mixed standard solution containing BRT and BRZ was prepared by transferring accurate volumes of each of the working solution of BRT as well as BRZ to a 10 mL volumetric flask and diluting it up to mark with mobile phase.

Preparation of sample solution

Accurately measured 1 mL of in-house suspension containing 0.2 % BRT and 1 % BRZ was taken and transferred to 100 mL volumetric flask. About 50 mL of methanol was added into the flask and sonicated for 15 minutes. Then, the final volume was made up to 100 mL with methanol to produce solution containing 20 µg/mL of BRT and 100 µg/mL of BRZ. From this stock, 1 mL of solution was taken and diluted up to10 mL with methanol to obtain a solution containing 2 µg/mL of BRT and 10 µg/mL of BRZ. The resulting solution was further diluted to get a final solution containing 0.8 µg/mL of BRT and 4 µg/mL of BRZ and then filtered through 0.2 µm filter to get a clear solution.

Method validation

The developed and optimized method was validated as per ICH guidelines [36] for various parameters such as specificity, system suitability, linearity and range, LOD, LOQ, accuracy, precision and robustness.

Specificity

The specificity of the method was assessed by comparing chromatograms obtained from drug standards with that obtained from sample solution.

System suitability

System suitability parameters like number of theoretical plates, resolution and tailing factor were evaluated by injecting six replicates of working standards containing 0.8 µg/mL of BRT and 4 µg/mL of BRZ. Then the % RSD was calculated.

Linearity and range

The linearity of the developed method was estimated using standard solutions of seven different concentrations in the range of 0.2–1.4 µg/mL for BRT and 1–7 µg/mL for BRZ. Each solution was injected in triplicate. A graph of average area vs. concentration was plotted and regression coefficients (R^2) for both the drugs were calculated. The linearity equations for both the drugs were obtained by linear regression analysis, using GraphPad Prism software.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ of BRT and BRZ were evaluated using standard deviation method. Calibration curves were plotted in the range of 0.025-0.2 µg/mL for BRT and 0.1-0.6 µg/mL for BRZ.LOD and LOQ of both the drugs were calculated using formula 3.3 σ/S and 10 σ/S, respectively, where σ is the standard deviation of intercepts and S is the slope of the calibration curve.

Accuracy

The accuracy of the proposed method was determined by standard addition method by calculating the percentage recoveries of both the drugs. The study was carried out at three different concentration levels. Known amount of standard solution of BRT and BRZ (0.4µg/mL and 2 µg/mL; 0.8 µg/mL and 4 µg/mL; 1.2 µg/mL and 6 µg/mL) were spiked into the prequantified sample solution of BRT and BRZ (0.1 µg/mL and 0.5 µg/mL); respectively. Area was measured in triplicates, concentrations of both the drugs were calculated and % recovery was determined at each level using following formula:

$$\% \, Recovery = \frac{Observedvalue}{Truevalue} * 100\%$$

Precision

The precision studies were carried out as inter-day and intra-day precision studies at three different concentration levels of test solution. The concentrations of BRT and BRZ at 50% level were 0.4 µg/mL and 2 µg/mL, respectively; at 100 % level were 0.8 µg/mL and 4 µg/mL, respectively and at 150 % level were 1.2 µg/mL and 6 µg/mL, respectively. Intraday precision studies were carried out on the same day at different time intervals whereas intraday studies were carried out on three different consecutive days. Area of both the drug at each concentration level was measured in triplicate and % RSD was calculated.

Robustness

The robustness of the method was evaluated by varying method parameters such as flow rate (1.0 mL/min, 1.2 mL/min); detection wavelength (259 nm, 261 nm); mobile phase composition (50:50, 49:51) and pH (3.7, 3.9). It was assessed by injecting the standard solution (0.8 µg/mL of BRT and 4 µg/mL of BRZ) six times and test solution (0.8 µg/mL of BRT and 4 µg/mL of BRZ) twice and calculating the values of % RSD. The data were evaluated using one-way analysis of variance (ANOVA).

Analysis of in-house suspension

An in-house suspension containing 0.2 % BRT and 1% BRZ was prepared, suitable dilution was done and then analyzed. The % content of each drug was determined using the following formulas:

$$\% \, ofBRT = \frac{A_{T1}}{A_{S1}} \times \frac{W_{S1}}{100} \times \frac{1}{10} \times \frac{0.8}{10} \times \frac{10}{W_{T1}} \times \frac{100}{1} \times \frac{10}{4} \times P_1$$

$$\% \, ofBRZ = \frac{A_{T2}}{A_{S2}} \times \frac{W_{S2}}{100} \times \frac{1}{10} \times \frac{4}{10} \times \frac{10}{W_{T2}} \times \frac{100}{1} \times \frac{10}{4} \times P_2$$

Where, A_{T1} and$A_{T2}$are the average area of test solutions of BRT and BRZ, respectively; A_{S1} and A_{S2} are the average area of standard solutions of BRT and BRZ, respectively; W_{T1} and W_{T2} are the weights of BRT and BRZ, respectively in the sample; W_{S1} and W_{S2} are the weight of standards of BRT and BRZ, respectively; P_1 and P_2 are the purity of standards of BRT and BRZ, respectively.

RESULTS AND DISCUSSION

Design of experiment

A 3^2 full factorial design was performed using 13 experimental runs for BRT as well as for BRZ. The dependent as well as independent variables of all runs are shown in Table 1. The proposed regression equations for various chromatographic responses of both the drugs are given in the Table 4.

It was observed that the best fitted model for BRT was the quadratic model. In case of BRZ for all the responses quadratic model was found to be the best fitted model except for tailing factor where in the best fitted model was linear (Table 5).A positive value represents an effect that favors the optimization, while a negative value indicates an inverse relationship between the factor and the response. In case of BRT, it is clear from the equations that the factor A (mobile phase composition) and factor B (flow rate) had negative effect on all the chromatographic responses. In case of BRZ, the factor A had negative effect on area, number of theoretical plates, resolution and retention time and it had a positive effect on tailing factor whereas the factor B had negative effect on all the chromatographic responses. Interaction of A and B had a negative effect on $Y_1$and Y_4 and had a positive effect on Y_2, Y_3 and Y_5 with reference to BRT. For BRZ, the square of the factor A^2 was having a positive impact while B^2 was having a negative impact on response area. The source sum of squares (Source SS) in ANOVA indicates that the contribution of factor A (mobile phase) (SS=993.31) is higher than factor B (flow rate) (SS =34.56) for optimizing the response term resolution.

Table 4: Regression equations for various chromatographic responses.

Drug	Regression equation
BRT	Y_1= 6897.08 – 272.60*A – 2480.63 *B – 6.20 * AB + 178.51 *A^2 + 2102.61 * B^2
	Y_2 = 5934.63 – 270.23 *A – 328.51 *B + 14.15 *A*B + 52.41 *A^2 + 279.20 *B^2
	Y_3 = 19.22 – 4.86 *A – 0.98 *B + 0.06 *A*B + 0.67 *A^2 + 1.22 *B^2
	Y_4 = 3.59 – 0.41 *A – 1.29 *B – 0.06 *A*B + 0.19 *A^2 + 1.07 *B^2
	Y_5 = 1.58 – 0.185 *A – 0.11 *B + 0.11 *A*B + 0.18*A^2 + 0.09 *B^2
BRZ	Y_6 = 4446.68 – 165.30 *A – 1591.73 * B + 78.65 *A*B – 46.69 *A^2 + 1132.11 *B^2
	Y_7 = 9678.51– 1262.89 *A – 1333.43 *B – 236.20 *A*B + 553.60 *A^2 + 1818.07 *B^2
	Y_8= 19.22 – 4.86 *A – 0.98*B + 0.06 *A*B + 0.67*A^2 + 1.23*B^2
	Y_9 = 8.81 – 3.12 *A – 3.29 *B + 0.79 *A*B + 0.95 *A^2 + 2.70 *B^2
	Y_{10} = 1.17 + 0.01 *A – 0.02 *B

The values of $R^2$for Y_1, Y_2, Y_3, Y_4 and Y_5 for full model in case of BRT were 0.9998, 0.9927, 0.9993, 0.9994, 0.8433; respectively whereas in BRZ were 0.9985, 0.9968, 0.9988, 0.9930 and – 0.0933; respectively (Table 5). For BRT, all model terms were found to be significant whereas in case of BRZ all model terms except tailing factor were found to be significant. In case of BRT, the calculated F values for full models of area, number of theoretical plates, resolution, retention time and tailing factor were 5865.76, 191.49, 2082.03, 2374.31 and 7.53; respectively whereas that of BRZ were 930.09, 432.40, 2082.03, 340.98 and 0.49; respectively.

3D response surface plots presented as Figure 3a–e for BRT and as Figure 4a–e for BRZ which were used to determine the relationship between the response and the factors. In case of BRT, the plot (Figure 3a) indicates that both the mobile phase (A) and flow rate (B) had a negative effect on area. With the decrease in flow rate, the area increases. It is evident from Fig. 3b, that an increase in mobile phase composition or flow rate decreases the number of theoretical plates. A response surface plot (Figure 3c, 4c) indicates the negative effect of both the factors on resolution. The retention time and tailing factor decreases with the increase in flow

rate (Figure 3d-e). In case of BRZ, both mobile phase (A) and flow rate (B) had a negative effect on area as well as on number of theoretical plates as shown in Figure 4a-b. It is obvious from Figure 4d that a decrease in flow rate causes an increase in retention time. When considering the response term tailing factor, the response surface plot (Figure 4e) indicates the positive effect of mobile phase composition on the response term.

Table 5: Regression analysis summary for the finally suggested models.

Drug	Response	Model	R^2	Adjusted R^2	Predicted R^2	SD	% CV	Adequate precision
BRT	Y_1	Quadratic	0.9998	0.9996	0.9977	42.38	0.53	191.25
	Y_2	Quadratic	0.9927	0.9876	0.9477	38.01	0.62	46.37
	Y_3	Quadratic	0.9993	0.9988	0.9944	0.12	0.61	144.28
	Y_4	Quadratic	0.9994	0.9990	0.9944	0.036	0.86	138.77
	Y_5	Quadratic	0.8433	0.7314	-0.2419	0.12	6.73	8.81
BRZ	Y_6	Quadratic	0.9985	0.9974	0.9849	64.60	1.31	80.06
	Y_7	Quadratic	0.9968	0.9945	0.9697	126.52	1.17	63.30
	Y_8	Quadratic	0.9993	0.9988	0.9944	0.12	0.61	144.28
	Y_9	Quadratic	0.9959	0.9930	0.9584	0.31	2.91	62.85
	Y_{10}	Linear	0.0889	- 0.0933	-0.8719	0.047	4.03	2.35

Method optimization

The final mobile phase ratio optimized for the simultaneous determination of BRT and BRZ was done using Design Expert ® (Version 9.0.4, Trial version) after interpreting the various response surface plots. In the optimization step, the effect of two factors i.e. mobile phase composition and flow rate on the various chromatographic responses were evaluated. The desirability plot for both the drugs was generated by the software. In case of BRT, the desirability factors of mobile phase and flow rate were found to be 0.906 and 0.240; respectively (Figure 3f) whereas incase of BRZ, they were found to be 0.976 and 0.846; respectively (Figure 4f). As per desirability factors, different combinations of methanol and acetate buffer at suggested flow rate were tried and responses for both the drugs were evaluated. The optimized mobile phase selected was methanol: 0.01M ammonium acetate buffer (pH 3.8) (49.5: 50.5, v/v) at flow rate of 1.1 mL/min, which resulted in desired resolution and peak symmetry and require low solvent consumption.

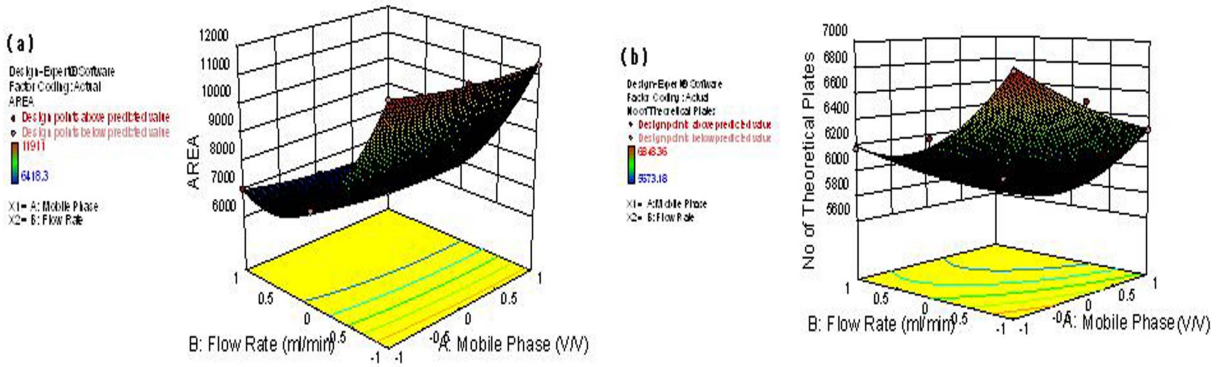

Figure 3. 3D surface plots of BRT for various chromatographic responses (a) area; (b) number of theoretical plates

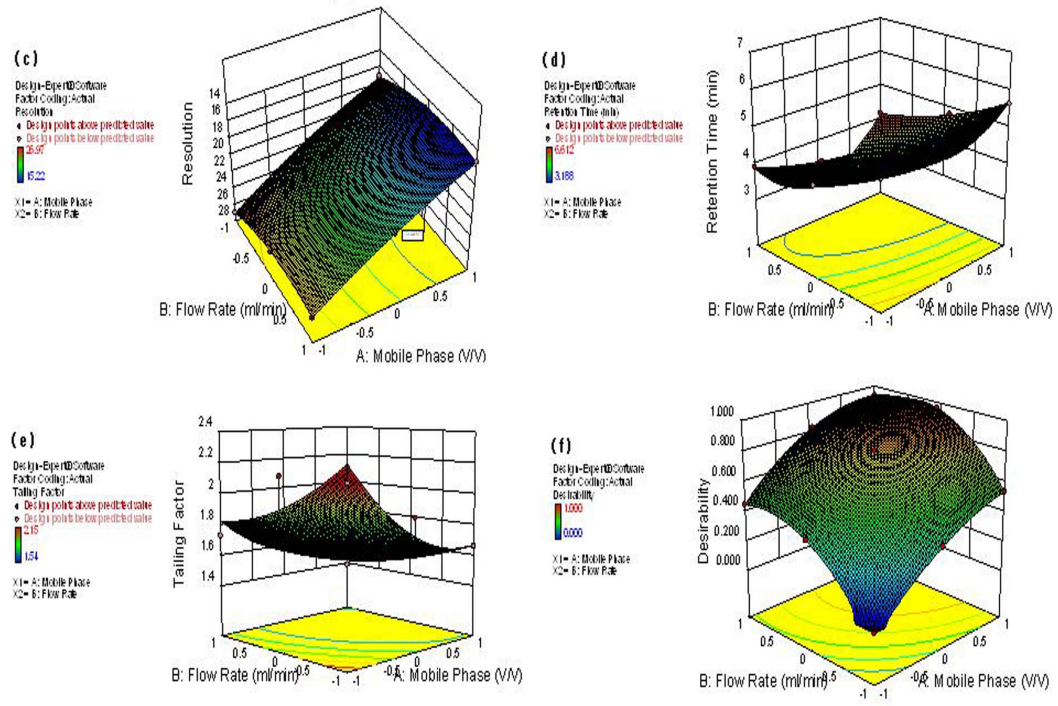

Figure 3. 3D surface plots of BRT for various chromatographic responses (c) resolution; (d) retention time; (e) desirability.

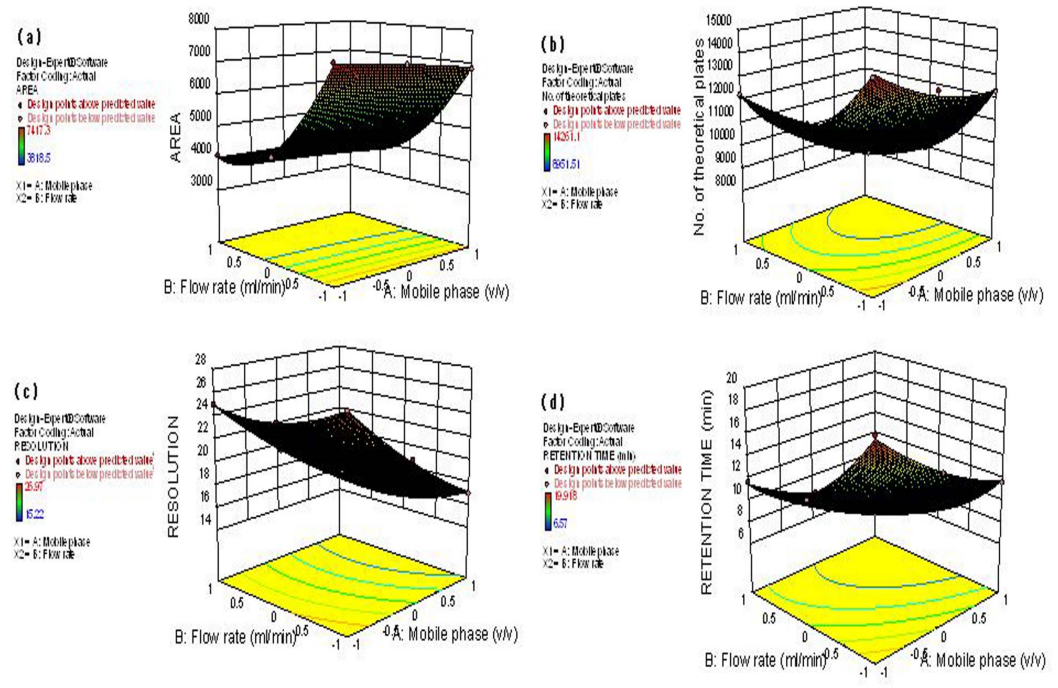

Figure 4. 3D surface plots of BRZ for various chromatographic responses (a) area; (b) number of theoretical plates; (c) resolution

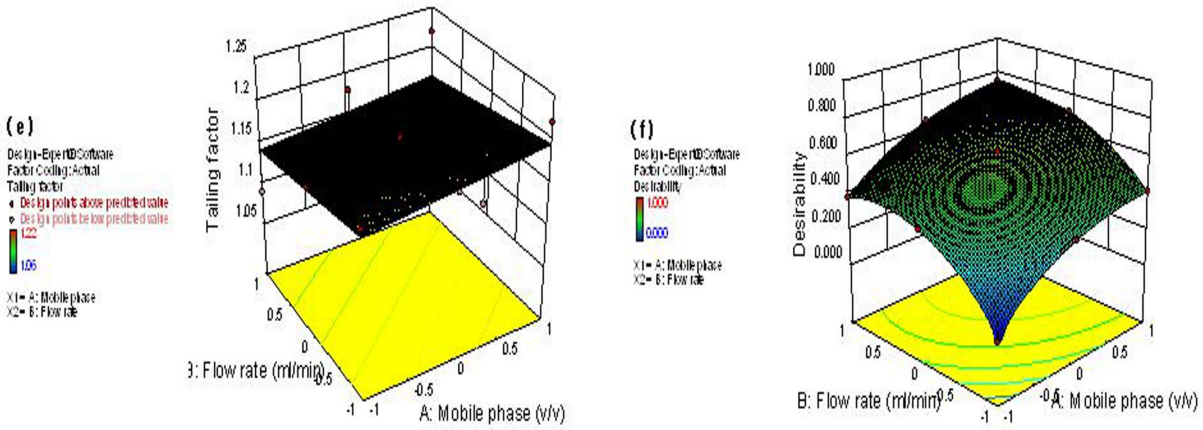

Figure 4. 3D surface plots of BRZ for various chromatographic responses (e) desirability.

Specificity

The proposed HPLC method was found to be specific as there was no interference found from the solvent, mobile phase or excipients present in the suspension (Figure 5 and 6)

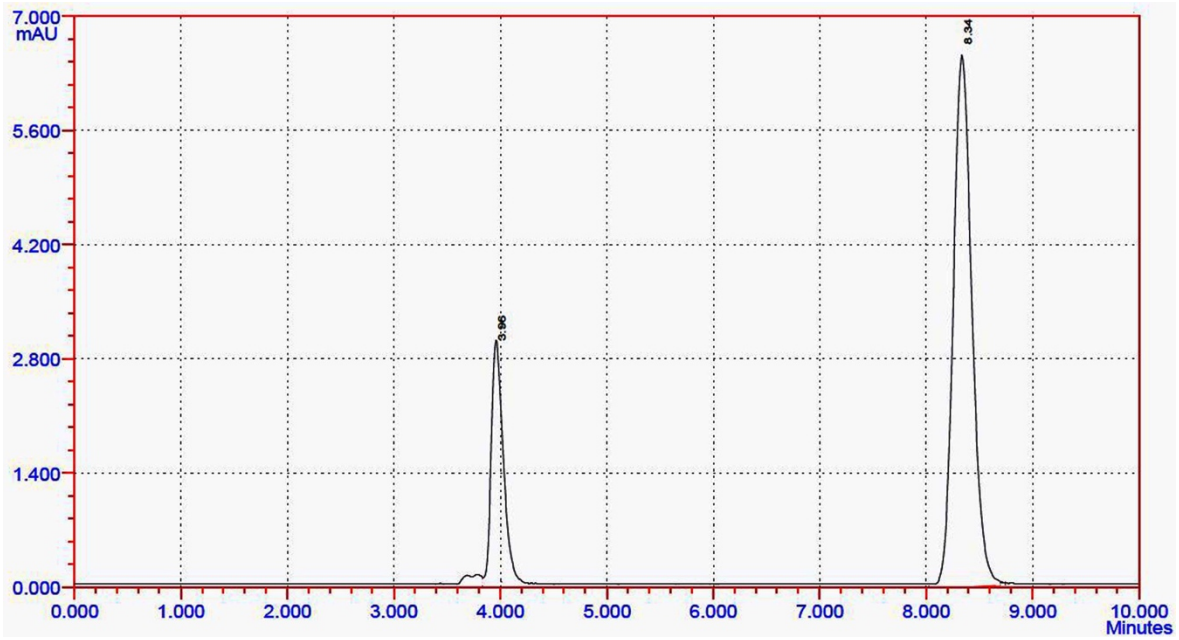

Figure 5. Representative chromatogram of standard BRT and BRZ using optimized mobile phase.

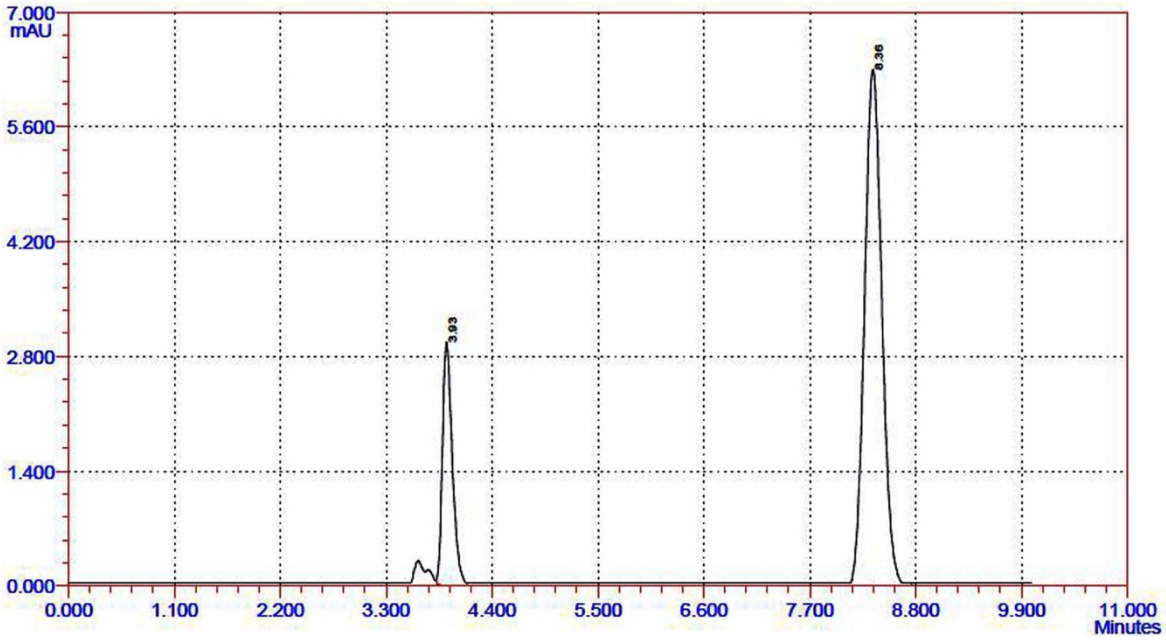

Figure 6. Representative chromatogram of BRT and BRZ in sample.

System suitability

The column efficiency as determined from the number of theoretical plates for both the drugs was found to be more than 4000; resolution was more than 14 and tailing for the same peak was found to be less than 2. Also the % RSD for all these parameters was found to be less than 2 %. System suitability analysis of both the drugs is represented in Table 6.

Table 6: System suitability parameters.

Parameters	BRT		BRZ	
	Mean± S.D[a]	%RSD[a]	Mean± S.D[a]	%RSD[a]
Number of theoretical plates	4906.63±69.84	1.42	8461.43±113.18	1.34
Resolution	14.57±0.17	1.15	14.57±0.17	1.15
Tailing factor	1.32±0.02	1.79	1.12±0.02	1.82

a = average of six determinations

Linearity and Range

The proposed method showed linearity over concentration range of 0.2 -1.4 µg/mL for BRT and 1 -7 µg/mL for BRZ with regression coefficients 0.9998 and 0.9993; respectively (Table 7). Statistically calculated F value for linearity regression was found to be F_{cal} 92271for BRT and F_{cal} 28594 for BRZ as compared to F_{Crit} 4.3807(DFn, DFd = 1.0, 19.0) indicating the statistical significance of method linearity.

Table 7. Regression analysis data for the proposed method

Parameters	BRT[b]	BRZ[b]
Wavelength (nm)	260	260
Linearity (µg mL^{-1})	0.2 -1.4	1 -7
Regression equation	Y= 5465x+ 82.97	Y= 4031x- 13.50
Slope	5465	4031
Intercept	82.97	13.50
Correlation coefficient (R^2)	0.9998	0.9993
LOD(µg mL^{-1})	0.0171	0.0296
LOQ(µg mL^{-1})	0.0518	0.0898

b = three determinations, LOD=Limit of detection, LOQ= Limit of quantification

Accuracy

The percentage recoveries of both the drugs were found to range between 99.04 – 101.67 % which are within the acceptance limit as shown in the Table 8.

Table 8: Accuracy of the proposed HPLC method.

% Level	Amount Present[c] (µg mL^{-1})		Amount recovered[c] (µg mL^{-1})		% Recovery ± S.D[c]	
	BRT	BRZ	BRT	BRZ	BRT	BRZ
50	0.41	1.98	0.41	1.96	100.80 ± 1.07	99.04± 0.30
100	0.80	4.00	0.80	3.99	100.67 ± 0.88	99.80± 0.28
150	1.21	5.96	1.23	5.95	101.67±0.19	100.02± 1.02

c = three determinations.

Precision

Intraday as well as intraday precision studies were carried out for evaluating the precision of the proposed method and the % RSD was found to be less than 2 at each level as represented in Table 9. Thus, the developed method was found to be precise.

Table 9: Intra-day and inter-day precision of the proposed HPLC method.

Drugs	Concentration (µg/mL)	Intraday Precision (% RSD)[d]		Interday Precision (% RSD)[d]	
		Day 1	Day 1	Day 2	Day 3
BRT	0.4	0.76	1.26	1.46	1.28
	0.8	0.38	0.28	0.65	0.70
	1.2	0.16	0.40	0.51	0.25
BRZ	2	0.12	0.39	0.65	0.42
	4	0.27	0.60	0.48	0.14
	6	0.83	0.88	0.48	0.19

d = three determinations

Robustness

In robustness study, % RSD was found to be less than 2 % in case of area of standard solutions and % content was found to be between 98-102 % (Table 10). Although the calculated F-value was higher than the critical F-value but the values of % RSD obtained for area, retention time and % w/w of drug were found to be less than 2.0. Hence, the developed method was robust.

Table 10: Evaluation of robustness for determination of BRT and BRZ.

Parameter	Area[e]		Average (% RSD) Retention time[e]		% Content (w/w)	
	BRT	BRZ	BRT	BRZ	BRT	BRZ
Flow rate (mL/min)						
1.0	4536.62(0.57)	16188.13(0.41)	4.07(0.65)	8.45(0.70)	100.83(0.25)	98.18(0.68)
1.1	4469.52(0.52)	15943.13(0.32)	4.00(0.26)	8.34(0.41)	99.15(0.52)	98.65(0.32)
1.2	4263.45(0.75)	15332.25(0.14)	3.73(0.62)	7.73(0.54)	101.22(0.14)	99.70(0.22)
Fcal/Fcri	44.67	127.58	117.45	114.63	2.29	8.96
Wavelength (nm)						
259	4543.83(0.81)	15861.75(0.31)	3.87(0.54)	8.13(0.49)	98.17(0.81)	98.50(0.46)
260	4469.52(0.52)	15943.13(0.32)	4.00(0.26)	8.34(0.41)	99.15(0.52)	98.65(0.32)
261	4357.82(1.40)	15884.48(0.55)	3.91(1.22)	8.39(1.62)	98.50(1.37)	98.15(0.78)
Fcal/Fcri	7.65	0.69	8.01	4.20	0.45	0.64
Mobile phase ratio (Methanol: Buffer, v/v)						
50:50	4466.98(0.94)	16044.40(0.35)	3.91(0.63)	8.14(0.36)	100.28(0.94)	98.99(0.14)
49.5:50.5	4469.52(0.52)	15943.13(0.32)	4.00(0.26)	8.34(0.41)	99.15(0.52)	98.65(0.32)
49:51	4456.77(0.69)	15935.43(0.17)	3.96(0.45)	8.87(0.46)	99.76(0.69)	99.23(0.31)
Fcal/Fcri	0.07	2.79	10.18	186.41	0.96	2.44
pH						
3.7	4431.27(0.98)	15908.47(0.31)	3.93(0.86)	8.11(0.86)	99.95(0.98)	98.88(0.36)
3.8	4469.52(0.52)	15943.13(0.32)	4.00(0.26)	8.34(0.41)	99.15(0.52)	98.65(0.32)
3.9	4455.45(0.75)	15737.18(0.23)	3.92(0.98)	8.81(0.69)	99.21(0.75)	98.95(0.11)
Fcal/Fcri	0.52	1.26	3.42	65.23	0.54	0.30

e = six determinations

Assay

The prepared in-house suspension was analyzed using the developed method. The content of BRT was found to be 99.17 % and that for BRZ was found to be 98.33 % (Table 11). Thus, the above developed method can be applied for the routine analysis of formulations containing BRT and BRZ.

Table 11: Results for analysis of in-house suspension.

Amount taken (µg mL^{-1})		Amount found (µg mL^{-1}) ^{g} ± S.D		% w/w ^{g}± S.D	
BRT	**BRZ**	**BRT**	**BRZ**	**BRT**	**BRZ**
0.8	4	0.81 ± 0.06	4.00 ± 0.01	99.17 ± 0.18	98.33 ± 0.34

g = Average of three determinations

CONCLUSION

A simple, rapid, sensitive, specific, accurate and precise RP-HPLC method has been developed for the first time and optimized utilizing QbD for the simultaneous determination of BRT and BRZ. The method is rapid as the run time is relatively short (10 min) within which the two drugs are well resolved. The main aim of implementing analytical QbD in method optimization was to identify the failures and the critical quality attributes so as to establish a design space such that there is no requirement of revalidation in case of any changes in method parameters. The QbD was applied in HPLC method development so as to verify robustness of the method. The developed HPLC method was suitable for routine quality control analysis.

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