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QbD based approach to RP-HPLC method development and validation of Bupivacaine hydrochloride in bulk and in-house developed nanostructured lipid carriers

Sarfaraz KHAN[1] *, Furquan KHAN[1]

[1] Department of Quality Assurance, Y. B. Chavan College of Pharmacy, Aurangabad, 431001, India.
* Corresponding Author. E-mail: sarfrazkhan3648@gmail.com (S.K.); Tel. +91-9923803455.

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ABSTRACT: The present study describes the principles of systematic Quality by design (QbD) approach for the development of RP-HPLC method for the quantification of Bupivacaine hydrochloride (BUP) in bulk and in-house developed nanostructured lipid carriers (NLCs). Initially analytical target profile (ATP), and critical analytical attributes (CAAs) were identified. Primary assessment studies were checked using Plackett-Burman Design. Further optimization studies were performed by applying Box-Behnken design. The Shimadzu C-18 column (250mm x 4.6mm i.d., 5µm particle size) was utilized for reversed-phase chromatographic separation with a mobile phase comprising a mixture of acetonitrile (ACN) and 0.1% ortho phosphoric acid (OPA) (pH 2.04) in 69.45:30.55 (% v/v). The flow rate was 0.805mL/min at a λ max of 214 nm and an injection volume of 12µL. The new developed method was validated according to the guidelines given by International Conference on Harmonization which revealed linearity between 25 to 80µg/ml and r^2 = 0.999. The result of % RSD was 0.38 and 0.44 respectively for high degree of intraday and interday precision. As per the new method the LOD and LOQ is 0.900µg/ml and 2.72µg/ml, respectively. Further the validated method was also applied for the estimation of BUP in NLCs formulation, which showed no interference of any formulation excipients. The studies demonstrated that the new method is rapid, simple, selective, and reproducible for the estimation of pure drug and in-house developed NLCs.

KEYWORDS: Bupivacaine hydrochloride; Quality by design; Nanostructured lipid carriers; HPLC, Validation.

1. INTRODUCTION

Bupivacaine is extremely used local anesthetic to treat the pain associated with surgical procedures or other severe and persistent pain [1]. Bupivacaine hydrochloride is chemically designated as 2- piperidine carboxamide, 1- butyl-N-(2,6- dimethylphenyl)-, monohydrochloride (Figure 1) [2]. Bupivacaine is from amide group and it works by inhibiting voltage gated Na^+ channels which results in inhibition of action potentials in nociceptive fibers and which blocks the progression of pain impulses [3].

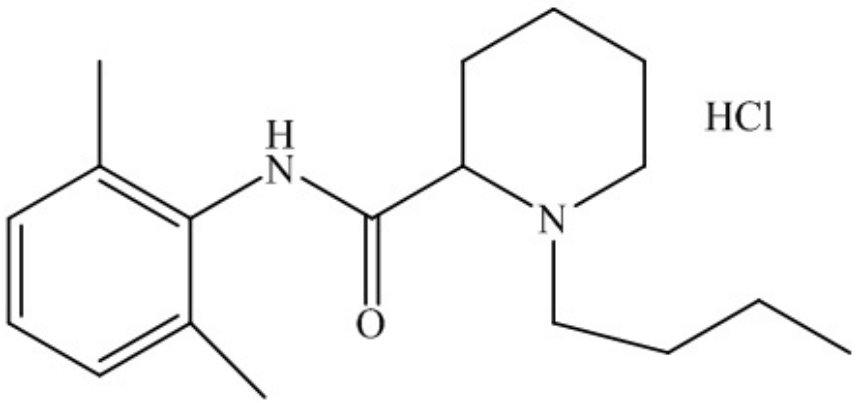

Figure 1. Structure of Bupivacaine Hydrochloride.

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Recent advancement in nanotechnology has developed many drug delivery systems one of them is NLCs. NLCs are fabricated by solid lipid, liquid lipid and surfactant. NLCs are the excess most encouraging drug delivery system as it increases the anesthetic effect. It has other advantages like enhancement of drug loading capacity, improved physical stability, protection against initial drug burst release and biocompatibility. NLCs can be synthesized by using different methodologies such as solvent emulsification/evaporation, micro emulsions, high pressure homogenization and solvent injection etc. [4-8].

Through literature survey it is found that there are many published methods for the determination of Bupivacaine levels in bulk drug, human plasma, urine and in mixtures using UV [9], HPLC [10-11], HPLC-UV, LC-MS [12-15], UPLC [16-17]. However, these previously reported methods have certain drawbacks like low sensitivity, low recovery, long retention time. Apart from this till date no analytical quality by design (AQbD) approach for systematic quantification of Bupivacaine in bulk is reported. In addition, this work will focus on developing validated method for the determination of Bupivacaine in NLCs by HPLC, as till date there is no AQbD developed method is available for the same.

Therefore, in present work we have concentrated on developing a new QbD based HPLC method that is rapid, simple, selective, and reproducible for pure drug and in-house developed NLCs.

In the recent years the application of AQbD in the analytical method development is essential to develop an accurate, precise and robust chromatographic methods for quantitative estimation of drugs and nano-formulations. By using design-of experiments (DoE) the association between input variables and parameters are obtained. The series of phases involved in this systematic approach is defining analytical target profile, critical analytical attributes, and screening of critical method parameters. Initial assessment was done by using Plackett-Burman design and further optimization was done by using Box-Behnken design.

2. RESULTS

2.1. Preliminary method development

Initially, different ratios of mobile phase compositions were attempted by utilizing water, acetonitrile, methanol, ortho phosphoric acid, with varying flow rate (ranging between 0.8 to 1.2mL/min). Taking into consideration the peak shape, suitable system parameters like retention time, number of theoretical plates, tailing factor etc., the mobile phase consists of ACN and 0.1% OPA (70:30) v/v) was establish to be most acceptable for analysis. Further the optimization of different parameters within the design space was achieved by using Plackett-Burman design.

2.2. Number of Theoretical plates, Tailing factor and Retention time

A number of theoretical plate counts show method performance and suitability, hence considered as CAA. It was concluded that ACN concentration and flow rate are significant model factors and pH of mobile of phase, detection wavelength, and injection volume are insignificant factors for number of theoretical plates (Figure 2 and table 1). Figure 3 depicts the normal and residual versus run plots which gives the information about model probability. Figure 4 (A) (3D counter plots) depicts the influence of mobile phase and flow rate on HETP which concludes proportionate relation with ACN concentration while inverse relation with mobile phase flow rate.

Tailing factor is regarded as CAA as method efficiency is dependent on it. Lower the peak tailing, highest the efficiency of analytical method. It was concluded that ACN concentration and pH of mobile phase are significant model factors and flow rate, detection wavelength, and injection volume are insignificant factors (Figure 2 and table 1). Figure 3 depicts the normal and residual versus run plots which gives the information about model probability. It was concluded from figure 4 (B) which depict the influence of mobile phase and pH on peak tailing (3D counter plots). Lower pH and higher ACN concentration give minimum tailing effect with maximum method efficiency.

For retention time it was concluded that ACN concentration, pH of mobile of phase and flow rate are significant model factors and detection wavelength, and injection volume are insignificant factors (Figure 2 and table 1). Figure 3 depicts the normal and residual versus run plots for the retention time. Figure 4 (C) which depict the influence of ACN concentration and flow rate on retention time (3D counter plots), which shows optimum concentration of ACN and flow rates for better retention.

Table 1. ANOVA Results for Plackett-Burman Design with P-value.

Theoretical plate		Tailing factor		Retention time	
Factors	P-values	Factors	P-values	Factors	P-values
A- ACN Conc.	**<0.0001**	A-ACN	**0.0022**	A-ACN	**0.0044**
B- Flow rate	**<0.0001**	C-Wavelength	0.2455	B- Flow rate	**<0.0001**
D-pH	0.0882	D-pH	**0.0199**	C- Wavelength	0.2347
G-G	0.0653	E-Injection Vol	0.2239	D-pH	**0.0237**
Adjusted R^2	0.9710	Adjusted R^2	0.7156	Adjusted R^2	0.9999
Predicted R^2	0.9457	Predicted R^2	0.5346	Predicted R^2	0.9994

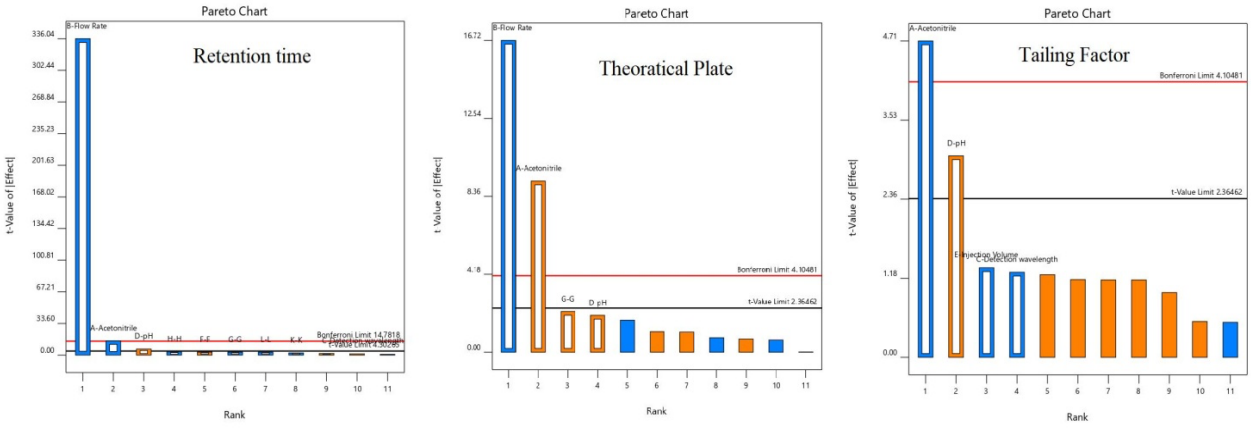

Figure 2. Pareto chart indicating the influence of various CAAs on retention time, number of theoretical plates and tailing factor.

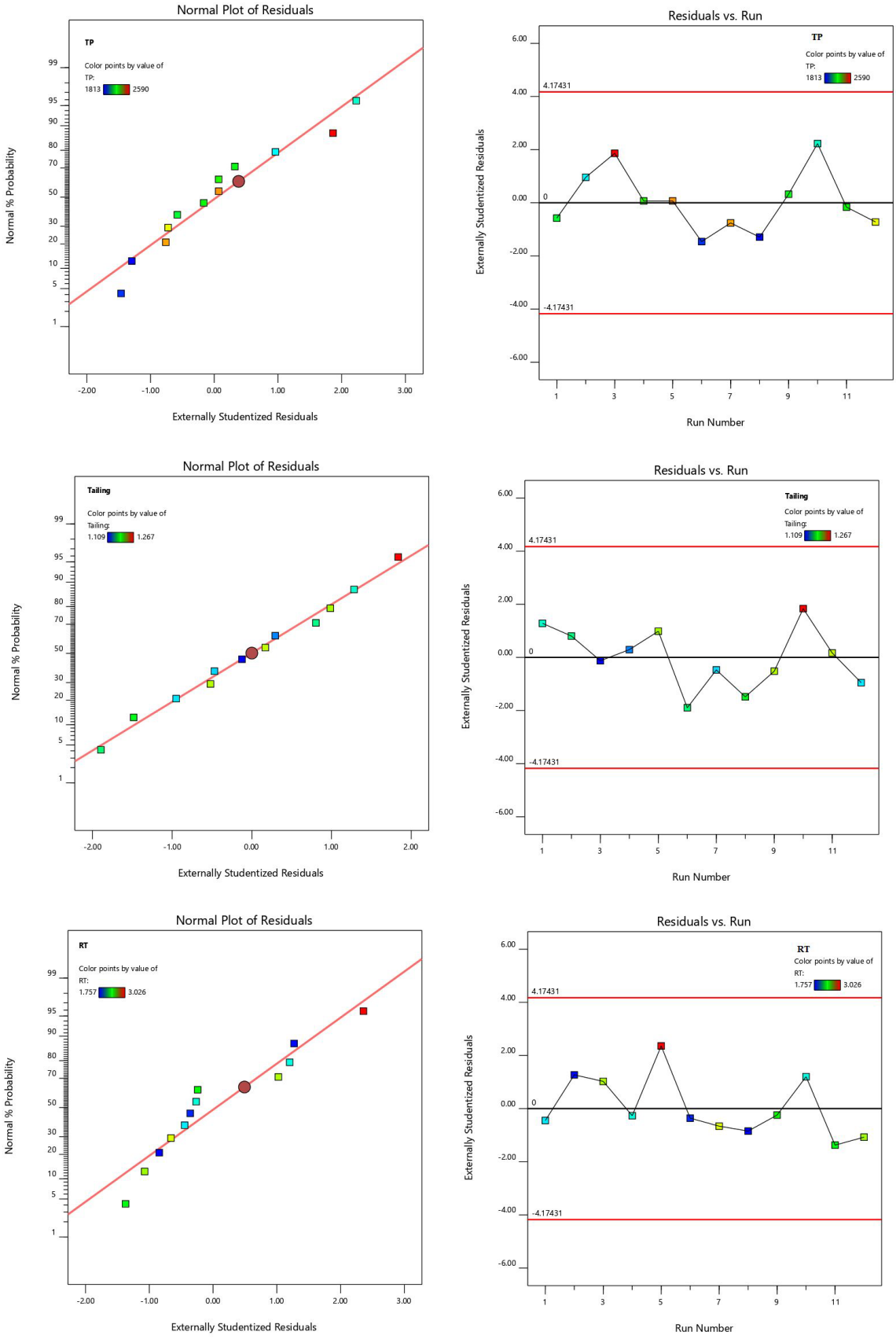

Figure 3. Normal plots of residuals and residuals vs run charts for number of theoretical plates, tailing factor and retention time.

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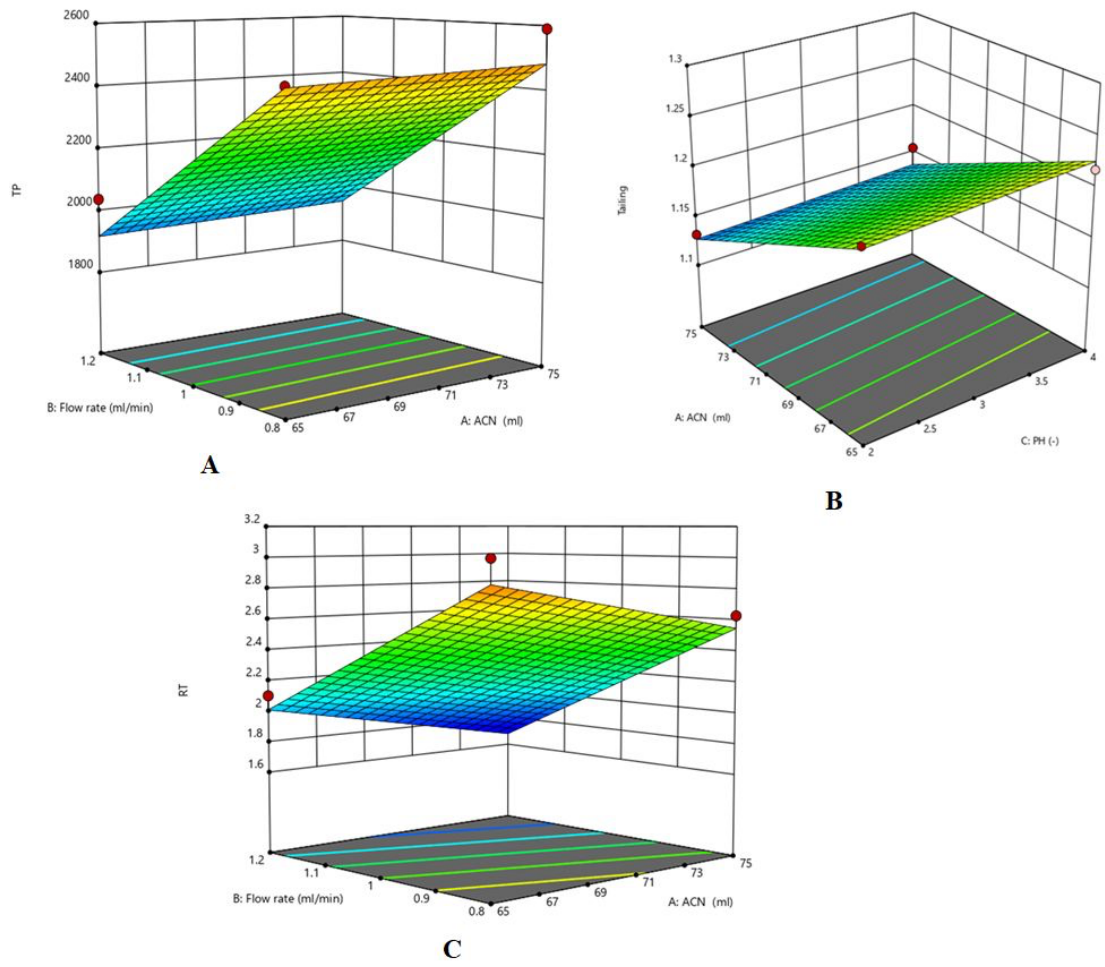

Figure 4. 3D response contour plot depicting the influence of CMPs i.e. ACN and flow rate on the number of theoretical plates (A). The influence of CMPs i.e. ACN and pH on the tailing factor (B). The influence of CMPs i.e. ACN and flow rate on the retention time (C).

2.3. Optimized method conditions

The numerical optimization was attempted to obtain optimum method conditions which helps to achieve desired goals i.e., peak tailing, optimum retention time, maximum theoretical plate count which yielded desirability value close to 1. The optimized method condition was observed at mobile phase ratio containing a mixture of ACN and 0.1% OPA (pH 2.04) in 69.45:30.55 (% v/v), the flow rate was 0.805mL/min and injection volume of 12µL. Box Behnken design were employed to evaluate prediction points of different factors and predicted mean of different responses. The flag plot and the optimum method parameters were shown in figure 5. Data is represented in table 2 and 3.

Table 2. Data of Predicted mean from Box Behnken design.

Factor	Name	Prediction points	Low Level	High Level
A	ACN	69.46	65.00	75.00
B	Flow rate	0.8050	0.8000	1.20
C	PH	2.05	2.00	4.00

Table 3. Data of Predicted mean from Box Behnken design and R^2 Values.

Response	Predicted mean	**Adjusted** R^2	**Predicted** R^2
Retention time	2.73794	0.9434	0.9074
Number of Theoretical plates	2494.63	0.8890	0.8183
Tailing Factors	1.1571	0.8314	0.7241

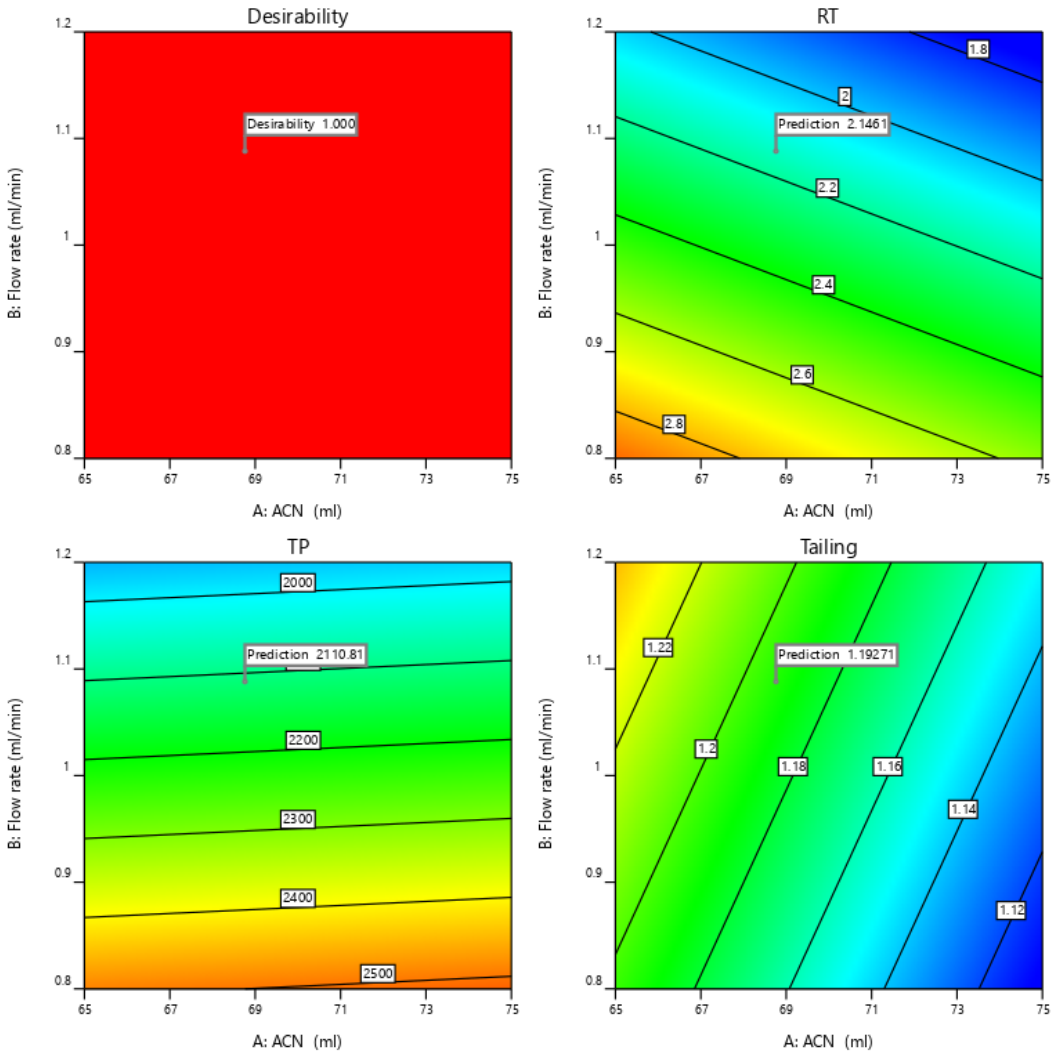

Figure 5. Plots indicating optimal analytical design space region.

2.4. Method development

2.4.1. Linearity and range

The standard calibration curve observed was linear above the concentration range of 25–80µg/mL with a high degree of coefficient of correlation (r^2 = 0.999). Figure 6 depicts the linear calibration plot of BUP with the regression equation shown as y = 37241x + 60776.

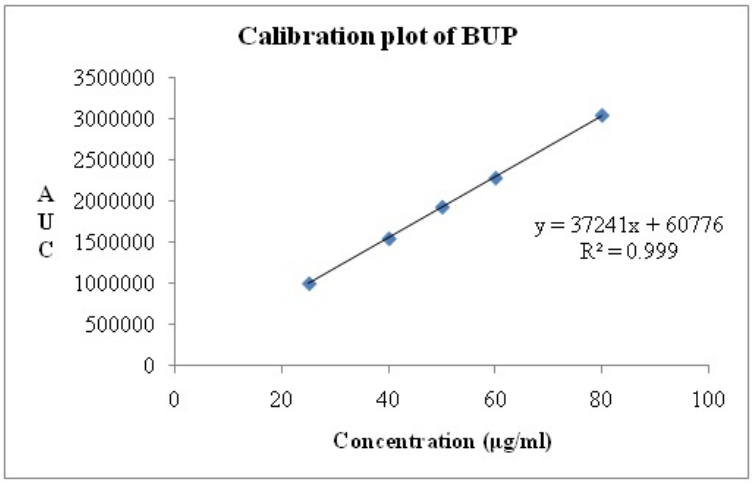

Figure 6. Calibration plot of BUP.

2.4.2. Accuracy

Accuracy data for the standard BUP (25µg/ml) concentration performed by recovery study at 3 levels, i.e., 80%, 100%, and 120% exhibit good recovery between 98–102% and a mean % RSD of less than 2. Table 4 illustrates % recovery data obtained by the proposed HPLC method.

Table 4. Accuracy data of different samples of BUP by developed HPLC method.

Drug Concentration (µg/ml)	Amount of Standard Added (µg/ml)	Amount Recovered (µg/ml) ± S.D.	% Recovery	% RSD
25	20	44.65 ±0.251	99.22	0.5642
25	25	49.84±0.146	99.68	0.2948
25	30	55.48±0.224	100.8	0.4041

2.4.3. Precision

As mentioned in table 5 the inter-day and intra-day precision data for different samples of BUP was in the range of 99.22 and 100.4% which is within the acceptable limits (% RSD < 2%). These results corroborate higher degree of precision of the developed method.

Table 5. Precision studies data of BUP.

Precision Studies	Concentration (µg/ml)	Amount recovered (µg/ml)	Average weight recovered ± S.D.	% RSD	% recovery
Interday	30	30.19 29.78 29.83	29.93 ± 0.18	0.6101	99.76
	40	39.87 39.74 39.47	39.69 ±0.16	0.4197	99.22
	50	50.07 49.86 49.68	49.68 ±0.15	0.3195	99.36
Intraday	30	30.12 29.92 30.33	30.12 ±0.16	0.5556	100.4
	40	39.78 39.97 39.69	39.81 ±0.11	0.2931	99.52
	50	50.04 49.68 49.86	49.86 ±0.14	0.2947	99.72

2.4.4. LOD and LOQ

The sensitivity of the developed method was determined in terms of LOD (0.900µg/ml) and LOQ (2.72µg/ml) which ratify high sensitivity of developed method for quantification of BUP.

2.4.5. Robustness

The robustness studies indicated that after minute alteration in each parameter, like the concentration of ACN, flow rate and volume of injection, showed lack of any noticeable variations in tailing factor, number of theoretical plates, and retention time. The results are represented in table 6. The values of % RSD were within the acceptable specifications (< 2%). These results signify that the new method is highly robust.

2.4.6. System suitability

The system suitability results confirmed lack of significant difference in the peak area and number of theoretical plates after six replicate injections. The values of % RSD for peak area and number of theoretical plates were found to be 0.324 and 0.663 respectively.

2.4.7. Analysis of in-house-developed nanostructured lipid carriers

The developed analytical RP-HPLC method was employed for estimation of in-house developed NLCs. The percent recovery achieved was 99.51%. In the chromatogram (Figure 7) insignificant change in the time of drug retention was observed. The absence of additional peaks in the chromatogram ratifying that there is no interference of the polymers used in formulation.

3. DISCUSSION

In the current work the analytical quality-by-design RP-HPLC method were established for the analysis of BUP in bulk and in-house-developed NLCs (Figure 7). Initially ATPs of the chromatographic method were identified which helps in the recognition of CAAs, namely, a theoretical plate count number, tailing factor and retention time. Five independent chromatographic factors were selected and assessed by using Plackett Burman design to observe their main interference and interactions on the identified CAAs. The three factors namely the mobile phase composition, flow rate and pH of mobile phase solution were found to be highly influential variables (p-value < 0.05) on CAAs. Further Box Behnken design was applied to obtain optimized chromatographic conditions by using response surface optimization methodology. The quality by design approach successfully developed the method for determination of BUP by HPLC and validated as per ICH guidelines Q2 (R1). The developed analytical method of BUP exhibited high degree of precision and sensitivity as the % RSD values for intraday and interday precision was found to be less than 2% and LOD and LOQ values were 0.900µg/ml and 2.72µg/ml, respectively. Hence, the optimized method of analysis can be effectively used for the routine estimation of BUP in bulk and in house developed NLCs.

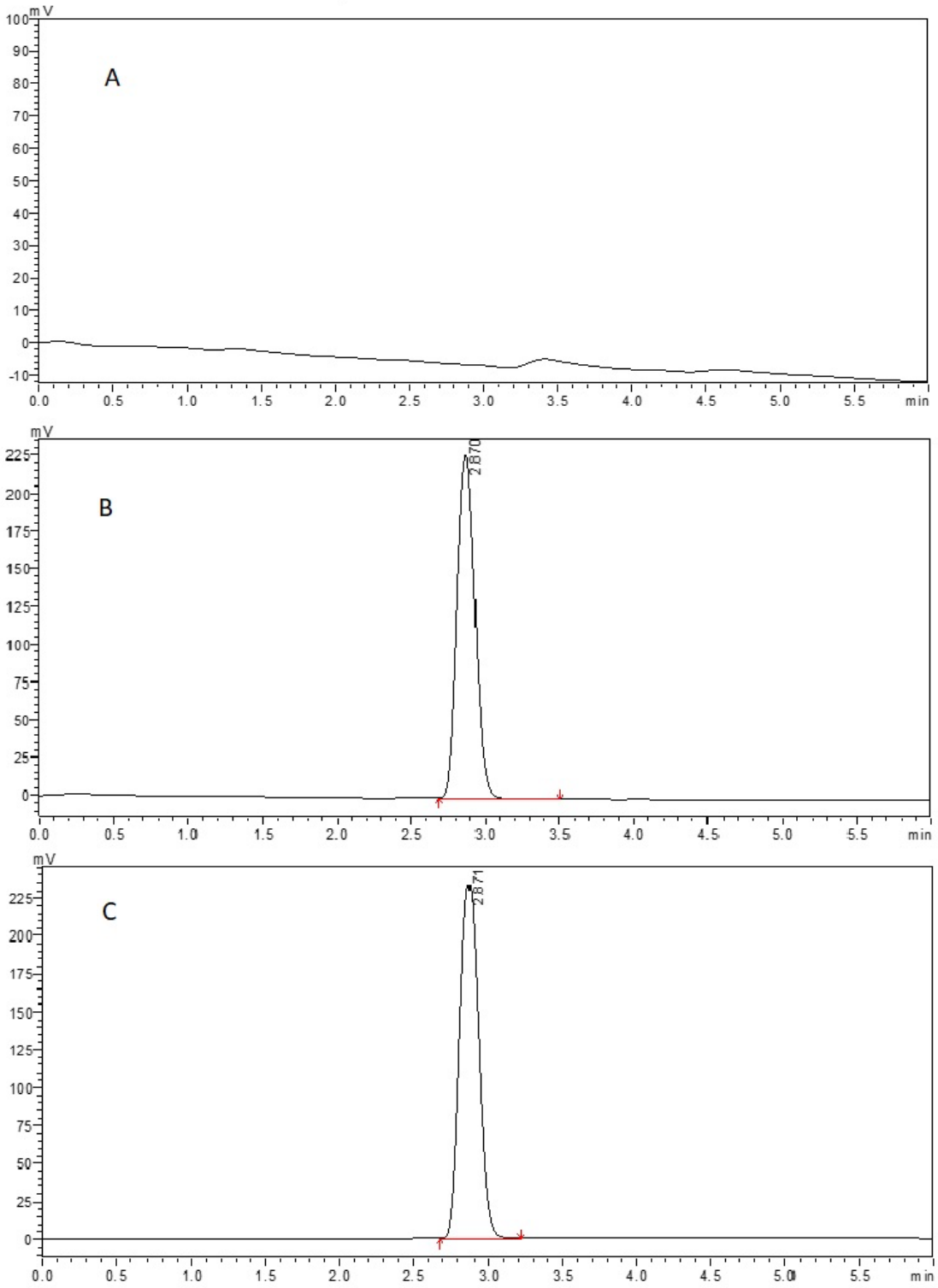

Figure 7. (A) Chromatogram of Blank (B) Chromatogram of BUP at Optimized conditions with Rt 2.870 min, (C) Chromatogram of BUP in in-house developed NLCs with Rt 2.871 min.

4. CONCLUSION

The validated method of analysis is a unique, simple, accurate, precise, robust and economical analytical RP-HPLC method for the determination of BUP. All the validated parameters of developed AQbD RP-HPLC method were observed within the given specifications as shown in Table 6. The developed and validated method is found acceptable for routine analysis of BUP in bulk and in-house developed NLCs. The absence of additional peaks in the chromatogram ratifying that there is no interference of the common polymers used in

formulation. The excellent linearity, accuracy and enhanced precision can be useful further in pharma industry in quality control department for routine analysis.

Table 6. Results of validated parameters of BUP.

Parameters	Values		
Absorbance maxima (nm)	214		
Mobile phase (Isocratic mode)	Acetonitrile: 0.1% OPA pH 2.04 (69.45 : 30.55)		
Linearity	$R^2 = 0.999$		
Regression equation	y = 37241x + 60776		
Range (µg/ml)	25-80		
LOD (µg/ml)	0.900		
LOQ (µg/ml)	2.72		
Retention time (min)	2.870		
Flow rate (mL/min)	0.805		
Robustness (n=3)			
Factors	Retention time (Min)	Tailing factor	Number of theoretical plates
Flow rate (ml/min)			
0.705	2.869	1.144	2462
0.805	2.870	1.096	2452
0.905	2.876	1.121	2458
Volume of Injection (µl)			
11	2.871	1.114	2474
12	2.870	1.096	2452
13	2.870	1.102	2468
Acetonitrile Concentration (ml)			
67.45	2.870	1.108	2448
69.45	2.870	1.096	2452
71.45	2.871	1.124	2458

5. MATERIALS AND METHODS

5.1. Chemical and reagents

Bupivacaine Hydrochloride (BUP) was provided as gift sample from Harman Finochem limited, Aurangabad, India. Acetonitrile and Ortho Phosphoric acid of HPLC grade (Merck, Mumbai, India). All other reagents and solvents utilized were of analytical grade and HPLC grade.

5.2. Instrumentation

Analytical method development was performed on Shimadzu comprising of Autosampler (SIL-20AC HT), quaternary gradient pump (LC-20 AD), an UV-Visible wavelength detector (SPD-20 A) connected to software Lab solutions (software Version DB 6.110).

5.3. Chromatographic condition

The Shimadzu C-18 column (250mm x 4.6mm i.d., 5µm particle size) was used for reversed-phase chromatographic separation with a mobile phase solution containing a mixture of ACN plus 0.1% OPA (pH

2.04) in 69.45:30.55 (% v/v). The rate of flow was 0.805mL/min at a λ max of 214 nm and an injection volume of 12µL. The mobile phase solution was filtered through 0.45µ membrane filter and degassed by ultrasonication for 10 mins before use. The temperature of column was kept at 25°C.

5.4. Preparation of stock solution (Primary standard)

The stock solution (Primary standard) BUP (i.e. 1,000µg/mL) was produced by accurately dissolving 100mg of BUP in 100ml of mobile phase. This primary standard solution was further diluted to obtain various working standards ranging from 25µg/mL to 80µg/mL and filtered through a syringe filter (0.22-µm) before subjecting to chromatographic analysis.

5.5. Preparation of solution of in-house developed NLCs

A solution of NLCs containing BUP were diluted by using distilled water and centrifuge at 5000 RPM for 15 min. From supernatant BUP was extracted by using methanol and analyzed after filtering through 0.22 µm Millipore filter [18].

5.6. Defining ATP and CAAs

ATP is defined to prepare an optimum analytical method which is a central approach to the application of AQbD. All the parameters that affect performance and quality of the method are identified as ATP. The method parameters that are directly affecting the ATP are identified as CAA. To get favorable analytical separation, the peak tailing, theoretical plate count and retention time were identified as CAA for proposed HPLC Method [19].

5.7. Plackett-Burman design

After defining the ATP and CAAs, a Plackett-Burman design at two levels was applied to obtain the Critical Method Parameter (CMPs). The Qbd was initiated by drawing the Ishikawa fishbone diagram (Figure 8). From Ishikawa fishbone diagram five independent chromatographic factors were selected and assessed. In initial experimental design 12 runs were carried out. Table 7 displays the information of experimental runs for selected factors with their screening levels. The effect of shortlisted factors on CAAs was studied by assessing the design. A linear polynomial model was adopted by fitting the linear model terms and obviating the interaction term(s). The Pareto charts and Half-normal plots were employed for the identification of most influential factors [20].

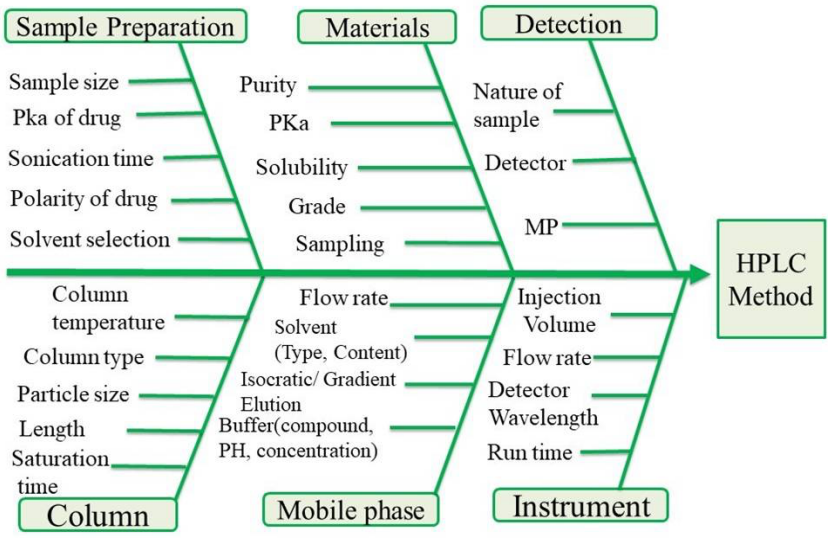

Figure 8. Ishikawa Fish bone diagram.

Table 7. Plackett-Burman Design matrix for analysis of method variables and process parameters.

Run	ACN Con. (ml)	Flow rate ml/min	Wavelength (nm)	pH	Injection volume (µl)	F6	F7	F8	F9	F10	F11
1	65	1.2	220	4	8	-1	-1	1	-1	1	1
2	75	1.2	210	2	8	1	-1	1	1	-1	1
3	65	0.8	210	2	8	-1	-1	-1	-1	-1	-1
4	75	1.2	220	2	8	-1	1	-1	1	1	-1
5	65	1.2	220	2	12	1	1	-1	-1	-1	1
6	75	1.2	210	4	12	1	-1	-1	-1	1	-1
7	75	0.8	220	4	12	-1	-1	-1	1	-1	1
8	75	0.8	210	2	12	-1	1	1	-1	1	1
9	75	0.8	220	4	8	1	1	1	-1	-1	-1
10	65	0.8	220	2	12	1	-1	1	1	1	-1
11	65	0.8	210	4	8	1	1	-1	1	1	1
12	65	1.2	210	4	12	-1	1	1	1	-1	-1

5.8. Method development by applying experimental design

Using factor screening studies, factors *viz.*, mobile phase ratio, flow rate and pH of mobile phase were selected as the influential CMPs. The Box-Behnken design (BBD) were employed for systematically optimizing CMPs at three different levels i.e., low (-1), medium (0), and high (+1) [21-22]. Table 8 shows the design matrix according to BBD with a total of 12 experimental runs including quintuplicate trials at the centre point (0, 0) runs. Sequences of the experimental run were assessed for method CAAs, i.e. peak tailing, number of theoretical plate count and retention time.

Table 8. Design matrix as per Box-Behnken Design for optimization of parameters for analysis of BUP.

Run	ACN Conc. (ml)	Flow rate (ml/min)	pH	Retention time (Min.)	Tailing factor	Number of theoretical plates
1	75	1	4	2.057	1.155	2138
2	75	1.2	3	1.775	1.165	2006
3	75	0.8	3	2.628	1.109	2590
4	75	1	2	2.110	1.132	2252
5	65	0.8	3	3.026	1.216	2462
6	70	1.2	2	1.833	1.168	1874
7	70	0.8	2	2.678	1.144	2453
8	70	1.2	4	1.757	1.181	1813
9	65	1	4	2.404	1.218	2175
10	65	1.2	3	2.101	1.267	2037
11	65	1	2	2.436	1.220	2210
12	70	0.8	4	2.610	1.146	2386

5.9. Optimization of data analysis and validating model

Data analysis optimization was performed by employing Placket burman as a primary while Box Behnkan as a secondary screening model using Design Expert® ver. 12.0.3.0 32 bit software (M/s Stat-Ease Inc., MN 55413, USA). The said models were utilized to estimate various studied responses, together with the inspected variables through series of experiments. The dependent variables were identified by defining polynomial equation with statistical significance (P-value) less than 0.05 to confirm various parameters like the regression coefficient (R_2), predicted error sum of squares (PRESS), and lack of fit analysis. In secondary screening, 3D- surface responses were used to explain the relationship, possible interactions, and effects between various CMPs, if any. The numerical optimization and desirability functions along with graphical optimization helps to evaluate the optimum chromatographic conditions and efficient method performance by recognizing the analytical design space with location of various CAAs consistent with the desired acceptance criteria (R_t and Peak tailing should be minimum with maximum AUC and HETP (height equivalent to theoretical plate).

5.10. Analytical method validation

The developed optimized HPLC analytical method was validated according to the guidelines recommended by ICH Q2 (R1) [23]. All the validation studies were performed in triplicate (n=3).

5.10.1. Linearity

Linearity of BUP was investigated by analyzing a series of dilutions in concentration ranging from 25–80µg/mL. The calibration curve was obtained by plotting peak area and concentration (Figure 6), using regression line equation.

5.10.2. Accuracy

The methods accuracy was evaluated by calculating percent recovery from 25µg/mL standard solution spiked with 80% (20µg/mL), 100% (25µg/mL), and 120% (30µg/mL) of additional amount of BUP. The accuracy data is examined by calculating SD and % RSD (Table 4).

5.10.3. Precision

The intra-day and inter-day precision of the new set method was analyzed by using three different concentrations of BUP (i.e., 30, 40 and 50µg/mL) at different time intervals on the same day and by repeating the same on the next day. The data were evaluated by calculating Mean percent recovery, SD, and % RSD (Table 5).

5.10.4. Limit of detection and limit of quantitation

According to ICH guidelines, LOD i.e. the lowest amount of drug concentration which can be identified and LOQ is the lowest amount of drug concentration which can be quantified. The LOD and LOQ were measured by using following formulae:

$LOD = 3.6\sigma/S$

$LOQ = 10\sigma/S$

where σ represents standard deviation, S represents the slope.

5.10.5. Robustness

The robustness was assessed by evaluating the method after minor modifications in the operating conditions such as volume of ACN (±2% v/v), injection volume (± 50%), flow rate (± 10%). The results of robustness are presented as by mean percent recovery and % RSD. The acceptable limit for estimated % RSD of peak area was less than 2.

5.10.6. System suitability

System suitability was assessed by performing analysis of six replicates of standard solution of BUP subsequently by calculation of SD and % RSD for the retention time and peak area.

5.10.7. Application of the developed new analytical method in pharmaceutical formulation

The in-house-developed NLCs formulation was analyzed by utilizing new developed as well as validated analytical method for determination of BUP. The NLCs were produced by minor modification in previously described method that is Lipid melt-emulsification technique combined with solvent injection technique [24]. Further, BUP was extracted by using methanol to prepare the working concentration of 50µg/ml after proper dilution. The solution was then subjected for analysis of the drug after filtering through 0.22µm Millipore filter paper.

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