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Development and validation of a rapid ultra-high performance liquid chromatography method for the assay of benzalkonium chloride using a quality-by-design approach

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ABSTRACT

A rapid robust reversed-phase UHPLC method has been developed for the analysis of total benzalkonium chloride in preserved drug formulation. A systematic Quality-by-Design (QbD) method development approach using commercial, off the shelf software (Fusion AE®) has been used to optimize the column, mobile phases, gradient time, and other HPLC conditions. Total benzalkonium chloride analysis involves simple sample preparation. The method uses gradient elution from an ACE Excel 2 C18-AR column (50 mm × 2.1 mm, 2.0 μm particle size), ammonium phosphate buffer (pH 3.3; 10 mM) as aqueous mobile phase and methanol/acetonitrile (85/15, v/v) as the organic mobile phase with UV detection at 214 nm. Using these conditions, major homologs of the benzalkonium chloride (C12 and C14) have been separated in less than 2.0 min. The validation results confirmed that the method is precise, accurate and linear at concentrations ranging from 0.025 mg/mL to 0.075 mg/mL for total benzalkonium chloride. The recoveries ranged from 99% to 103% at concentrations from 0.025 mg/mL to 0.075 mg/mL for total benzalkonium chloride. The validation results also confirmed the robustness of the method as predicted by Fusion AE®.

1. Introduction

Benzalkonium chloride (BAK), a quaternary ammonium excipient, is a mixture of alkylbenzyldimethylammonium chloride with the formula $[C_6H_5CH_2N(CH_3)_2R]Cl$, where R is an alkyl group varying from C_8H_{17} to $C_{18}H_{37}$ [1] (Fig. 1). It has three main application purposes: as a biocide, a cationic surfactant, and a phase transfer agent in the chemical industry. Its applications are extremely wide, ranging from disinfectant in formulations to microbial prevention in the oilfield service industry. In preserved drug formulation, BAK is commonly used as a preservative [2–6], and it has been reported that homologs C12 and C14 are the most common components and together comprise at least 70% on the BAK [7]. Per ICH and FDA guidelines, preservatives in pharmaceutical formulations should be quantitated during stability studies and release of the product. BAK should also be analyzed in both developmental and commercial formulations during stability studies and product release. Due to its vast use a fast method is necessary to save analysis time. In addition, due to the extensive use of BAK methods particularly in pharmaceutical industry, often such analysis methods are run in multiple laboratories requiring a robust method that will produce consistent results among different labs.

Chromatographic method development can be a time consuming and subjective process. In today's fast paced and highly competitive industrial settings, fast and robust HPLC method development becomes increasingly important. Most method development is done using a manual, one-factor-at-a-time (OFAT) process where the approach is to vary one system parameter at a time and examine the resultant performance. This procedure may be continued until no further improvement is obtained, at which time another parameter is selected for study. This process can be improved by applying a Quality-by-Design (QbD) strategy that develops analytical HPLC methods [8–10] to meet performance requirements using sound statistical experimentation principles. Commercial, off the shelf software like Fusion AE® automates LC method development according to rigorous Quality-by-Design (QbD) concepts. To our knowledge, QbD concepts which are described in ICH Q8, FDA/EMA guidance and by other authors [11–15] have not been applied in the development of any BAK method. In addition, very few articles were found in the literature [16,17] using a QbD method development tool probably due to the fact that such tools/capabilities are relatively new. Efficient

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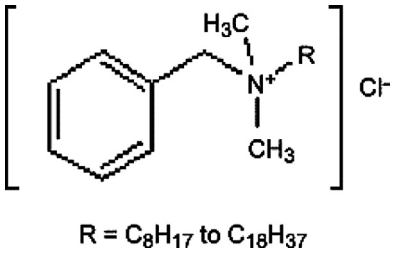


Fig. 1. Representation of BAK.

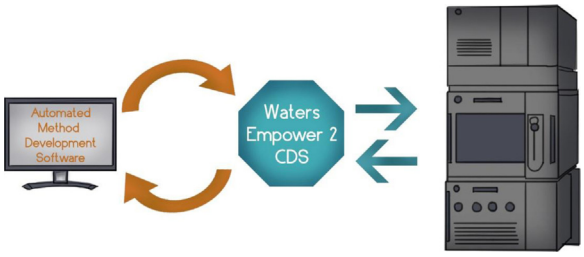


Fig. 2. Fusion AE® exports designed methods and sequences directly to Empower 2 and imports results data for analysis and modeling.

experimental protocol design, execution, and various optimizations used in this study would provide sound scientific foundation for the method development. In addition, the approach used for method development in this study ensures reliability by calculating and confirming LC method robustness prior to validation.

UHPLC is quickly becoming popular in the pharmaceutical industry due to the advantages of having high peak efficiency and shorter runtime without compromising separation. Several HPLC [7,19–22] and electrophoretic [23,24] methods have been developed for BAK determination in preserved drug formulation. However, these methods typically have long runtimes, and most of them use a higher wavelength in the range of 240–270 nm, which typically results in low detection sensitivity. Some methods, including USP method, use reversed-phase CN column, which in our experience have reproducibility and column stability issues [25–27]. The most commonly used USP method has long runtime (>30 min) and a low detection sensitivity (4 mg/mL). To our knowledge, the best method reported in literature [21] has runtime of 7 min with good sensitivity (0.07 mg/mL). Unlike the USP method which uses a Cyano bases reverse phase column, this new improved method used specially modified octadecyl (C18) bonded silica phase.

The objectives of this work are to: (1) develop a simple, ultrafast (≤2.0 min runtime) and robust reversed-phase UHPLC method using QbD approach for routine determination of the total BAK content, and (2) validate the method for a typical preserved drug formulation.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade methanol, acetonitrile, o-phosphoric acid and ACS grade ammonium hydroxide (28.0–30.0%) were purchased from Burdick & Jackson (Morristown, NJ). Nanopure water available in the laboratory was filtered using a Milli-Q water filtration system. BAK was purchased from FeF Chemicals (sum of C12 and C14 homologs is ≥99%).

2.2. Equipment

The UHPLC system used for method development and method validation was the Waters UPLC H-Class (manufactured by Waters, Milford, MI) UPLC system with a UV and a PDA detector. The signal was monitored and processed using Empower 2 software (Waters, Milford, MA). Method development experiments were designed using Fusion AE method development software from S-Matrix (Eureka, CA).

2.3. Method

Fusion AE® method development software was used to develop a QbD method (see Fig. 2). Fusion AE® is an automated experimentation software platform. It contains five integrated activities (work environment): design of experiments, data entry/analysis, best answer searches, visualization graphics, and reporting toolkit.

Columns with different selectivity were used to find the best columns for the separation using Fusion AE® experiment design. Aqueous mobile phases at different pH and different organic mobile phases were tested per Fusion AE® systematic design to find the optimal mobile phase conditions. Gradient was also optimized using Fusion AE® to obtain best and most robust separation with shortest run time.

In the final method, the selected chromatographic column was ACE C18-AR (50 mm × 2.1 mm, 2.0 μm particle size). The mobile phase consisted of ammonium phosphate (pH 3.3; 10 mM) as aqueous mobile phase and methanol/acetonitrile (85/15, v/v) as the organic mobile phase. The flow rate of the mobile phase was kept constant at 1.0 mL/min. The column temperature was maintained at 45 °C and the optimum wavelength was monitored at 214 nm. The injection volume was 3 μL.

2.4. Preparation of standard and sample solutions

A working standard solution of 0.05 mg/mL of BAK in water/methanol (50/50, v/v) was prepared for assay. Preserved drug formulation containing 200 μg/g BAK was diluted to 50 μg/g BAK and injected during sample analyses and validation.

Table 1
Initial screening of column using 10 mM ammonium phosphate as mobile phase A and methanol or acetonitrile as mobile phase B.

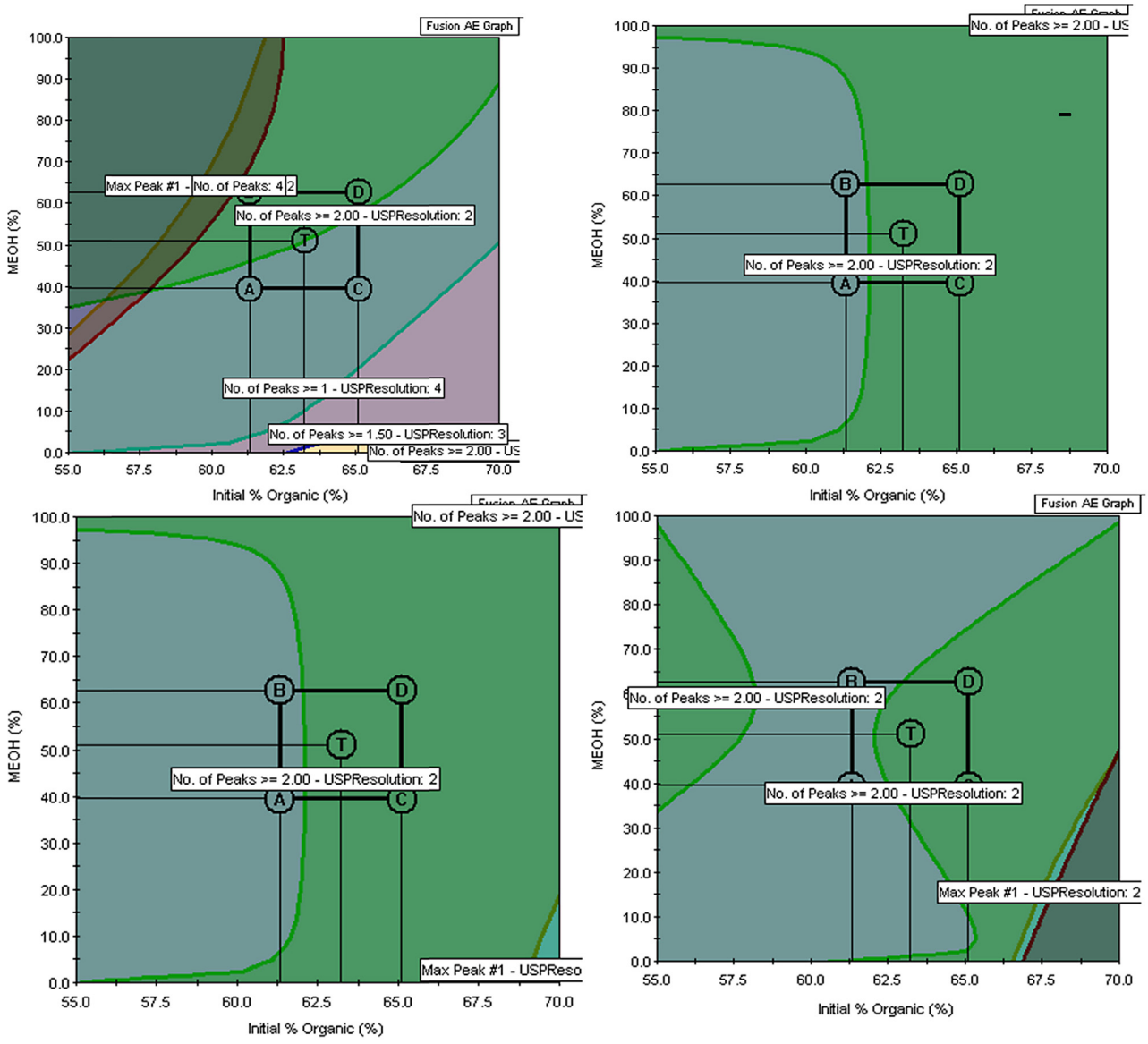
Column	Mobile phase B = ACN		Mobile phase B = methanol	
2.1 mm × 50 mm	Resolution, unknown 1-C12	USP tailing of C12 peak	Resolution, unknown 1-C12	USP tailing C12
Zorbax Eclipse plus C18, 1.8 μm	1.3	1.6	1.87	1.60
Zorbax SB-Aq, 1.8 μm	Not resolved	NA	2.30	2.7
Acquity BEH-C18, 1.7 μm	1.76	2.4	3.2	2.6
Acquity HSS-C18, 1.7 μm	Not resolved	NA	Not resolved	NA
Acquity BEH-Shield, 1.7 μm	3.0	2.3	3.0	2.1
Zorbax SB-Phenyl, 1.8 μm	Not resolved	NA	Not resolved	NA
ACE C18-AR, 2 μm	2.0	2.7	4.0	2.0
ACE-C18-PFP, 2 μm	1.8	1.8	3.4	1.7
ACE-C18, 2 μm	2.4	2.2	4.4	1.9
ACE-phenyl, 2 μm	2.0	2.5	Not resolved	NA

2.5. Method validation

Method was validated on the following criteria: specificity, linearity, precision, robustness and accuracy based on ICH guidelines (ICH Q2 (R1)).

2.5.1. Specificity

Specificity is the ability to unequivocally assess the analyte in the presence of other potential components, typically including impurities, degradants, matrix, etc. In order to determine the specificity of the method, the following solutions were injected: diluent,



Name	Lower bound	color
No. of Peaks ≥ 1.50 - USPResolution	3.0	Violet
No. of Peaks ≥ 2.00 - USPResolution	2.0	Green
No. of Peaks ≥ 1.00 - USPResolution	4.0	Orange
Max Peak #1 - USPResolution	2.0	Teal

Fig. 3. Acceptable performance region using different columns, various mobile phase B composition and different starting mobile phase conditions. The lack of any unshaded region indicates that all the conditions shown in the table above were not met under any method conditions tested.

drug formulation containing active drug without BAK, standard solution of BAK and sample solution prepared from a preserved drug formulation.

2.5.2. Linearity

In order to verify the linearity of the detector, a minimum of five concentration levels equivalent to 0.025, 0.0375, 0.05, 0.0625 and 0.075 mg/mL of total BAK, corresponding to 50–150% of BAK in the standard solution, were prepared and injected.

2.5.3. Accuracy of the method

The accuracy experiment was performed over the range of 50%, 100% and 150% of the label claim of a typical BAK concentration in sample, representing respectively 0.025, 0.050 and 0.075 mg/mL of total BAK. Three samples at each concentration level were prepared and injected.

2.5.4. Precision

2.5.4.1. Repeatability. The repeatability of the method was evaluated by the analysis of total BAK (preservative) in the drug solution. Six sample preparations were performed and each one was injected once.

2.5.4.2. Intermediate precision. In order to evaluate the intermediate precision of the UHPLC method, the assay of total BAK (preservative) in the preserved drug formulation, was analyzed on a different day, using a different preparation of mobile phase, a different HPLC system, a different column as well as a different analyst performing the analysis. The same lot of the drug formulation was used in this study for a comparison purpose.

2.5.4.3. System precision. System precision was determined from five replicate injections of the BAK standard solution (0.05 mg/mL). The reproducibility of the sum of the homologs peak areas for each of the two homolog peaks and the resolution between each adjacent homolog peak was evaluated.

2.5.5. Robustness

2.5.5.1. Variations in method parameters. The effects of small changes (approximately ±5–10%) of operating parameters such as mobile phase composition, pH of the buffer, mobile phase B

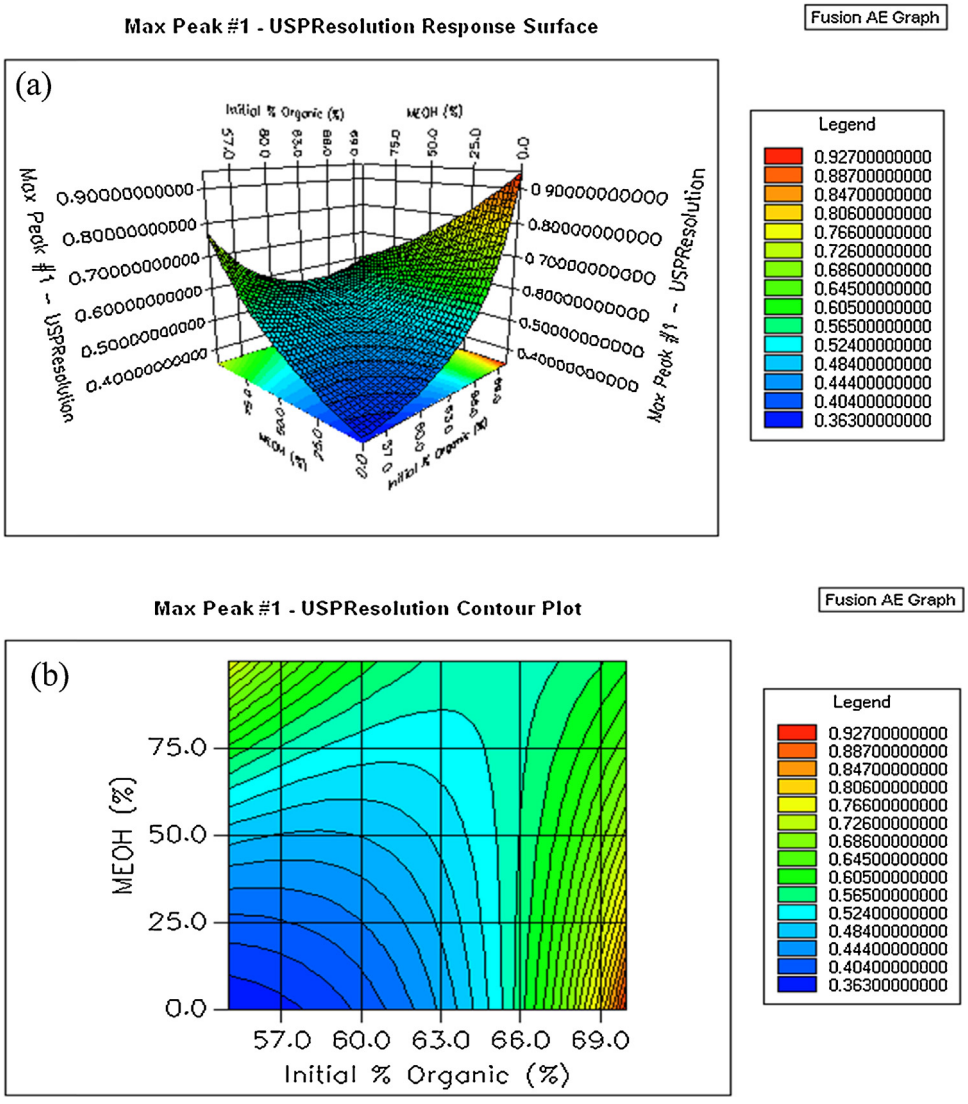


Fig. 4. (a) Response surface of the USP resolution of the maximum peak (BAK C12 peak) separation from its closest peak in ACE C18-AR column using 0.02% phosphoric acid in water as mobile phase A, (b) contour plot of the USP resolution of the maximum peak (BAK C12 peak) separation from its closest peak in C18-AR column using 0.02% phosphoric acid as mobile phase A. These plots indicate the need to improve method condition to obtain baseline separation.

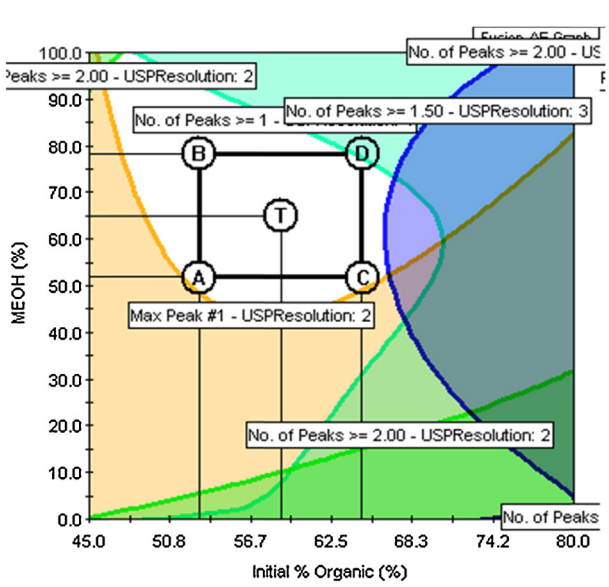
composition, starting mobile phase condition, gradient time, injection volume and column temperature, etc. were tested in order to assess method's sensitivity to minor changes in operating conditions. One method parameter was changed during each experiment while keeping the rest of the parameters same as proposed method parameters. The BAK standard solution and the sample solution were injected for each condition tested.

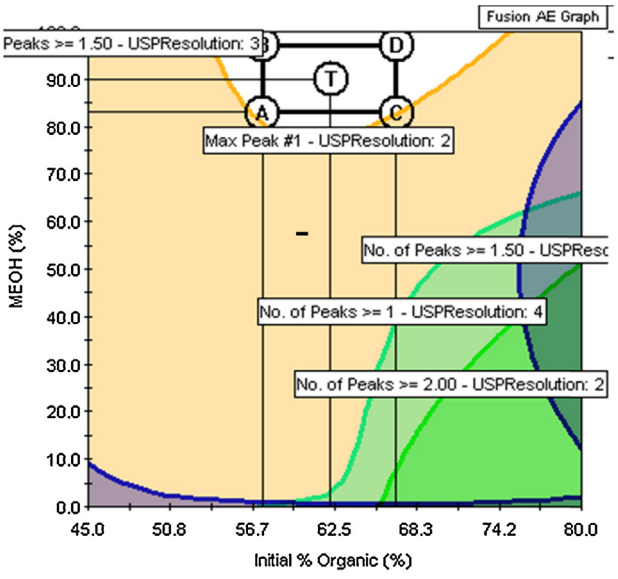
3. Results and discussion

3.1. Optimization of chromatographic conditions

Different HPLC methods have been reported for the analysis of BAK [19–22]. Of these methods, the columns used are CN columns [22] or more conventional reversed-phase columns, C-8 and C-18 [19–21]. With the CN column, typical chromatographic conditions for mobile phase consist of a buffer with a pH of 2.5–7.0 with acetonitrile and/or methanol as organic solvent. In this study, a cyano column was not explored due to potentially shorter lifetime and reproducibility issues.

Achieving best separation with fastest possible runtime with highest possible sensitivity was our goal during the method development. Separation between two peaks which is measured by resolution between them, can be improved most by improving selectivity, which is the ratio of retention factors of one analyte versus other. To achieve best separation choosing the right column with optimum selectivity is key. After choosing the right column, mobile phase must be optimized again to optimize





Variable	Upper bound	Lower bound	Center point
Initial % Organic	53.0	64.7	58.8
MeOH% in Mobile phase B	51.7	78.2	64.9

Variable	Upper bound	Lower bound	Center point
Initial % Organic	56.6	68.3	62.5
MeOH% in Mobile phase B	87.7	99.2	93.4

Name	Lower bound	color
No. of Peaks ≥ 1.50 – USP Resolution	3.0	Violet
No. of Peaks ≥ 2.00 – USP Resolution	2.0	Green
No. of Peaks ≥1.00 – USP Resolution	4.0	Teal
Max. Peak #1-USP Resolution	2.0	Orange

Fig. 5. Acceptable performance region using different columns, various mobile phase B compositions and different starting mobile phase conditions. For both ACE C18-AR and ACE-C18 unshaded region were found indicating that that all the conditions shown in the table were met in the unshaded region. The center point (T) of the black-outlined box is the method condition that should be chosen. Points A, B, C and D indicate the method robustness.

separation with fastest runtime without compromising sensitivity. To our knowledge use of such systematic approach of maximizing selectivity without compromising sensitivity is completely missing in the current literature for the development BAK HPLC method. Initially, 10 columns were quickly screened on a linear gradient using Fusion AE®. This screening was performed using 10 mM Ammonium phosphate in water at pH 3.2 as mobile phase A and methanol as mobile phase B. During this screening maximum four peaks were observed in the BAK lot used in this study. Two major peaks observed were BAK C12 and C14 homologs. In addition, two minor peaks were also observed (labeled as unknown impurities 1 and 2). The results of this screening were shown in Table 1. Four columns with different selectivity were chosen from this screen due to either their good separation ability or lower peak tailing: (1) Waters BEH C18 (50 mm × 2.1 mm, 1.7 μm particle size) and (2) Waters BEH Shield C18 (50 mm × 2.1 mm, 1.7 μm particle size) (3) ACE C18 (50 mm × 2.1 mm, 2.0 μm particle size) and (4) ACE C18-AR (50 mm × 2.1 mm, 2.0 μm particle size).

Further method development experiments were designed by Fusion AE® by varying mobile phase A pH, composition of mobile phase B (ACN to MeOH ratio), starting mobile phase composition, and gradient time. Column temperature and flow rate were kept constant throughout the optimization (45 °C and 1.0 mL/min). Temperature was kept constant since change in temperature known to have minimum effect on the separation of BAK homologs [22]. The maximum column efficiency is generally achieved when the linear velocity is between 3 and 7 mm/s in UHPLC type (≤2 μm silica particle diameter) reverse phase columns [28,29]. In our study, we have chosen 1.0 mL/min which is equivalent to 4.8 mm/s of linear velocity within the column. Separation of four peaks was monitored and optimized. The following limits of acceptability were set: number of peaks with USP resolution ≥2.0 must be 2; number of peaks with USP resolution ≥1.5 must be 3; number of peaks with USP resolution ≥1.0 must be 4; USP resolution between the largest peak and its closest peak must be ≥2.0.

Only acidic mobile phases were explored in order to obtain lower peak tailing. The acidic mobile phases chosen for exploration

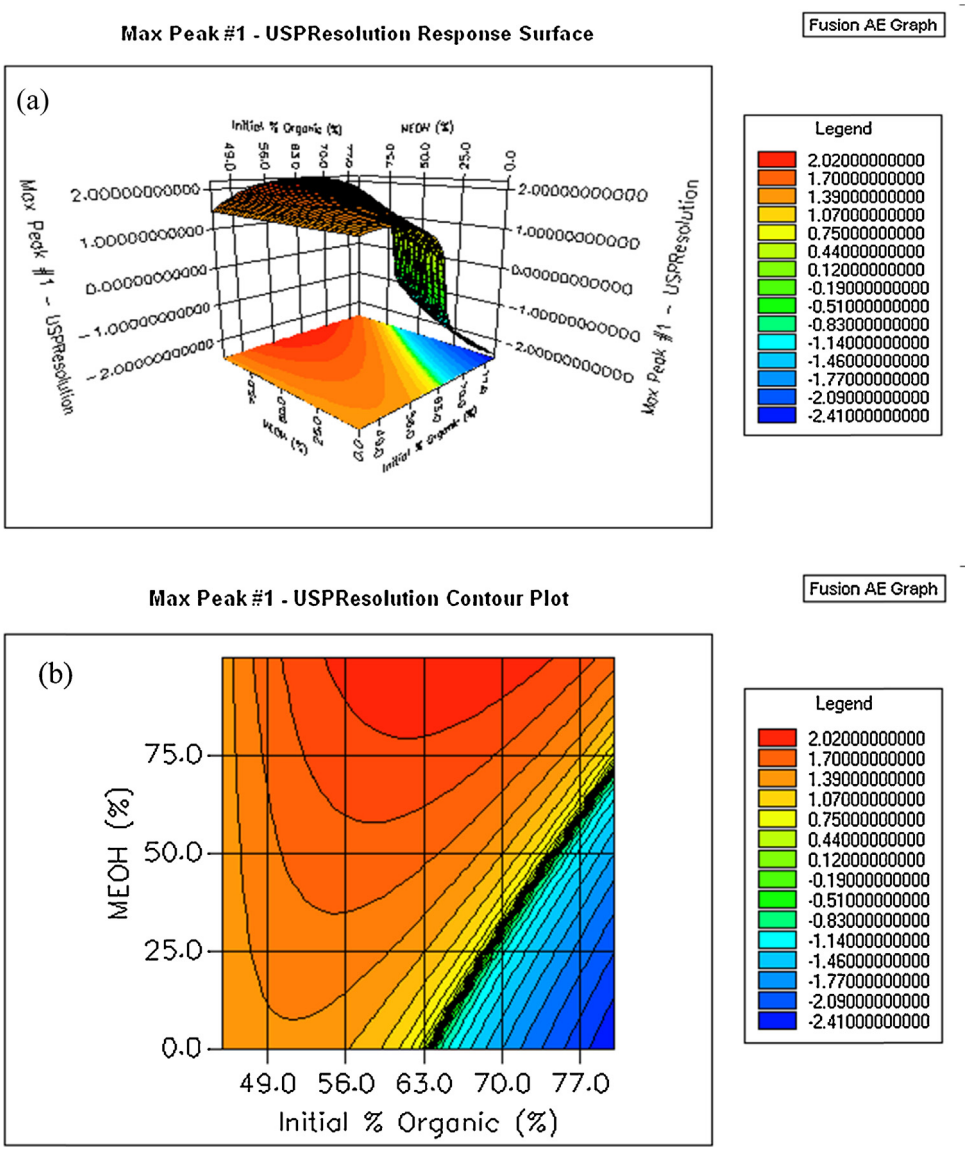
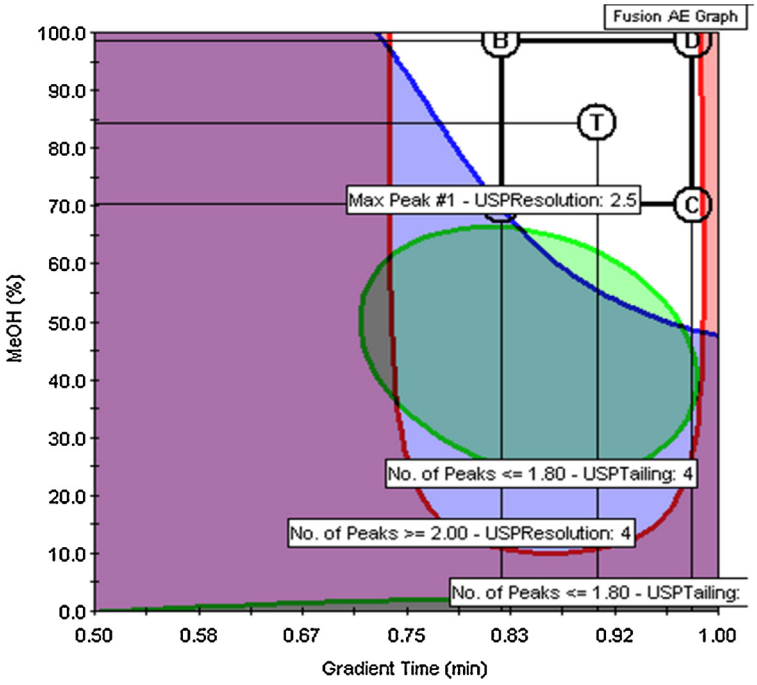


Fig. 6. (a) Response surface of the USP resolution of the maximum peak (BAK C12 peak) separation from its closest peak in C18-AR column using 10 mM pH 3.3 ammonium phosphate buffer as mobile phase A, (b) contour plot of the USP resolution of the maximum peak (BAK C12 peak) separation from its closest peak in ACE C18-AR column. These plots indicate that resolution of the maximum peak (BAK C12 peak) separation from its closest peak can be more than 2 (dark red region). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were 0.02% phosphoric acid and 10 mM ammonium phosphate at pH 3.3.

Initial evaluation was made with 0.02% phosphoric acid as mobile phase A with different combinations of mobile phase B (acetonitrile and methanol) and different % initial organic mobile phase B. This evaluation did not provide enough separation with any of the columns tested as shown in Fig. 3. Unshaded region shows conditions that meet or exceed the limits of acceptability. No unshaded region was seen in Fig. 3. The 3D plots in Fig. 4 show combined effect of variables such as % initial organic and % methanol against USP resolution. The color gradient represents the magnitude of the interaction and the curvature indicates the type of interaction. As we can see, the USP resolution for C12 peaks was always below 1 thus not providing enough separation. However, with 10 mM ammonium phosphate at pH 3.3 as the mobile phase A, all the imposed separation criteria was met for both ACE C18 and ACE C18-AR column as shown in Figs. 5 and 6. Such design spaces were also found for BEH C18 and BEH Shield C18 column. This result clearly showed that 10 mM ammonium phosphate in water at pH 3.3 is better mobile phase A than more acidic 0.02% phosphoric acid in water as mobile phase A for any of the four columns explored. So, further optimization was conducted using 10 mM ammonium phosphate in water at pH 3.3 as mobile phase A. Although all four columns showed good separation using 10 mM ammonium phos-



Variable	Upper bound	Lower bound	Center point
Gradient Time	0.83	0.98	0.91
MeOH	69.9	98.7	84.3

Name	Lower bound	Color
No. of Peaks ≥ 2.00 – USP Resolution	4.0	Violet
Max peak #1- USP Resolution >= 2.50	1.0	Orange
No. of Peaks ≤1.80-USP tailing	4.0	Green

Fig. 7. Acceptable performance region using ACE C18-AR, varying gradient time and methanol % in the mobile phase B. The center point (T) of the black-outlined box is the method condition that should be chosen. An excellent condition for separation was found when the following parameters were used: initial gradient starting with 60% mobile phase B containing 84% methanol and 16% acetonitrile and gradient time of ~0.9 min.

phate in water at pH 3.3 as mobile phase A, Ace C18 and Ace C18-AR column were chosen for further evaluation, due to much lower back pressure and lesser run time than BEH C18 and BEH Shield C18.

For the next step of optimization, 10 mM of ammonium phosphate buffer was used as mobile phase A, the % initial organic mobile phase was kept constant at 60% with variations in gradient time and % methanol in mobile phase B. Fig. 7 shows acceptable performance region using ACE C18-AR varying gradient time (0.5–1 min) and methanol % (0–100) in the mobile phase B. As mentioned earlier, the unshaded region, if there is any, shows the condition that meets or exceeds the limits of acceptability of critical responses. The limits of acceptability were: number of peaks with USP resolution ≥2.0 must be 4; USP resolution between the largest peak and its closest peak must be ≥2.5; and number of peaks with USP tailing ≤1.8 must be 4 (since four peaks are present in the BAK used in this study). The unshaded region is the design space in terms of the mean performance region of the critical method parameters. In Fig. 7, the center point (T) of the black-outlined box is the method condition that should be chosen for optimal robustness of the method. Points A, B, C and D indicate the method robustness.

The results from the optimization step provided an excellent condition for separation when the following parameters were used: Initial gradient starting with 60% mobile phase B containing 84% methanol and 16% acetonitrile and gradient time of ~0.9 min (Table 2). Fig. 8 indicates that resolution of the maximum peak (BAK

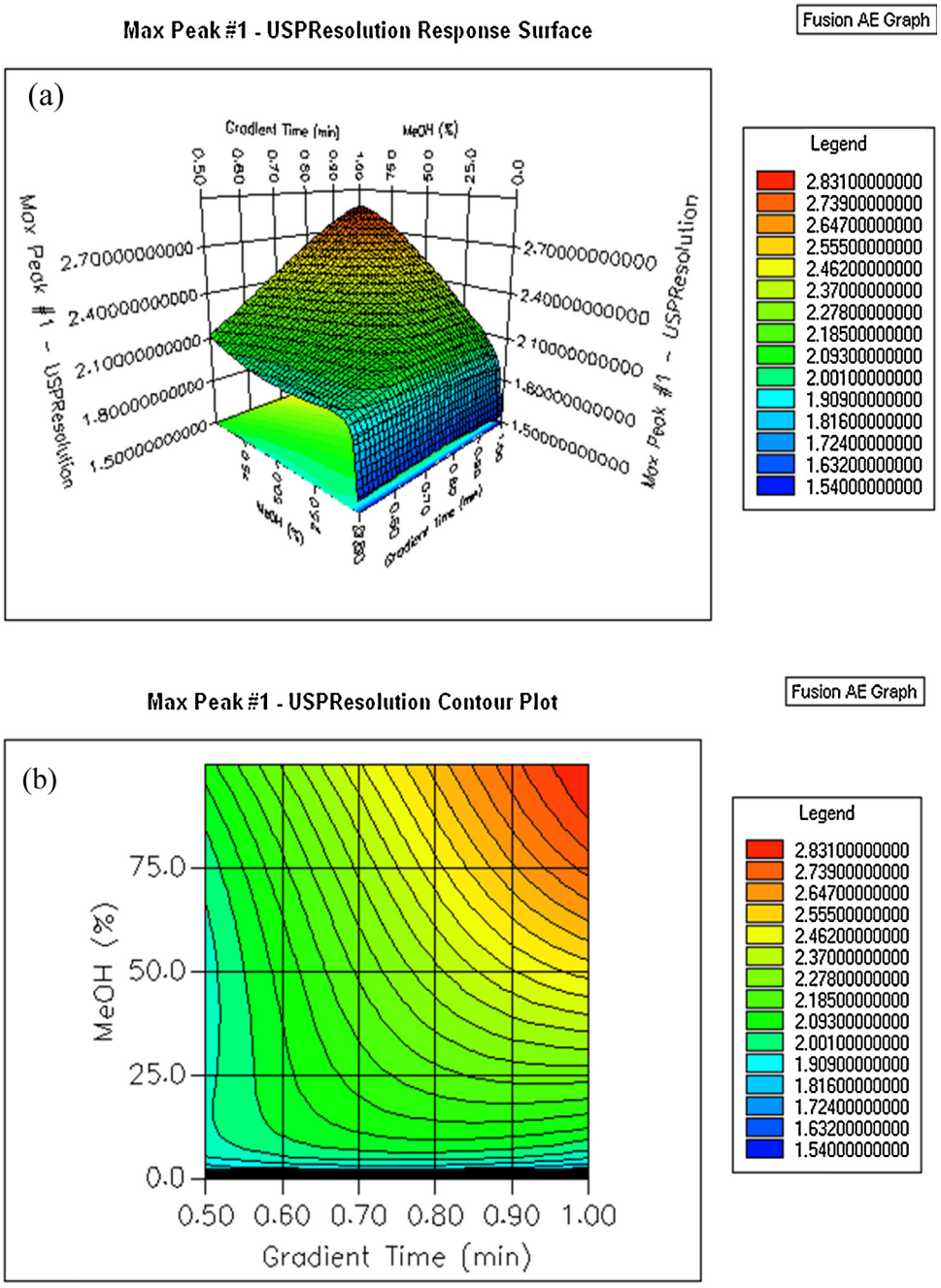


Fig. 8. (a) Response surface of the USP resolution of the maximum peak (BAK C12 peak) separation from its closest peak in C18-AR column using 10 mM pH 3.3 ammonium phosphate buffer as mobile phase A, (b) contour plot of the USP resolution of the maximum peak (BAK C12 peak) separation from its closest peak in C18-AR column using 10 mM pH 3.3 ammonium phosphate buffer as mobile phase A. These plots indicate that resolution of the maximum peak (BAK C12 peak) from its closest peak can be more than 2 when the target (T) was chosen as per Fig. 7.

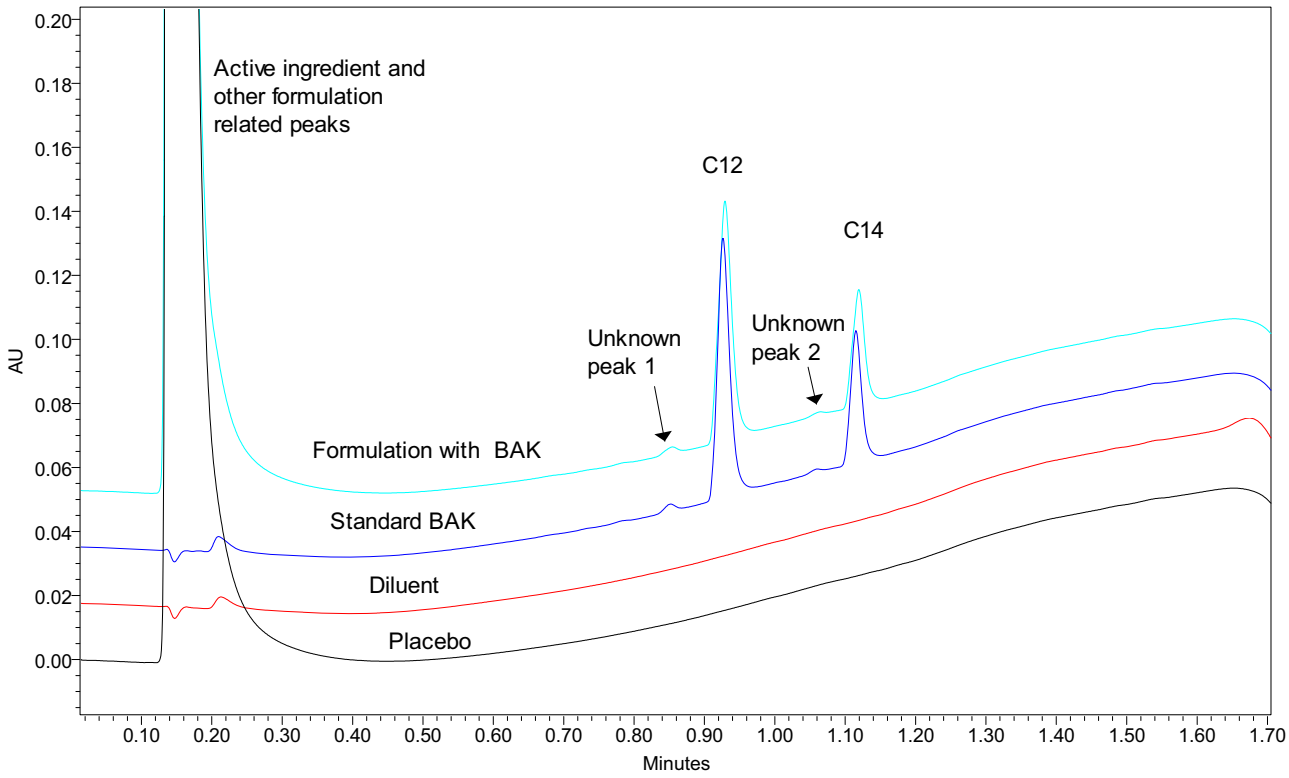


Fig. 9. Overlaid chromatograms of placebo, diluent, standard and sample by optimized method condition using ACE C18-AR column (2.1 × 50 mm, 2.0 μm) where mobile phase A is pH 3.3, 10 mm ammonium phosphate, mobile phase B (ACN:MeOH = 15:85) and % B increased to 95% in 0.9 min.

C12 peak) separation from its closest peak can be more than 2 when the target (T) condition was set as in Fig. 7. Since at 1.0 mL/min flow rate, the back pressure of the column was approximately 8000 psi, a higher flow rate can be employed, if necessary, to reduce the run time. In the final method, the flow rate was set at 1.0 mL/min with good sensitivity, very short runtime and sufficiently low back pressure to allow good column lifetime. The chromatograms are shown in Fig. 9.

3.2. Optimization of samples preparation

Various ratios of water/methanol have been tested as the diluent, and the recoveries of multiple preserved formulations containing different actives have been determined. Water/methanol (50/50, v/v) was found to provide good recovery of BAK for drug solutions.

Table 2
UHPLC condition of the final method.

UHPLC settings	
Column:	ACE Excel C18-AR, 2.1 mm × 50 mm, 2.0 μm
Mobile phase A:	10 mM ammonium phosphate buffer at pH 3.3
Mobile phase B:	ACN:methanol (15/85, v/v)
Pump mode:	Gradient
Flow rate:	1.0 mL/min
Column temperature:	45 ± 2 °C
Autosampler temperature:	Room temperature
Injection volume:	3 μL
Detector wavelength:	214 nm, AUFS set at 2.0000
Run time:	2.0 min

Gradient Program for waters acquity H-class or equivalent

Time	% A	% B	Curve
0.00	38	62	–
0.90	5	95	6
1.30	5	95	6
1.31	38	62	6
2.0	38	62	6

3.3. Validation

3.3.1. Specificity

To demonstrate method specificity and lack of interference from other formulation excipients and impurities, formulation containing no BAK was tested. The formulations tested showed no interference with BAK peaks (Fig. 9). In addition, the BAK peaks found to be pure by peak purity analysis using Empower 2 software.

3.3.2. Accuracy

Accuracy for BAK was determined by spiking known amount of BAK into BAK placebo formulation at concentrations of 0.025 mg/mL, 0.05 mg/mL and 0.075 mg/mL, corresponding to 50, 100 and 150% of the BAK LS, respectively. Triplicate spikes were performed for all levels. The results are summarized in Table 3 and met the acceptance criteria specified in the same table.

3.3.3. Standard Linearity

Standard linearity for BAK was generated from 0.025 mg/mL, 0.05 mg/mL and 0.075 mg/mL, corresponding to 50–150% of the BAK Working Standard concentration. The 95% confidence interval for the line includes the origin justifying the use of a single point standard for quantitation of BAK. The results meet the acceptance criteria and are summarized in Table 3.

3.3.4. Method repeatability

Six replicate sample preparations of the preserved drug formulation were analyzed according to method. The precision (standard deviation from mean result) meet the acceptance criteria and are summarized in Table 3.

Table 3
Method validation result and acceptance criteria: accuracy, linearity, precision, intermediate precision and range.

Method validation element	Result	Acceptance criteria
Accuracy		
50% of the BAK LS (200 ppm)	101 ± 1.9	80–120
100% of the BAK LS (200 ppm)	101 ± 1.0	80–120
150% of the BAK LS (200 ppm)	102 ± 0.5	80–120
Standard linearity		
50–150% of BAK LS (200 ppm)		
Correlation coefficient (r)	0.999	0.990
Slope	1449.5	Report
Y-intercept (Y_0)	2190.4	Report
Y-value at 100% of the BAK LS (Y_{100})	143,727	Report
$(Y_0/Y_{100}) \times 100\%$	Origin is included by 95% confidence interval	≤5, or origin included by 95% confidence interval
Repeatability		
BAK corresponding to 100% of the BAK LS ($n = 6$)	0.1	≤10
Intermediate precision		
BAK corresponding to 100% of the BAK LS		
Day-to-Day repeatability		
Day 1		
Mean LS	98	Report
%RSD	0.20	≤10
Day 2		
Mean LS	101	Report
%RSD	0.16	≤10
Ratio of the mean, Day 2/Day1	1.03	0.90–1.10
Operator-to-Operator repeatability		
Operator A		
Mean LS	99	Report
%RSD	0.46	≤10
Operator B		
Mean LS	98	Report
%RSD	0.20	≤10
Ratio of the mean, Operator B/Operator A	0.98	0.90–1.10
Range	50–150% of BAK LS	Meet acceptance criteria for accuracy, linearity and precision

3.3.5. Intermediate precision

3.3.5.1. Day-to-Day repeatability. Day-to-Day repeatability was evaluated by a single analyst on different days with freshly prepared standards, samples and mobile phase. Two replicates of the same formulation were separately prepared and analyzed according to method by the same analyst on different days. The results meet the acceptance criteria and are summarized in Table 3.

3.3.5.2. Operator-to-Operator repeatability. Operator-to-Operator repeatability was evaluated by different analysts on different day using a different instrument and HPLC column with freshly prepared standards, samples and mobile phase. Two replicates of the same formulation were separately prepared and analyzed according to method by Operator-A and Operator-B. The results meet the acceptance criteria and are summarized in Table 3.

3.3.6. Range

The range of the method for BAK quantitation was determined and reported based on data obtained from linearity, accuracy, and precision studies. The method has been demonstrated to be linear, accurate and precise for quantitating BAK from 0.025 to 0.075 mg/mL, corresponding to 50–150% of the BAK LS.

3.3.7. Robustness

By using Fusion AE® for method development, Quality-by-Design (QbD) principles governed the experimental approach and Design of Experiment (DOE) methodology was used in constructing the experimental design. Fusion AE® predicts a design space where method will be able to meet specified method performance goals. So, a verification of robustness of the method is necessary to make sure design space prediction by Fusion is accurate. About ±5–10% change in the various method parameters were made (see Table 4) and a sample is quantified against the bracketing working standard. All results met the acceptance criteria for method robustness. For example, when column temperatures were varied from 40 °C to 50 °C and % Label Strengths were 99% at 40 °C, 99% at 45 °C and 100% at 50 °C. Such small variation in % of Label Strength values meet our preset acceptance criteria and indicate that method is robust to column temperature variations. Similarly, it was found that the method is robust to variation in mobile phase A molarity, pH of the mobile phase, injection volume of sample, starting mobile phase composition, final mobile phase composition, mobile phase B composition (% of methanol) and detection wavelength. The results are summarized in Table 4.

Table 4
Method validation result and acceptance criteria: Robustness.

Method validation element	Result*	Acceptance criteria
Robustness		
Effect of column temperature on %LS		
45 °C (X_1), 40 °C (X_2), 50 °C (X_3)	99, 99, 100	Report
X_2/X_1, X_3/X_1	1.00, 1.01	0.90–1.10
Effect of mobile phase A molarity on %LS		
10 mM (X_1), 9 mM (X_4), 11 mM (X_5)	99, 100, 99	Report
X_4/X_1, X_5/X_1	1.01, 1.00	0.90–1.10
Effect of mobile phase pH on %LS		
3.3 (X_1), 3.0 (X_6), 3.6 (X_7)	99, 99, 99	Report
X_6/X_1, X_7/X_1	1.00, 1.00	0.90–1.10
Effect of injection volume on %LS		
3 μL (X_1), 2.5 μL (X_8), 3.5 μ L (X_9)	99, 97, 97	Report
X_8/X_1, X_9/X_1	1.02, 1.02	0.90–1.10
Effect of starting mobile phase composition (%B)/gradient slope on %LS		
62% B (X_1), 66% B (X_{10}), 58% B (X_{11})	99, 98, 99	Report
X_{10}/X_1, X_{11}/X_1	0.99, 1.00	0.90–1.10
Effect of final mobile phase composition (%B)/gradient slope on %LS		
90% B (X_1), 81% B (X_{12}), 99% B (X_{13})	99, 99, 99	Report
X_{12}/X_1, X_{13}/X_1	1.00, 1.00, 1.00	0.90–1.10
Effect of mobile phase B composition (% of methanol) on %LS		
85% (X_1), 87% (X_{14}), 83%(X_{15})	99, 98, 98	Report
X_{14}/X_1, X_{15}/X_1	1.01, 1.01	0.90–1.10
Effect of detector wavelength on %LS		
214 nm (X_1), 212 nm (X_{17}), 216 nm (X_{18})	99, 99, 98	Report
X_{17}/X_1, X_{16}/X_1	1.00, 1.01	0.90–1.10

* %LS values for each robustness condition were within ±2 standard deviation and only mean robustness values presented here.

4. Conclusion

In conclusion, a simple and rapid reversed-phase HPLC method has been systematically developed using Fusion AE® method development software. Response surface plots graphically illustrated the major effects of mobile phase pH, mobile phase B composition, % of initial organic mobile phase, gradient slope and time on the separation. This method has been successfully applied in the separation of BAK homolog and the total BAK determination in a preserved drug formulation. The Quality-by-Design (QbD) approach of method development is recommended by FDA and provides the method developer a very clear picture of the robustness during the development process. Unlike a traditional method development approach, a much better method condition can be chosen in the development process since the design space is very clear in each step. Furthermore, the robustness of in traditional method development is mostly unknown until the validation is finished. Using the QbD approach shown here, a clear idea of robustness of the method is

already available before going for validation. Multiple preserved drug formulations containing different active drugs have been analyzed with success using this analytical method. The validation results confirmed the usefulness and the robustness of the method. The method was found to be precise, accurate and linear at concentrations ranging from 0.025 mg/mL to 0.075 mg/mL for total BAK. The method developed in this study using Ace C18-AR phase has a runtime of 2.0 min and 0.05 μg/mL sample concentration. This new method shows >10 times improvement in terms of runtime and >80 times improvement in sensitivity compared to most commonly used US Pharmacopeia method for benzalkonium chloride analysis.

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