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The use of the Box–Behnken experimental design in the optimisation and robustness testing of a capillary electrophoresis method for the analysis of ethambutol hydrochloride in a pharmaceutical formulation

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Abstract

Box–Behnken experimental designs do not appear to be extensively used in optimisation of analytical methods using capillary electrophoresis (CE). This paper describes the use of the Box–Behnken experimental design to optimise the factors affecting the separation of ethambutol hydrochloride (EB), its impurity 2-amino-1-butanol and the internal standard (phenylephrine hydrochloride) in a CE method for a pharmaceutical tablet assay. The three factors studied simultaneously were: buffer pH, buffer concentration and applied electric field, each at three levels. The method was optimised with respect to three responses: resolution between peaks, theoretical plate count and the migration time of the EB peak. A statistical programme, which applies a multiple response optimisation algorithm, was used to calculate and optimise the three responses simultaneously. The optimum conditions were established to be 58.0 mM sodium borate buffer at pH 9.50 and an applied electric field of 412 V/cm. The robustness of the method was also determined and confirmed using a second Box–Behnken design, as part of the validation exercise. System suitability values for the method were derived from the regression surface analysis. The CE method for a pharmaceutical tablet formulation was further validated according to current regulatory requirements, with respect to linearity and range, precision, specificity, accuracy and limit of quantitation. The optimised method gives a fast and efficient separation under 4 min, with complete resolution between the three peaks, and represents an improvement over the existing USP method. It can be concluded that the Box–Behnken experimental design provides a suitable means of optimising and testing the robustness of a CE pharmaceutical method. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Box–Behnken; Capillary electrophoresis; Experimental design; Optimisation; Ethambutol; Pharmaceutical analysis; Robustness testing

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1. Introduction

Optimisation of a capillary electrophoretic separation can be difficult due to the wide array of parameters and variables that must be controlled to achieve separation selectivity and meet other performance criteria. The advantages in using a multi-variate approach to optimising an analytical method include reductions in the number of experiments, improved statistical interpretations (particularly with the current availability of statistical software packages) and reduced overall retention time requirements. Furthermore, interaction effects between parameters can be investigated with multivariate experiments, which would be impossible to do with a univariate approach [1,2]. The use of multivariate statistical analysis to optimise CE analytical methods has been investigated by Altria et al. [3], Morris et al. [4] and Persson-Stubberud et al. [5]. The experimental designs that have been utilised in capillary electrophoresis (CE) are Plackett–Burman, overlapping resolution mapping, fractional factorial designs, simplex designs and central composite designs. The application of Box–Behnken designs has been recorded in optimisation of food technology processes [6], microbiological studies [7] and pharmaceutical formulation development work [8], amongst others. The Box–Behnken design was employed by Hows et al. to optimise a CE method used in the screening of food products for veterinary drug residues [9]. The method was successfully optimised for the simultaneous determination of sulphonamide, dihydrofolate reductase inhibitor and β-lactam residues in animal tissue. However, the use of Box–Behnken experimental design does not appear to be reported in optimisation work of CE pharmaceutical methods. In addition, the Box–Behnken experimental design does not appear to have been utilised for robustness testing of CE analytical methods either.

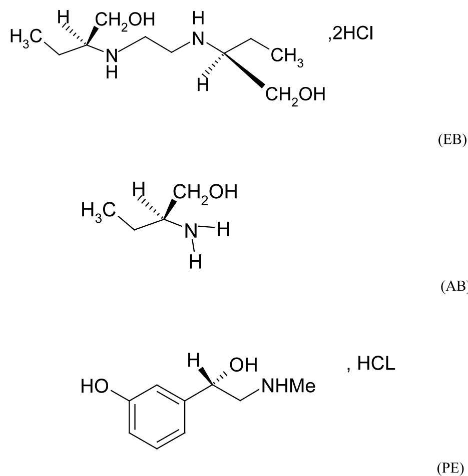

Fig. 1. Structures for ethambutol hydrochloride (EB), 2-amino-1-butanol (AB) and phenylephrine hydrochloride (PE).

As with central composite designs, Box–Behnken designs are response surface methods used to examine the relationship between one or more response variables and a set of quantitative experimental parameters [10]. Response surface methods are often used once preliminary screening has been carried out, using designs such as factorial designs, to determine which factors significantly affect the response. They are also used when curvature in the response surface is suspected. However, central composite designs usually have axial points outside the 'cube' (unless alpha, the axial spacing needed to ensure orthogonality, is specified as less than or equal to one). Box–Behnken designs do not have axial points, thus all design points fall within the safe operating zone. These designs also ensure that all factors are never set at their high levels, simultaneously [10,11]. Furthermore, Box–Behnken designs have fewer design points. Also, each factor requires only three levels instead of the five required for central composite designs (unless alpha is equal to one), which may be experimentally more convenient and less expensive to run than central composite designs with the same number of factors.

Ethambutol hydrochloride (EB) [(*S*,*S*)-*N*,*N′*-ethylenebis (2-aminobutan-1-ol) dihydrochloride] (Fig. 1) is an antibacterial agent used with other antituberculous agents in the primary treatment of pulmonary and extrapulmonary tuberculosis to suppress emergence of resistance to the other agents in the regimens [12]. EB is administered orally and is usually formulated as a tablet. EB degrades to 2-amino-1-butanol (Fig. 1) and the amount of this degradant is limited by a number

of pharmacopoeial monographs to be not greater than 1% of the content of ethambutol hydrochloride. The pK_a values for ethambutol are 6.3 and 9.5 (20°) [13].

The British Pharmacopeia (BP) [14] and the United States Pharmacopeia (USP) [15] approved methods for the determination of ethambutol hydrochloride content in tablets consist of chloroform extractions followed by a non-aqueous titration. This type of analytical technique is not only cumbersome due to the handling of a toxic organic solvent, but it also lends itself to high experimental error and it is non-selective. Furthermore, the 2-amino-1-butanol is assayed separately by a fluorescence method. In the interest of finding a more efficient alternative analytical method, which would simultaneously assay ethambutol hydrochloride and 2-amino-1-butanol, a CE method was developed and published in the USP Pharmacopeial Forum in 1997 [16]. However, the method employs a sodium borate concentration of 100 mM at pH 10.0, producing high currents (>100 μA) at the designated method parameters, resulting in excessive Joule heating. Moreover, the Pharmacopeial Forum method does not include the use of an internal standard, which has been recommended in a chapter on CE published in the European Pharmacopoeia (EP) Forum [17] and in the USP general chapter on CE [18], to compensate for variations in injection volume, inherent with CE instruments.

The aims of this study were to (i) test the use of the Box–Behnken design to optimise a CE separation; (ii) test the use of the Box–Behnken design to determine the robustness of a CE separation, and (iii) improve the existing USP method for the analysis of ethambutol hydrochloride, its impurity, 2-amino-1-butanol and an internal standard (phenylephrine hydrochloride).

2. Experimental

2.1. Instrumentation

The CE system used was a 3D CE (Hewlett Packard, Waldbron, Germany) with photo diode array detector, controlled via ChemStation software. The capillary used was a 50 μm internal diameter; 48.5 cm length (40 cm to detector or effective length) bare fused silica supplied by Hewlett Packard. The capillary temperature was held constant at 25 °C (air cooling mechanism). The statistical software used to evaluate the experimental design results was Minitab version 12.1.

2.2. Chemicals and reagents

All reagents used were of analytical grade. All aqueous solutions were prepared with HPLC grade water (Milli-Q, Millipore, Bedford, MA, USA). The running buffer solution was prepared by dissolving the appropriate amount of boric acid H_3BO_3 (analytical reagent, AnalaR, Merck P/L, Australia) and adjusting to the desired pH with 5 M NaOH (BDH AnalaR-grade). The buffer solution was then degassed and filtered through 0.45 μm Millex-HV filter discs (Millipore). Ethambutol hydrochloride and phenylephrine hydrochloride (Sigma) were purchased from Bio Scientific P/L, Australia. The 2-amino-1-butanol (TCI Tokyo Kasei Organic Chemicals) was purchased from Laboratory Supply P/L, Australia. The samples and reference standard materials were kindly supplied by Wyeth Australia Pty. Ltd.

2.3. Procedure

2.3.1. Method screening and optimisation

2.3.1.1. Reference standard and buffer solutions. The reference standard and internal standard solutions were prepared by dissolving the prescribed amounts in Milli-Q water. A combined reference and internal standard solution was prepared to contain the following approximate concentrations: 3 mg/ml of ethambutol hydrochloride, 0.03 mg/ml of 2-amino-1-butanol (impurity) and 0.075 mg/ml of phenylephrine hydrochloride (internal standard). The combined reference standard solution was filtered through 0.45 Millex-HV filter discs (Millipore) and introduced hydrodynamically at 25 mbar for 3 s, followed by running

buffer solution for 2 s, also at 25 mbar. The compounds were detected at 200 nm. Prior to each injection, a pre-analysis capillary wash was programmed to flush with 0.1 M NaOH for 1 min, Milli-Q water for 1 min and running buffer solution for 1 min The capillary was initially conditioned with 0.1 M NaOH for 30 min.

2.3.1.2. Experimental design. Three experimental parameters, or factors, were varied at three levels: sodium borate buffer concentration (mM), buffer pH, and applied electric field (V/cm). These parameters were chosen as they were considered to have the most significant effect on the separation efficiency of this method. The levels were selected based on knowledge of the system acquired from initial experimental trials. To arrive at the operational 'safe zone' for the buffer pH, a uni-variate set of experiments was conducted, as it was found that selectivity and resolution were markedly affected by pH variations. All experiments were carried out in duplicate, i.e. the reference standard solution was injected twice for each experiment. The inclusion of centre points provided a more precise estimate of experimental error and provided a measure for the adequacy of the model (lack of fit). It also enabled the determination of the significance of the main, interaction, and quadratic effects, i.e. which coefficients in the second order model were significantly non-zero.

Three response factors were utilised for the determination of the optimum method conditions. The first one was the resolution factor (R_s) between the ethambutol hydrochloride and the phenylephrine hydrochloride peaks. From previous experiments, these were the two peaks most difficult to separate. The 2-amino-1-butanol peak was well resolved from the ethambutol peak in all experiments. The second response factor was retention time (R_t) measured by the migration time of the ethambutol hydrochloride peak. Finally, the number of theoretical plates (N) for the ethambutol hydrochloride peak was selected as the third response factor as it was the most affected by changes in experimental conditions. For each injection, the CHEMSTATION software method was programmed to automatically calculate the three response factors: R_s, R_t and N. A multiple response optimisation algorithm, part of MINITAB 12 software, was used to determine the optimum method parameters, where the responses were peak resolution (R_s), retention time (R_t), and number of theoretical plates (N).

2.3.2. Method validation

2.3.2.1. Sample, reference standard and buffer solutions. The sample solution was prepared by accurately weighing an amount, equivalent to 3 mg/ml ethambutol hydrochloride, from a homogenous sample of ground tablets, into a 100 ml volumetric flask. Approximately 60 ml of Milli-Q water was added and the solution ultrasonicated for 30 min. An aliquot of the internal standard solution was pipetted into the flask, to produce 0.075 mg/ml of final phenylephrine hydrochloride concentration. The solution was made up to volume with Milli-Q water and mixed thoroughly. The combined reference standard solution was prepared as per Section 2.3.1.

The method was subsequently validated by following the validation protocol for analytical methods in the Eudralex and the International Conference for Harmonisation (ICH) Guidelines [19,20]. The method's linearity, precision, limit of quantitation, limit of detection, specificity and system suitability were determined. For the Accuracy and Precision test, a placebo was prepared by mixing all the raw materials in the tablet formulation minus the active ingredient, in this case ethambutol HCl. For the assay of ethambutol hydrochloride, the sample and standard solutions were introduced, in duplicate, hydrodynamically at 25 mbar for 3 s, followed by running buffer solution for 2 s, also at 25 mbar. The standard and sample solutions were re-run separately, with injections at 50 mbar for 10 s (increased sample loading), for the 2-amino-1-butanol impurity content determination.

2.3.2.2. Experimental design. The robustness of the method was determined by evaluating the effect of small changes in the experimental parameters on the ethambutol hydrochloride assay, expressed as % label claim (%LC) ethambutol HCl. These changes represent the typical errors

encountered in a laboratory. The surface response diagrams generated during the optimisation of the method provided an initial indication of the parameter ranges that should be tested in the robustness evaluation. A three factor, at three levels, Box–Behnken experimental design was also used for testing the robustness of the analytical method. The following parameter ranges, from the optimum set of conditions, were used to develop a Box–Behnken design at three levels: Buffer pH = ±0.5 pH unit; Buffer Concentration = ±6 mM (±10%); Voltage = ±3 kV (±15%).

3. Results and discussion

3.1. Method screening and optimisation

3.1.1. Preliminary experiments and factor selection

The compound selected in this research as the internal standard was phenylephrine hydrochloride because its electrophoretic mobility is similar to the run buffer (for good peak symmetry), it migrates close to (but is resolved from) ethambutol hydrochloride, it has a detector response similar to that of ethambutol hydrochloride, and it is commercially available in high purity. Fig. 1 shows the molecular structure of phenylephrine hydrochloride. Its pK_a values are 8.9 (–OH–) and 10.1 (–NH–) at 20 °C [13].

Initial method screening to determine the most significant factors did not require an experimental design approach due to the method background provided by the USP Pharmacopeial Forum [15]. The USP method parameters are: 100 mM sodium borate at pH 10.0, applied voltage of 12 kV, 75 μm × 40 cm effective length-fused silica capillary (Beckman), column temperature 25 °C; no internal standard was used. The standard and sample solutions were prepared in dilute buffer solution (10 mM borate). Under these conditions the retention time for the ethambutol hydrochloride assay is approximately 7 min.

The pH of the running buffer affects both the electroosmotic flow and the electrophoretic mobility. The electroosmotic flow (EOF) increases with increasing buffer pH. In developing a CE method, the buffer pH should be initially tried at a value near the pK_a of the solutes to be separated [21–23]. However, for basic compounds such as ethambutol and phenylephrine, working at a high pH could result in peak tailing and poor method robustness. Resolution, selectivity and peak shape can be dramatically affected by changes in pH; small differences in pK_a can cause a critical difference in the separation of similar compounds. Univariate experiments at 63 mM borate buffer and 20 kV voltage were carried out at pH values of 9.00, 9.30, 9.60, 9.80, 10.00 and 10.50 to determine the operational 'safe zone'. From these experiments, the pH range tested in the Box–Behnken design was determined to be 9.00–9.80.

Increasing the ionic strength of the buffer decreases the magnitude of the EOF, hence increasing the retention time [21–23]. It also counteracts electromigrational dispersion. As a result, resolution improves and there is an increase in the number of theoretical plates. When excessive, Joule heating contributes to zone broadening. A buffer concentration range (25–75 mM) was, therefore, investigated in the Box–Behnken design. Increasing the voltage results in shortening retention time and improving the separation efficiency and resolution [21–23]. However, as voltage is further increased, excessive Joule heating results in band broadening. Hence, maximum resolution is obtained by maintaining the voltage below the level at which Joule heating becomes a limiting factor. The range of 15–25 kV was hence investigated in the Box–Behnken design. Table 1 shows the experimental conditions for each run in the Box–Behnken design. Examples of electropherograms from experimental conditions away from the optimum are shown in Fig. 2. The impurity 2-amino-1-butanol migrated approximately 1 min earlier than ethambutol HCl, in all experimental conditions away from the optimum.

3.1.2. Response surface regression analysis

The response data obtained for the resolution factor (R_s), number of theoretical plates (N) and retention time (R_t) are given in Table 1. By using a fitted full quadratic model Eq. (1), a response

Table 1
Optimisation method parameters for Box–Behnken experimental design and average response results for resolution factor (R_s), plate count (N) and retention time (R_t)

Run order	Buffer pH	[Buffer] (mM)	Voltage (kV)	R_s	N	R_t (min)
1	9.00	30.0	20	2.60	18 712	2.32
2	9.80	30.0	20	0.20	27 061	2.77
3	9.00	70.0	20	3.90	31 923	2.79
4	9.80	70.0	20	0.10	55 864	3.28
5	9.00	50.0	15	3.29	26 498	3.67
6	9.80	50.0	15	0.40	38 282	4.95
7	9.00	50.0	25	3.35	21 370	1.89
8	9.80	50.0	25	0.00	37 757	2.26
9	9.40	30.0	15	5.00	19 301	3.51
10	9.40	70.0	15	8.77	39 686	4.93
11	9.40	30.0	25	5.39	19 800	1.97
12	9.40	70.0	25	9.40	43 139	2.08
13	9.40	50.0	20	7.26	27 912	2.99
14	9.40	50.0	20	7.40	23 361	2.85
15	9.40	50.0	20	7.48	30 825	2.95

surface regression analysis for each response factor was performed using coded units. Table 2 shows the values calculated for the coefficients and P-values (P-value is the probability of the null hypothesis). Using a 5% significance level, a factor is considered to affect the response if the coefficients differ from zero significantly and the P-value < 0.050 [2].

$$y_{1i} = \beta_0 + \beta_1 x_{1i} + \beta_2 x_{2i} + \beta_{11} x_{1i}^2 + \beta_{22} x_{2i}^2 + \beta_{12} x_{1i} x_{2i} + r_i \quad (1)$$

From Table 2, it can be seen that the resolution between ethambutol hydrochloride and phenylephrine hydrochloride, as expected, was most significantly affected by the pH of the buffer, with a P-value of 0.009. The borate buffer concentration was also a significant factor, with a P-value of 0.032. Interaction effects were not found to be significant for the resolution factor.

The number of theoretical plates, or plate count, for the ethambutol peak was significantly affected by the buffer pH (P-value of 0.001) and buffer concentration also (P-value of 0.000). This is to be expected as both parameters are increased: both will increase the sodium ion concentration in the background electrolyte, and consequently reduce the extent of electromigration dispersion. In addition, it was found that the interaction between buffer pH and buffer concentration was also significant, giving a P-value of 0.044. The retention time (R_t) measured by the migration time of the ethambutol peak was significantly affected by all three parameters: buffer pH, buffer concentration and voltage, with P-values of 0.002, 0.002 and 0.000, respectively. The interaction effects of pH and voltage (P-value of 0.028), and buffer concentration and voltage (P-value of 0.007), were also significant. In total, three interaction effects were found to be significant, illustrating the benefits of performing multivariate analysis when optimising a CE analytical method.

Response surface diagrams were produced for each response. Since the model has more than two factors, one factor was held constant for each diagram, therefore, a total of nine response surface diagrams were produced; three for each response. Fig. 3 shows the response surface diagrams for buffer concentration versus buffer pH for each response (R_s, N and R_t), keeping the voltage constant at 20 kV.

3.1.3. Optimisation calculations

The final optimum experimental parameters were calculated using the Minitab Response Surface Optimiser function, which allows for com-

promise among the various responses. This function searches for a combination of factor levels that jointly optimise a set of responses by satisfying the requirements for each response in the set. The optimisation was accomplished by: obtaining the individual 'desirability' (d) for each response, combining the individual desirabilities to obtain the combined or composite desirability (D), and finally by maximising the composite desirability and identifying the optimal factor settings. The

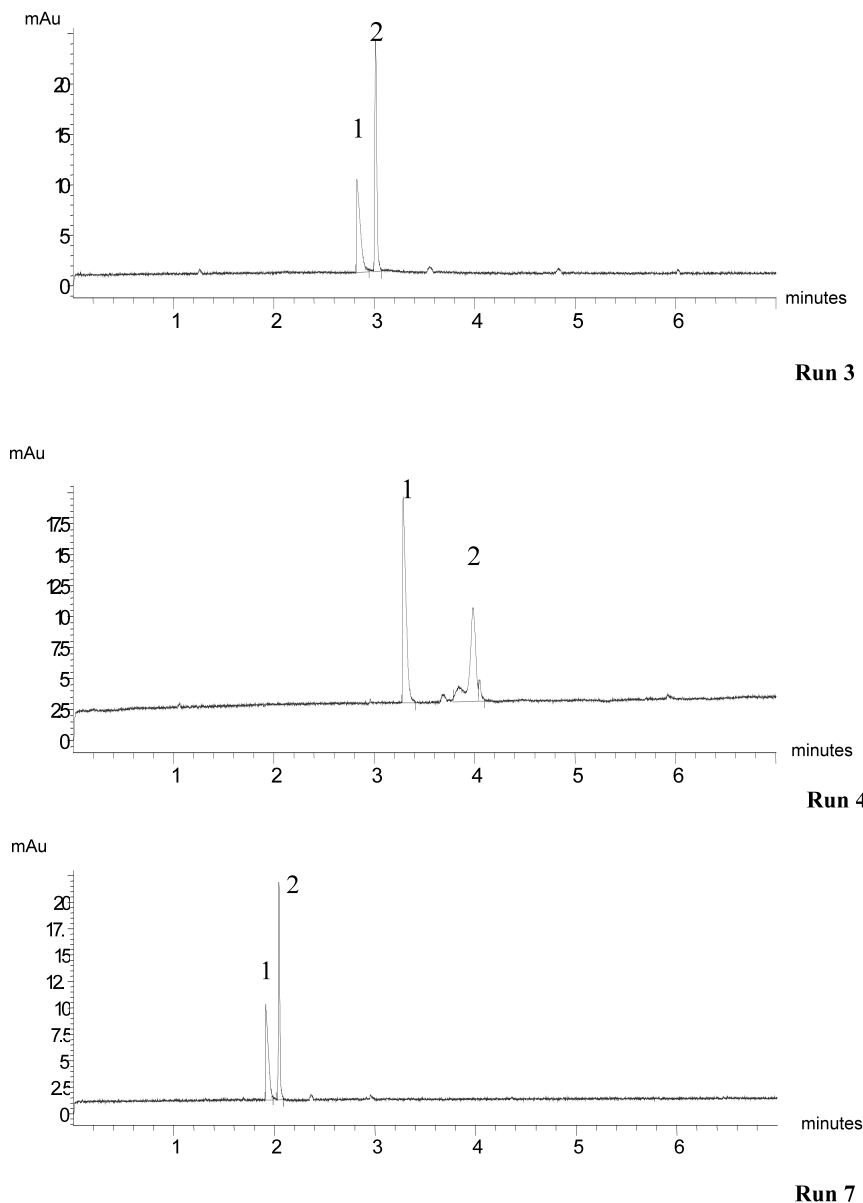

Fig. 2. Electropherograms of sample solution at various experimental conditions away from the optimum. Run 3 conditions: 70.0 borate buffer at pH 9.00 and 20 kV voltage; Run 4 conditions: 70.0 mM borate buffer at pH 9.80 and 20 kV voltage; Run 7 conditions: 50.0 mM borate buffer at pH 9.00 and voltage 25 kV; Peak 1, ethambutol hydrochloride, peak 2, phenylephrine hydrochloride.

Table 2
Regression coefficients and the associated probability values (P-value) for each response

Term	Resolution factor (R_s)		Plate count (N)		Retention time (R_t) (min)	
	Coefficient	P-value	Coefficient	P-value	Coefficient	P-value
Constant	7.378	0.000*	27 365.8	0.000*	2.931	0.000*
pH	−1.559	0.009*	7557.6	0.001*	0.323	0.002*
[Buffer]	1.117	0.032*	10 717.2	0.000*	0.316	0.002*
Voltage	0.085	0.831	−212.7	0.844	−1.108	0.000*
pH × pH	−5.524	0.000*	3259.5	0.084	−0.035	0.671
[Buffer] × [Buffer]	−0.144	0.806	2764.2	0.127	−0.107	0.671
Voltage × Voltage	−0.095	0.871	351.3	0.826	0.299	0.227
pH × [Buffer]	−0.340	0.553	3898.0	0.044*	0.012	0.874
pH × Voltage	−0.114	0.840	1150.6	0.465	−0.229	0.028*
[Buffer] × Voltage	0.061	0.913	738.4	0.633	−0.328	0.007*

Asterisks denote most significant factors and interaction effects (P-value <0.05).

measured responses are transformed to a dimensionless desirability (d) scale. The scale of the desirability function ranges between $d=0$, for a completely undesirable response, to $d=1$ for a fully desired response above which further improvements would have no importance.

The individual desirabilities (d) for each response were obtained by specifying the goals and boundaries required for each response. There were three goals to choose from: minimise the response, target the response, or maximise the response. Upper and lower boundaries for each goal also needed to be specified. A weight factor, which defines the shape of the desirability function for each response, was then assigned for each response. Weights must be between 0.1 and 10, with larger weights corresponding to more important responses and smaller weights to less important responses. After the individual desirabilities were calculated for each response, they were combined to provide a measure of the composite desirability of the multi-response system. This measure of the composite desirability is the weighted geometric average of the individual desirabilities or the responses [11].

A target value of 7.0 was assigned to the resolution factor response, with a weight factor of 6.0 and importance value of 5.0. The retention time (R_t) and plate count (N) were given lower weight factors, see Table 3. In CE analysis, migration times are inherently short; therefore, a lower weight factor was given to R_t whilst still aiming at minimising the response. The number of theoretical plates is typically high in CE however, in this particular separation the ethambutol hydrochloride peak shape and sharpness needed to be maximised, therefore, N was assigned the second highest weight. The individual desirabilities were calculated and the composite desirability (D) for the optimal solution was determined to be 0.8815. The optimal calculated parameters were: buffer pH 9.50, buffer concentration 57.77 mM and voltage 21.24 kV. Based on these calculations, the following experimental parameters were set as the optimum: buffer pH 9.50, buffer concentration 58.0 mM borate and a voltage of 20 kV (applied electric field 412.4 V/cm).

The response surfaces (Fig. 3) were evaluated to obtain an initial indication of the robustness of the method. Fig. 3(a) clearly shows that the pH of the buffer must be maintained between 9.00 and 9.70 in order to achieve suitable resolution ($R_s >$ 1.0) between the ethambutol hydrochloride and phenylephrine hydrochloride peaks. A buffer concentration range of 40–60 mM and voltage range of 15–20 kV (response surface diagrams not shown) would provide fast and efficient separations. The information obtained from these surface diagrams was helpful in establishing parameter ranges in the subsequent robustness testing of the method. To confirm the correctness of the calculated optimum parameters and pre-

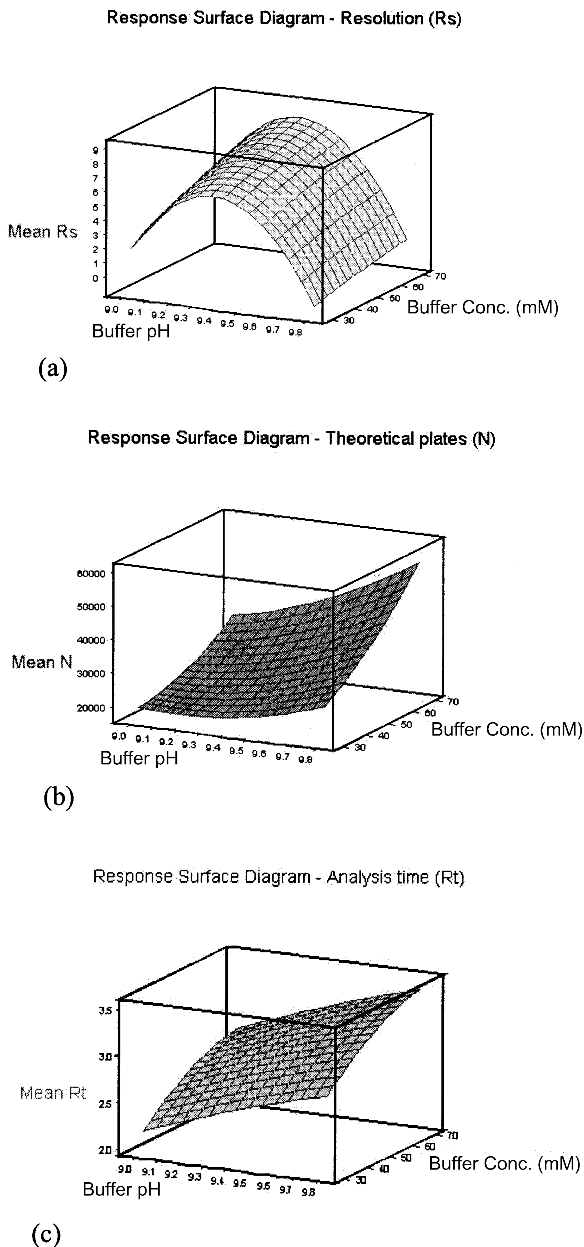

Fig. 3. Response surface diagrams for buffer pH and buffer concentration at a constant voltage of 20 kV: (a) resolution factor, R_s; (b) number of theoretical plates, N; and (c) retention time, R_s.

dicted responses, an ethambutol hydrochloride tablet (400 mg per tablet) was analysed using the optimum conditions. An electropherogram of the sample is presented in Fig. 4 (2-amino-1-butanol peak not shown as it was analysed separately, migrating at approximately 2.5 min). A resolution factor of 7.9 was obtained and the plate count was 37 000. These results compared well with the predicted values using Minitab 12.1, of 7.0, and 35 000, respectively.

3.2. Method validation

3.2.1. Robustness test

For an analytical method to be robust, it must be able to demonstrate that it can produce quantitative results despite small changes in the experimental parameters, which may occur in a typical testing laboratory. Hence, the ethambutol HCl assay (%LC) was used as the response factor. The assay values were calculated from normalised peak area ratios, which involved the division of the normalised peak area for ethambutol by the normalised area for phenylephrine. The calculated assay values (%LC) are shown in Table 4, as well as the experimental parameters followed for each run in the Box–Behnken design developed to test the robustness of the method. The response factors R_s, N and R_t were also measured during the robustness test work, for future calculation of the system suitability values, and are, therefore, included in Table 4. For these calculations, the retention time (R_t) was measured as the migration time of the last peak, phenylephrine HCl.

A response surface regression analysis for the ethambutol HCl assay (%LC) response factor was performed, using coded units, by applying a fitted full quadratic model Eq. (1). Table 5 provides the regression coefficients and the associated probability values (P-value) for ethambutol HCl assay (%LC) response. As can be seen from the values, there were no statistically significant parameters affecting the %LC response, since all P-values were >0.05. The same is applicable to the quadratic terms; hence there were no significant interaction effects. These results indicated that the analytical method was robust since the applied variations to the experimental parameters did not produce any statistically significant effects on the ethambutol HCL assay (%LC). Therefore, it was

Table 3
Multiple response optimisation settings and desirability values

Response	Goal	Lower	Target	Upper	Weight	Importance	Desirability (d)
R_s	Target	6.0	7.0	8.0	5.0	5.0	0.7770
N	Larger	20 000	30 000	40 000	3.0	3.0	1.0000
R_t	Smaller	2.9	2.9	3.5	2.0	2.0	1.0000

Composite desirability $(D) = 0.8815$.

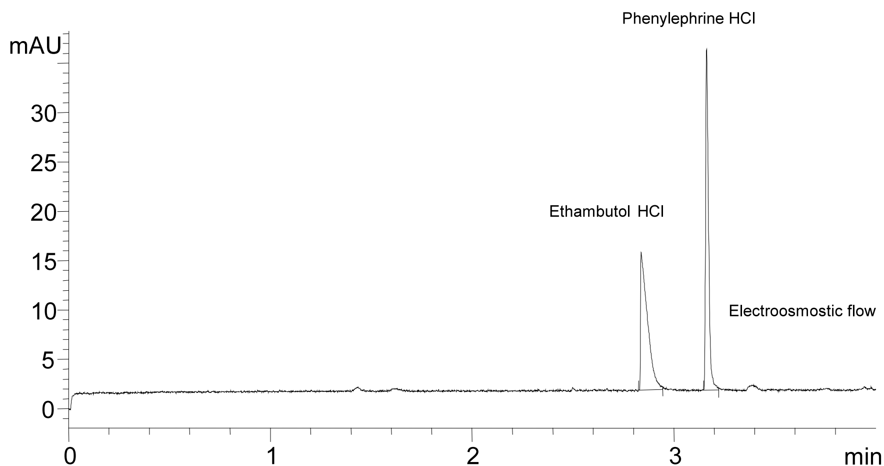

Fig. 4. Electropherogram of ethambutol hydrochloride assay sample solution under optimal experimental conditions (58.0 mM borate buffer at pH 9.50 and an applied electric field of 412.4 V/cm, sample injection 25 mbar for 3 s; for other conditions refer to Section 2).

Table 4
Experimental parameters and response values for robustness testing by a Box–Behnken design

Run order	Buffer pH (21 °C)	[Buffer] (mM)	Voltage (kV)	Etham assay (%LC)	R_s	N	R_t (min)
1	9.3	52.2	20	97.28	5.56	15 703	3.057
2	9.7	52.2	20	98.55	6.23	21 179	3.364
3	9.3	63.8	20	98.78	5.58	15 785	3.065
4	9.7	63.8	20	102.79	6.72	32 396	3.518
5	9.3	58.0	18	98.64	5.85	16 675	3.567
6	9.7	58.0	18	97.78	7.34	22 276	4.043
7	9.3	58.0	22	98.52	5.69	16 236	2.690
8	9.7	58.0	22	98.14	5.50	22 840	2.909
9	9.5	52.2	18	99.19	7.20	17 226	3.735
10	9.5	63.8	18	98.97	8.17	20 063	3.971
11	9.5	52.2	22	97.66	7.70	20 240	2.910
12	9.5	63.8	22	99.82	4.07	20 731	2.870
13	9.5	58.0	20	98.54	8.20	23 238	3.406
14	9.5	58.0	20	98.01	7.56	18 894	3.383
15	9.5	58.0	20	100.83	7.50	18 564	3.380

demonstrated that the CE method for the ethambutol HCl assay is robust.

3.2.2. System suitability

Response surface regression analysis for R_s, N and R_t factors were performed, also applying the fitted full quadratic model. From the regression models, 95% prediction intervals were calculated for the response factors at the optimum experimental conditions, shown in Table 6. This demonstrated that the Box–Behnken experimental design utilised for the robustness testing of the method could be further employed to calculate the system suitability values for the selected response factors.

3.2.3. Linearity

Three reference standard solutions were prepared containing 80, 100 and 120% LC of ethambutol HCL and their normalised peak area ratios for ethambutol HCL/phenylephrine HCl were measured in triplicate, respectively. From the regression analysis of 'normalised peak area ratio' versus 'ethambutol HCl %LC' it was shown that the method was linear within this concentration range, with a correlation value of 99.9%, a Y-intercept of 0.0036 and a slope of 0.0101. The standard deviation (S.D.) of the regression line was 0.0047.

Table 5
Regression coefficients and the associated probability values (P-value) for ethambutol HCl assay (%LC) response

Term	Coefficient	P-value
Constant	112.157	0.000
pH	0.933	0.394
[Buffer]	1.774	0.136
Voltage	−0.102	0.923
pH × pH	−0.262	0.804
[Buffer] × [Buffer]	0.542	0.611
Voltage × Voltage	−0.814	0.453
pH × [Buffer]	0.895	0.412
pH × Voltage	0.157	0.882
[Buffer] × Voltage	0.777	0.472

Table 6
Predicted system suitability values range (95% predicted interval) for R_s, N and R_t responses

Response factor	Predicted response range (95%PI)
R_s	5.92–9.58
N	12 340–28 124
R_t	3.33–3.45 (min)

3.2.4. Specificity and solution stability

Heat, oxidation, acid hydrolysis and alkaline hydrolysis stressed samples were analysed to ensure the method could separate the peaks of interest from any breakdown products. Photodiode array scans performed on the chromatographed stressed samples showed that there were no interferences in the detection of the peaks corresponding to ethambutol HCl, phenylephrine HCl and 2-amino-1-butanol, confirming the specificity of the method. In addition, sample solutions, which had been allowed to stand for 48 h, were shown to be stable.

3.2.5. Accuracy and precision

Spiked placebos equivalent to 100.0%LC ethambutol HCl were analysed in duplicate, resulting in assay results of 100.8% and 99.5%LC, giving an average result of 100.2%LC and coefficient of variation of 0.92%. The repeatability (intra-assay precision), intermediate precision (ruggedness) and reproducibility (inter-laboratory precision) were determined, analysing six replicates for each test. The average assay results for the repeatability, intermediate precision and reproducibility tests were 100.2, 100.4, and 99.9% LC, respectively. The coefficients of variation were 1.45, 1.03 and 1.10%, respectively. These results showed that the method is accurate and precise.

3.2.6. Limit of quantitation (LOQ) for 2-amino-1-butanol

In order to achieve a signal to noise ratio greater than 10:1 for the 2-amino-1-butanol impurity (i.e. S:N > 10:1), as well as provide reproducible normalised peak areas (%SD < 2%), the sample loading was increased by increasing the injection parameters from 25 mbar for 3 s to 50 mbar for 10 s. The normalised peak areas from ten injections using these injection parameters

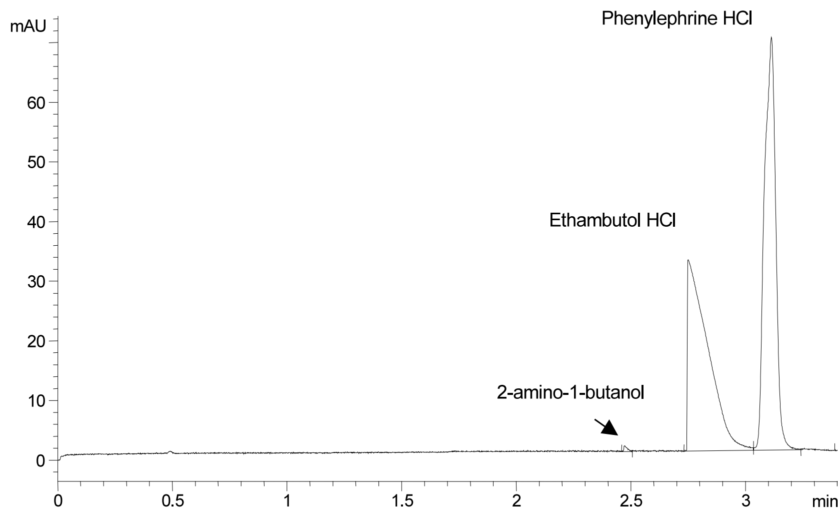

Fig. 5. Electropherogram of reference standard solution injected with higher sample loading (50 mbar for 10 s) for the impurity test (2-amino-1-butanol). Experimental conditions were 58.0 mM borate buffer at pH 9.50 and applied electric field 412.4 V/cm, for other conditions refer Section 2.

yielded a coefficient of variation %SD of 0.82%. Therefore, the impurity could be analysed using the same sample preparation and instrument parameters used in the main component assay, with a higher sample loading. Fig. 5 shows an electropherogram of the reference standard solution injected at 50 mbar for 10 s.

4. Conclusion

The Box–Behnken experimental design can be utilised for the optimisation and robustness testing of a CE pharmaceutical method. Optimisation of the three parameters buffer pH, buffer concentration and applied electric field, required only 15 experiments. This multivariate analysis approach enabled the identification of interaction effects between the experimental parameters. The Box–Behnken experimental design was also used to determine the robustness of the method by running 15 experiments with small measured variations in the three parameters pH, buffer concentration and applied electric field. The response surface regression analysis results showed that the method was indeed robust. System suitability values could be further derived from the robustness data response surface regression models, providing ranges of predicted response values for R_s, N and R_t. In addition, a CE method for the analysis of ethambutol HCl, and its impurity 2-amino-1-butanol, in a tablet formulation, was optimised to separate all peaks within 4 min and was validated to comply with existing regulatory requirements. This resulted in an improvement on the existing USP method, providing potential benefits to commercial laboratories testing large numbers of samples.

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