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Optimization of the capillary electrophoresis separation of ranitidine and related compounds

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Abstract

Experimental designs were used as part of a strategy to determine the significant factors controlling separation and the optimum conditions for analysis of ranitidine and related compounds by using capillary electrophoresis. Two-level fractional factorial designs were employed to screen for the significant factors in the separation with six factors studied. It was necessary to use two fractional factorial designs due to significant overall curvature in the first of the designs used in the screening. Central composite designs were applied to determine the optimal conditions for the significant factors. Multi-linear regression and canonical analysis were used in the analysis. The optimization was based on a response function which takes into account all the peaks in the electropherogram. The pH value and the voltage were found to be the most significant factors.

Keywords: Chemometrics; Factorial design; Experimental design; Optimization; Pharmaceutical analysis; Ranitidine

1. Introduction

Capillary electrophoresis (CE) can provide very simple method development and, in many cases, an adequate separation can readily be obtained. However, in some cases, obtaining a satisfactory separation can be more difficult, especially if the resolution of particular peak pairs is critical. It is in these cases that experimental design may be used to solve the problem. It allows a large number of factors to be screened simultaneously to determine which ones have a significant effect on the separation. Depending on the design chosen, the resulting response model can show the relationship of each factor towards the response as well as the interactions between the factors. These factors can then be optimized to give the best possible separation in a relatively low number of experiments, often resulting in a saving in both time and consumables. Response models from experimental designs such as central composite designs [1] can give an initial indication of the robustness of a method [2], from the change in response with a change in a factor level.

In the pharmaceutical industry, there is increasing interest in CE due to the high separation efficiencies that can be obtained, the short analysis times that can result, the relatively low cost of the instrumentation and the suitability of CE for the types of compounds of pharmaceutical interest [3]. Experimental designs have been used previously with CE in such areas as the analysis of porphyrins [4], fungal metabolites [5], heterocyclic amines, carcinogenic and mutagenic compounds found in cooked foods [6] and in several

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other areas including pharmaceutical analysis, as shown in a review by Altria et al. [2].

A general strategy for optimization is to first screen for important variables, using a two level factorial or fractional factorial design, then to optimize the factors, preferably using a central composite design [7]. The linear model obtained from a two-level design for screening can lead to erroneous conclusions concerning the significance of a factor [8]. If the levels chosen are either side of the optimum, as is the case for points A and B in Fig. 1, then it is possible for a significant variable to be predicted as being insignificant. Box and Draper [1], p. 189, recommend a procedure for an overall curvature check that 'supplies an unbiased estimate of the sum of the pure quadratic effects'. In this procedure, the centre points are included in the design. The overall curvature is determined by c (Eq. (1)). Unfortunately, if significant curvature is present, the information available is insufficient to determine the variables with significant second order effects. This test for curvature does not give information on which variable has a significant optimum. It only indicates that the linear model is inadequate.

$$c = \{\text{average response in two-level factorial runs}\} - \{\text{average response for runs at the centre}\}. \quad (1)$$

An alternative is to use a three-level factorial design. In Jones' study [9], three level Plackett–Burman designs had some main effects confounded with each other. In comparison, a two-level design may sometimes have the main effects confounded with a number of two factor and higher multiple-factor interactions but not with each other. Vander Heyden et al. [10] proposed a three-level design that omitted the first or second half of the factors of a three-level Plackett–Burman design. The main effects and second order effects were not confounded with themselves or with each other. The second order effects were confounded with the two-factor interactions. This could be a problem as, according to Vander Heyden et al. [10], the second order and two-factor interactions often have the same order of magnitude.

This study involves the optimization of the CE separation of ranitidine hydrochloride and four related compounds. Ranitidine (Fig. 2) is a histamine h_2-receptor antagonist that is used in the treatment of gastric and duodenal ulcers and other related disorders and works by inhibiting the secretion of gastric acid. The ranitidine-related compounds, referred to as compounds 1–4, are degradation products, impurity compounds or metabolites. Few chemical data were available for these compounds, however, since the purpose of this study was to investigate the optimization procedure, this was not considered to be required. If the full information on chemical behaviour was known, it would reduce the necessity for an optimization involving all possible CE parameters and would result in having a more refined choice of factor ranges.

It is often of concern to the pharmaceutical industry to determine a small amount of one analyte in the presence of a large amount of another. It has previously been shown that a small amount of ranitidine-related compound 2 can be separated from a large amount of ranitidine hydrochloride [11]. Altria [12] has also shown that small amounts of ranitidine-related impurities in a degraded ranitidine syrup sample can be analysed in the presence of a large amount of ranitidine hydrochloride. In both cases, the high/low injection volume technique was used.

The optimization strategy involved a comparison of two two-level factorial designs to screen for

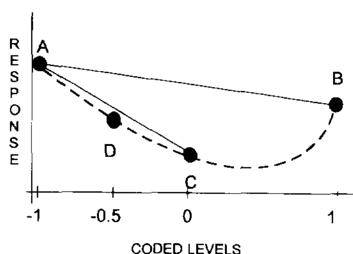

Fig. 1. Factor main effect model. Curved line is the unknown actual response for changes with one factor. Line AB is the model obtained from the first screening and point C is the centre point for this screening. Line AC is the model obtained from the second screening and point D is the centre point for this screening.

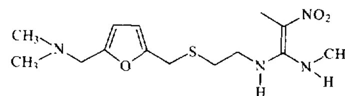

Fig. 2. Structure of ranitidine.

significant factors and central composite designs to optimize these factors. For an experimental response for each electrophoretic result, the chromatographic exponential function (CEF, Eq. (2)), an objective function that describes the quality of each experimental run in terms of separation and time of final elution, has been developed [13].

$$CEF = ((\sum_{i=1}^{n-1} (1 - e^{a(R_{opt} - R_i)})^2) + 1)(1 + \frac{t_f}{t_{max}}) \quad (2)$$

The optimum resolution, R_{opt}, was set at 1.5; the value a, which adjusts the weighting of the resolution term relative to the time term, was set at 3, and t_{max}, the maximum acceptable time, was set at 10 min. t_f is the final peak elution time, R_i is the resolution of the ith peak pair for each experimental result and n is the number of expected peaks. The absence of a distinguishable peak is treated as that peak having a resolution of zero. The objective is to minimise the CEF. An optimum electropherogram with all peaks resolved to at least R_{opt} and with a total elution time of less than t_{max} would have a CEF value of between 1 and $2n$.

The objective function can be considered to represent the 'quality' of the total electropherogram, which, in essence, gives equal weighting to all components. Thus, no attempt is made to neglect compounds that may not be 'critical' for the separation. This compares with a recent approach by Altria and Filbey [14] who appear to have chosen the resolution between a minor impurity peak and a major drug peak as one of the responses in a robustness study of a CE method for drug analysis.

2. Experimental

2.1. Instrumentation

The CE system used was a Lauerlabs Prince Capillary Electrophoresis system (Emmen, Netherlands) equipped with a Lauerlabs Lambda 1000 UV–Vis variable-wavelength detector. Data were acquired and manipulated using Shimadzu CLASS-LC10 software version 1.41 (Shimadzu Oceania, Sydney, Australia). All pH measurements were made using a Hanna Instruments Laboratory pH meter, Model HI 8519 (Woonsocket, RI, USA)

2.2. Column preparation

Uncoated fused-silica capillaries (50 μm I.D.×375 μm O.D.) were obtained from SGE International (Ringwood, Australia) and cut to suitable lengths. A detection window was created by removing a portion of the polyimide coating from the capillary at an appropriate distance from the outlet end of the capillary. Columns were prepared by etching with 1 *M* NaOH for 1 h prior to initial use and by flushing for a minimum of 15 min with each new buffer used. Columns were also flushed with 1 *M* NaOH at the end of each day and stored overnight in Milli-Q water (Millipore Australia, Lane Cove, Australia). Column lengths were 63 cm (50 cm effective length).

2.3. Capillary electrophoresis

Sample was injected using a 100-mbar hydrodynamic injection for 6 s. All other analysis conditions were set according to the screening and central composite designs. Where temperature was not a controlled factor, the runs were conducted at ambient temperature (23–25°C). The temperature was recorded for every run. Detection was at 214 nm.

2.4. Screening and central composite designs

Screening and central composite designs were generated, results analysed and linear and full quadratic models generated, respectively, by regression using Minitab for Windows version 10.5 (Minitab, State College, PA, USA). Surface plots were generated using Statistica version 4.5 (Statsoft, Hamburg, Germany). The optimum conditions were calculated by canonical analysis [1], using Mathcad version 5 (MathSoft, Cambridge, MA, USA).

2.5. Buffer preparation

Appropriate masses of analytical-reagent grade NaH_2PO_4 (BDH, Kilsyth, Australia) and α-cyclodextrin (α-CD; 99%, Sigma, St. Louis, MO, USA)

were dissolved, where appropriate, in a small quantity of Milli-Q water and an appropriate volume of HPLC-grade methanol (BDH), which were added before the solution was made up to volume with more Milli-Q water. The pH of the buffer solutions was adjusted using 1 *M* NaOH or 1 *M* HCl, as required.

2.6. Sample preparation

Samples were prepared in Milli-Q water. Ranitidine and related compounds were provided by Glaxo Australia. The analyte mixture used during the screening and optimization procedure contained ranitidine-related compound 1 (135 mg/l), ranitidine-related compound 2 (90 mg/l), ranitidine-related compound 3 (180 mg/l), ranitidine hydrochloride (RHCl, 265 mg/l) and ranitidine-related compound 4 (90 mg/l). Peak identities were confirmed by spiking sample solutions with the analyte being determined.

3. Results and discussion

The approach to screening for the present optimization study is to run a two-level (high/low) fractional factorial or Plackett–Burman design with centre points. If there is an indication of curvature (using Eq. (1)) then the design will be rerun using either the high or low level, along with the centre point of the previous design, as the new levels. A comparison of the linear models of the two designs will give an indication of the significant variables within the tested range. If there is a second order relationship, there will be a difference in the significance of the variable between the two screening models. For example, in Fig. 1, the main effect for the first screening design is represented by the straight line, AB. This factor is insignificant; however, the centre point C indicates that there is an overall curvature. This centre point is the response when all factors are set at their centre values. The second screening design gives as its main effect model, for the same factor, the line AC. The centre point this time is point D. The overall curvature has decreased and the magnitude of the coefficient (the gradient of the line) has increased in comparison to the first design. The comparison of the two designs indicates that this factor has a minimum within the region of the first design, not necessarily in the region of the second design. The curved line is a proposed second order response. This line will not be known unless an experimental design with at least three levels is run, such as a central composite design.

The six variables screened in the design were the percentage of methanol, α-cyclodextrin concentration, voltage, temperature, pH and buffer concentration. The factors were chosen on the basis of their potential to have a significant impact on the analysis being studied. There are many parameters to be considered when developing a CE method and many of these parameters can have significant interactions. These interactions often mean that the effect of a change in one variable on the overall separation will not be immediately obvious. The effects of changing many of the experimental parameters found in CE are discussed by McLaughlin et al. [15] and Li [16].

The run voltage used has a major effect on a CE separation as it influences the time required to complete the analysis and the efficiency of the separation. Since it changes the analysis time, the voltage will also affect the CEF value. The higher the voltage used, the quicker and more efficient the separation will be (usually). This may suggest that the optimum voltage would be the highest possible. This is not always the case, since joule heating may occur and the heat produced may be greater than the heat dissipation capabilities of the system. Resulting thermal gradients across the capillary may cause band broadening and hence a loss of efficiency. As a result of these conflicting effects, increasing the resolution by increasing the voltage is not always practical. The voltage must be quadrupled to double the resolution [17], p. 28, which means that the conditions at which joule heating occurs are approached rapidly.

Resolution is best improved by maximising the differences in the electrophoretic mobilities of the analytes. This can be achieved by manipulation of the buffer composition, pH and modifier content. Reduction of electroosmotic flow (EOF) will also improve the resolution. Organic modifiers such as methanol have been shown to cause a reduction in the EOF and also to increase the resolution in some

CE separations [18], p. 470. McLaughlin [15] discusses the effect of buffer composition and modifier content in CE, as do Weinberger [17] and Landers [18].

The pH of the buffer plays a significant role in CE separations. As the pH increases, more of the silanol groups at the capillary wall become ionized. The zeta potential increases, which increases the EOF (in the negative potential direction) and so, in this experiment, reduces the elution time. This will affect the value obtained for the CEF. The pH will also affect the charge observed on the analytes and thus affect the relative retention of the solutes and hence the selectivity of the analysis.

The use of CDs is common in chiral CE separations, as CDs exhibit chiral selectivity by virtue of forming inclusion complexes between the CD and the analyte. These inclusion complexes are dependent on the size and shape of the analyte molecules and the cavity size of the CD. This means that CDs can give a high degree of shape selectivity and have been employed in the run buffer for the isotachophoretic separation of inorganic anions, which was not a chiral separation [19]. CD was therefore included in the screening to see if the separation was affected by the shape selectivity of the CD towards the different solutes.

The temperature of the analysis can sometimes be important in CE, as fluctuations in the temperature can affect the viscosity of the buffer solution, leading to higher analyte electrophoretic mobilities and shorter analysis times. Temperature changes can also affect the pH of the buffer. The lower the temperature used the more effective the heat dissipation and therefore the wider the workable region before joule heating becomes a problem. Conversely, as many instruments are unable to provide adequate temperature control for ambient and sub-ambient temperatures, it can sometimes be beneficial to operate at a slightly higher temperature to minimise any fluctuations that can occur at temperatures that are near ambient temperature. A 10% increase in temperature can cause a 20% decrease in migration time ([17], p. 558; [18], p. 33).

The initial screening design was a 1/4 two-level fractional factorial design with six factors and four centre points. The design and levels are given in Table 1. The overall curvature obtained via Eq. (1) was extremely high (16 070) when compared to the average CEF of the centre points (11). A CEF value of 16 000 indicates that two components are completely overlapped. The positive value of c indicates that one or more variables was minimising within this range. A second set of screening runs was performed. The design was similar to the first except that the new levels were the minimum and the centre points of the first design. The curvature for this screening, although reduced (6535), was still significant. The average CEF was fourteen for the centre points. A second-order model was therefore needed for the optimization. For both screening designs the significance of a factor was determined using the results from regression analysis (Table 2). The table lists the regression coefficients, along with the standard errors of the coefficients, t-ratio for testing if the coefficients are zero, and the associated probability (P) value [20] for this test (the t-ratio is equal to the coefficient divided by the standard error). Using a 5% significance level, the coefficients are considered to be significantly different from zero (hence the factor significantly affects the response) if the P-value was less than 0.05. In both designs, the percentage of methanol and the buffer concentration were insignificant, with P values of 0.318 and 0.251, respectively, for the first design and 0.458 and 0.149, respectively, for the second design. These two factors were not considered in the optimization and were set at constant levels of 10% (v/v) methanol and 30 mM NaH_2PO_4. Although the methanol was not significant according to the screening designs, initial studies by Overall [11] indicated that having no methanol present was unsuitable, leading to broadened peaks. Hence, 10% methanol was chosen for all analyses. The temperature was insignificant at the 5% level for both screening designs but was significant within the 10% level for the second design. As there was still significant curvature in the design, this factor could have been included in the optimization. However, the Lauerlabs instrument was unable to effectively adjust the temperature over a sufficient length of the capillary (approximately 25–30% of the length) and, as a result, this variable was not included in the optimization. Separations were performed at the ambient laboratory temperature. Table 2 lists the coefficients and the P values for the pH, voltage and α-CD. In the first screening, voltage

Table 1
Design matrix for both screenings

Block	Methanol (%)	α-CD	pH	Voltage	Temperature	NaH_2PO_4
1	+	+	+	+	+	+
1	−	+	−	+	+	−
1	+	−	−	−	+	−
1	−	−	−	+	−	+
1	−	+	+	−	−	−
1	+	−	+	+	−	−
1	+	+	−	−	−	+
1	−	−	+	−	+	+
1	0	0	0	0	0	0
1	0	0	0	0	0	0
2	+	+	−	+	−	−
2	+	−	−	+	+	+
2	−	+	+	+	−	+
2	−	+	−	−	+	+
2	+	+	+	−	+	−
2	+	−	+	−	−	+
2	−	−	−	−	−	−
2	−	−	+	+	+	−
2	0	0	0	0	0	0
2	0	0	0	0	0	0

Factor	Screening 1 levels			Screening 2 levels		
	−1	0	1	−1	0	1
Methanol (%)	4	12	20	4	8	12
α-CD (m*M*)	1	5.5	10	1	3.25	5.5
pH	2	5.5	9	2	3.75	5.5
Voltage (kV)	10	20	30	10	15	20
Temperature (°C)	24	42	60	24	33	42
NaH_2PO_4 (m*M*)	20	35	50	20	27.5	35

Runs were performed in random order within each block.

was significant, pH was insignificant and α-CD was bordering on significant. In the second design, both voltage and α-CD were insignificant, whereas pH was significant. As discussed earlier, due to the significant changes in the coefficients (gradients of the main effects plots) and the *P*-values (significance) of the pH and voltage between the two screening designs, these two factors needed to be considered in the optimization. It was difficult to determine whether α-CD should be included. Since only two factors were identified as having to be included in the optimization out of the six that were studied, the α-CD was also included as the third factor.

In considering both designs, a five-level central composite design was used to optimize the variables,

Table 2
Coefficients, *t* ratios and probability values for the significant factors

	1st Screening			2nd Screening		
	Coefficient	*t* ratio	*P* value	Coefficient	*t* ratio	*P* value
pH	726	0.650	0.562	−4902	−3.277	0.022
Voltage (kV)	−6095	−5.456	0.012	−596	−0.398	0.707
α-CD (m*M*)	2957	2.647	0.077	−931	−0.622	0.561

Table 3
First central composite design

Run	Block	pH	Voltage (kV)	α-CD (m*M*)	T_r	R1,2	R2,3	R3,4	R4,5	CEF	Ln(CEF)
1	1	7.58	14	8	9.967	9.25	1.14	1.53	1.07	25.400	3.235
2	1	3.42	26	2	10.632	33.43	12.18	6.49	3.73	10.311	2.333
3	1	7.58	26	8	5.183	8.16	1.19	3.29	0	12 039.201	9.396
4	1	3.42	14	8	23.957	30.67	10.86	6.11	3.24	16.942	2.830
5	1	5.5	20	5	10.203	23.51	8.29	4.99	2.42	9.854	2.288
6	1	3.42	14	2	24.613	32.32	11.41	6.01	3.57	17.293	2.850
7	1	7.58	14	2	10.26	9.96	2.34	0.94	1.95	45.488	3.817
8[a]	1	3.42	26	8	0	0	0	0	0	31 697.199	10.364
9	1	5.5	20	5	9.724	23.37	8.01	4.78	2.4	9.606	2.262
10	1	7.58	26	2	4.831	10	1.79	3.1	0	11 757.084	9.372
11	1	5.5	20	5	8.583	20.46	7.1	4.21	2.2	8.863	2.182
12	1	5.5	20	5	8.522	20.03	6.89	4.13	2.16	8.783	2.173
13	2	5.5	20	0	6.859	12.38	4.02	2.25	2.03	7.474	2.011
14	2	5.5	9.9	5	14.798	14.53	4.38	2.43	2.01	11.145	2.411
15	2	5.5	30.1	5	5.124	12.93	4.12	2.4	1.94	6.664	1.897
16	2	5.5	20	10	7.195	12.25	3.58	2.07	1.48	6.312	1.842
17	2	5.5	20	5	7.371	14.84	4.7	2.8	2.05	8.013	2.081
18	2	5.5	20	5	7.357	15.26	4.86	2.83	2.08	8.059	2.087
19	2	2	20	5	10.876	24.22	1.64	3.72	0	16 548.749	9.714
20	2	9	20	5	6.625	6.64	2.01	0	0	26 351.811	10.179

[a]Column blockage occurred for this experiment even upon repetition.

pH, voltage and α-CD concentration. The experimental design and results are listed in Table 3. The large differences in response values make it difficult to adequately fit a response model to the data using multi-linear regression. To alleviate this problem, the natural log transformation [1] of the CEF responses was used. This transformation results in contracting the high end of the range and expanding the lower end of the range. As the CEF achieves an optimum at the lower end of the range, this means that the region around the optimum is expanded. The *P*-values and coefficients from the resulting response model (Table 4) indicate that α-CD is insignificant and could therefore be left out of the buffer system. Note that in Experiment 13 (Table 3), the resolution was still good in the absence of α-CD. The pH is the most significant variable followed by the interaction of pH and voltage. It is difficult from this model to locate the minimum (Fig. 3). As a result, another central composite experimental design with thirteen runs was carried out with a narrower pH range and the same voltage range, with the design and results given in Table 5. This new response model (Fig. 4) has a better defined optimum region. The minimum was calculated, using canonical analysis, to be at a pH of 4.87 and a voltage at 23.8 kV. Fig. 5 is the electropherogram obtained using these conditions. The effects of selection of pH or voltage at values away from the optimum is illustrated in Fig. 6. At a pH of 2, the resolution is very poor for two components, with only four peaks now observed (Fig. 6a). At lower voltages, the analysis time becomes

Table 4
Response model coefficients and probability values (*t* ratio) from the first central composite design

Factor		Coefficient	*P* value
Constant	b_0	2.1976	0.008
Block		−0.0464	0.902
pH	b_1	1.1258	0.038
Voltage	b_2	0.7841	0.123
α-CD	b_3	0.0013	0.998
pH*pH	b_{11}	2.7013	0.000
Voltage*Voltage	b_{22}	−0.0507	0.903
α-CD*α-CD	b_{33}	−0.1336	0.752
pH*Voltage	b_{12}	1.4814	0.048
pH*α-CD	b_{13}	−0.1771	0.787
Voltage*α-CD	b_{23}	0.1882	0.774

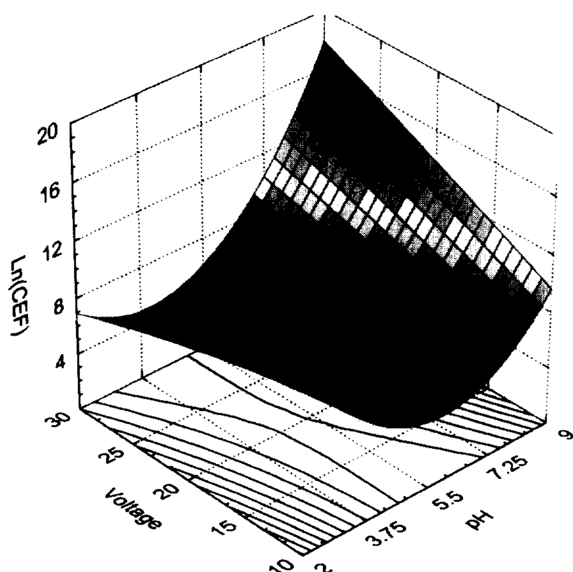

Fig. 3. Response model for the central composite design in Table 3. α-CD is held at 0 m*M*.

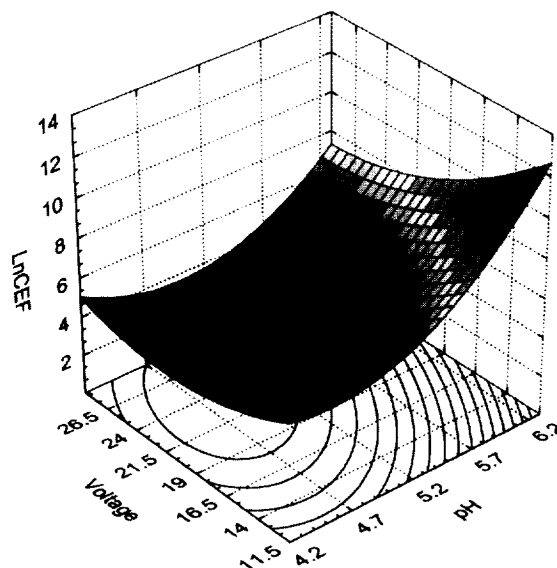

Fig. 4. Response surface model for the central composite design in Table 5.

significantly longer (Fig. 6b) and so, even though the resolution is good, in terms of the efficiency of the analysis, there is little advantage over the optimum electropherogram (Fig. 5).

The reproducibility of the system was determined by performing six runs under the optimum conditions. The reproducibility data are shown in Table 6. There is a significant improvement in the R.S.D. when adjusted migration times are considered, over unadjusted migration times. Reproducibility could possibly be further improved by extending the buffer equilibration time prior to analysis or using an effective form of temperature control.

4. Conclusion

Optimal conditions were obtained for the separation of ranitidine hydrochloride and four related compounds by capillary electrophoresis by applying

Table 5
Second central composite design

Run	Block	pH	Voltage (kV)	T_f	R1,2	R2,3	R3,4	R4,5	CEF	Ln CEF
1	1	5.25	11.5	10.618	11.08	3.58	1.45	0.45[a]	1 036.107	6.943
2	1	6.00	14	8.075	7.36	3.21	0.54	1.85	517.181	6.248
3	1	4.19	20	7.491	19.18	6.25	3.61	2.07	8.164	2.100
4	1	5.25	20	6.755	14.92	4.53	2.57	2.04	7.647	2.034
5	1	5.25	20	6.677	14.31	4.39	2.45	1.98	7.454	2.009
6	1	5.25	20	6.64	13.76	4.33	2.4	2.07	7.556	2.022
7	1	6.00	26	4.621	8.44	3	0.98	1.96	25.831	3.252
8	1	6.31	20	5.957	7.37	2.6	1.76	0	12 649.546	9.445
9	1	5.25	28.5	4.35	11.77	3.64	1.99	1.95	5.939	1.781
10	1	5.25	20	6.558	12.4	3.77	1.95	1.99	6.854	1.925
11	1	4.50	14	12.784	21.81	7.5	4.35	2.23	10.910	2.390
12	1	4.50	26	6.5	20.96	7.27	4.28	2.22	7.891	2.066
13	1	5.25	20	7.633	17.75	5.71	3.38	2.1	8.269	2.113

[a] Under these conditions, a small unidentified peak appeared in the electropherogram between peaks 3 and 4.

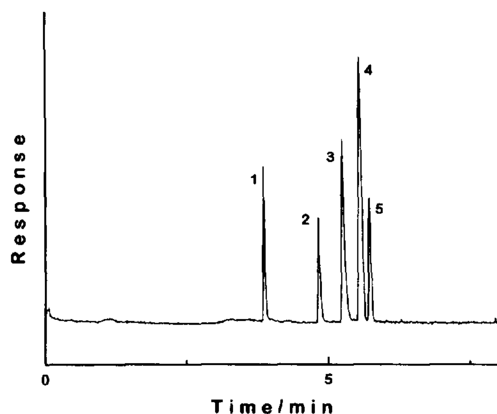

Fig. 5. Electropherogram of ranitidine hydrochloride and related compounds under the optimum conditions. Peak identities: 1 = ranitidine-related compound 1; 2 = ranitidine-related compound 2; 3 = ranitidine-related compound 3; 4 = ranitidine hydrochloride; 5 = ranitidine-related compound 4. Buffer conditions: 30 m*M* NaH_2PO_4, 10% (v/v) methanol, pH 4.87. Instrumental conditions: 23.8 kV, ambient temperature (24±1°C)

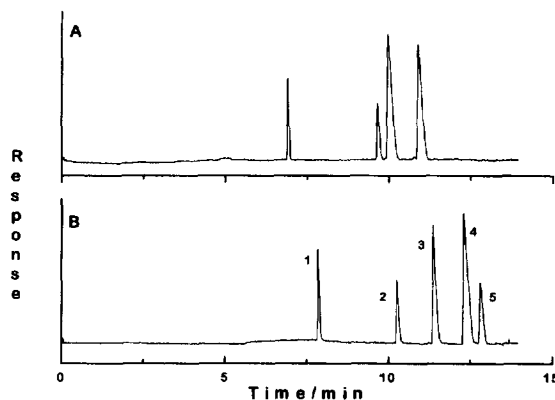

Fig. 6. The effect of pH and voltage on the separation of ranitidine and related compounds. Electropherogram A: pH 2, 20.0 kV. Electropherogram B: pH 4.5, 14.0 kV. Buffer conditions: 30 m*M* NaH_2PO_4, 10% (v/v) methanol, 5 m*M* α-CD. Instrumental conditions: ambient temperature (24±1°C). Peak identities for electropherogram B are as for Fig. 5.

Table 6
Reproducibility data at predicted optimum conditions

	t_{mig}/min	$t^{a}_{mig, adj}$/min	Area (%)[b]	Height (%)[c]
Peak 1	3.787 (1.91)[d]	0.681 (0.70)	41.96 (2.96)	53.91 (8.35)
Peak 2	4.727 (2.11)	0.849 (0.46)	30.15 (9.03)	34.31 (8.75)
Peak 3	5.118 (2.22)	0.920 (0.34)	65.21 (3.07)	61.20 (8.15)
Peak 4	5.395 (2.61)	0.969 (0.32)	100.00 (−)	100.00 (−)
Peak 5	5.564 (2.54)	1.000 (−)	33.60 (2.30)	46.64 (3.38)

Buffer: 30 m*M* NaH_2PO_4, 10% (v/v) methanol, pH 4.87.
[a] All $t_{mig, adj}$ values are calculated relative to t_{mig} for Peak 5.
[b] Area (%) values are calculated on adjusted areas (i.e. area/t_{mig}) and normalised with respect to Peak 4.
[c] Normalised with respect to Peak 4.
[d] %R.S.D. values are given in parentheses, $n=6$.

a strategy involving both fractional factorial and central composite experimental design. There was a need to apply two factorial designs to screen for significant factors, due to significant curvature in the first design. As a result, voltage and pH were determined to be the most significant factors and were optimized using a central composite design. The optimum conditions were determined to be pH = 4.87 at a run voltage of 23.8 kV, with buffer comprising 30 m*M* NaH_2PO_4 and 10% (v/v) methanol using a 63 cm (50 cm effective length)×50 μm I.D. capillary, giving completely resolved peaks within 6 min. The analysis was performed at ambient temperature.

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