ELSEVIER

Journal of Chromatography B, 697 (1997) 207–215

JOURNAL OF CHROMATOGRAPHY B

Fractional factorial design optimization of the separation of pilocarpine and its degradation products by capillary electrophoresis

Karin Persson*, Ove Åström

Pharmaceutical R&D, Astra Läkemedel AB, SE-151 85 Södertälje, Sweden

Abstract

The separation of pilocarpine and its degradation products by micellar electrokinetic capillary chromatography (MECC) has been optimized by using fractional factorial design of the experiments. Critical parameters were identified in a screening design, and an optimization design was used to optimize the separation. The optimal separation method was based on a borate buffer with sodium dodecyl sulfate (SDS). It is concluded that by using fractional factorial design it is possible to improve the separation of pilocarpine, its *trans* epimer, isopilocarpine and their hydrolysis products, pilocarpic acid and isopilocarpic acid. ©1997 Elsevier Science B.V. © 1997 Elsevier Science B.V.

Keywords: Fractional factorial design; Pilocarpine; Isopilocarpine; Pilocarpic acid; Isopilocarpic acid

1. Introduction

Pilocarpine, isolated from *Pilocarpus jaborandi* Holmes, is used as a solution of the monohydrochloride to reduce intraocular tension in the treatment of open-angle glaucoma, or to cause miosis.

The formulation is best tolerated when its acid–base equilibria are kept close to the physiological pH of the eye, 7.4. Pilocarpine is chemically stable in acid solutions up to pH 5 but degrades by epimerization to the *trans* epimer, isopilocarpine, with increasing alkalinity. The diastereomers reversibly hydrolyze to their respective acid forms when exposed to heat and/or acid–base-catalyzed hydrolysis, Fig. 1. Both the epimerization and the hydrolysis result in a loss of the pharmacologic and therapeutic effects of the alkaloid.

Several reports describe the separation of pilocarpine from its degradation products. Reversed-phase high-performance liquid chromatography (HPLC) [1–4] is used in some cases, and ion-exchange HPLC [5,6] in others. However, unsatisfactory re-

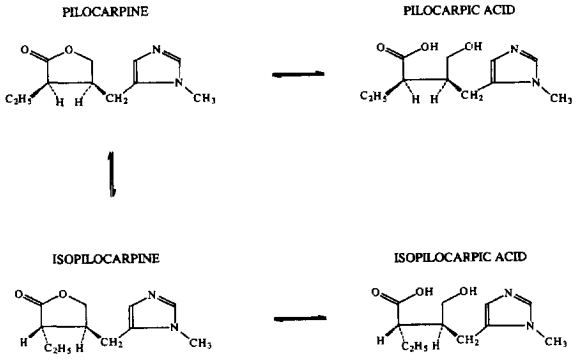

Fig. 1. Structure of pilocarpine and its degradation products.

*Corresponding author.

PII S0378-4347(97)00108-4

sults are obtained when selectivity, peak symmetry and retention times are considered.

Capillary electrophoresis (CE) is an increasingly applied technique for the separation of pharmaceutical stereoisomers, degradation products and impurities [7–10]. By using micellar electrokinetic capillary chromatography (MECC) it is possible to separate pilocarpine and its potential degradation products within a single analysis [11].

Baeyens et al. [12] reached a full baseline separation of pilocarpine and isopilocarpine with CE using the addition of β-cyclodextrin (β-CD) to a phosphate buffer, but did not succeed in separating the two acids.

Factorial designs are based on systematic multivariate optimization schemes instead of univariate procedures. In a statistical experimental design the variables, i.e., the factors, are thus varied at the same time, making it possible to distinguish between effects, e.g., responses, caused by a single variable or by interacting variables. Replicating center-points can be added to provide protection against curvature (quadratic effects) caused by interactions in the model and to obtain an independent estimate of the error. It is possible to reduce the number of experiments by focusing on the main effects and to run only a fraction of the complete factorial experiment, a so-called fractional factorial design.

Several reports [13,14] describe this approach in the optimization and robustness testing of CE-based methods. Some examples of different designs used in HPLC and CE are the Plackett–Burman design [15–17], the central composite design [18] and the fractional factorial design [19,20].

The aim of the present study was to optimize the separation of pilocarpine, isopilocarpine and their hydrolysis products, pilocarpic acid and isopilocarpic acid, respectively, by using a fractional factorial design of the experiments. The optimal separation method should have peak symmetries close to 1.0, the shortest possible migration time for the last peak, pilocarpine, and complete resolution, $R_s > 1.5$, between all four peaks.

A screening design, where pH was kept constant, was used to identify critical parameters and another design, where pH was included, was used to optimize the separation. The latter was based on the results of the screening.

2. Experimental

2.1. Equipment

Capillary electrophoresis was performed on a Hewlett-Packard ^{3D}CE instrument (Walbronn, Germany), with a built-in diode-array detector. The data were recorded with the matching ^{3D}CE ChemStation software.

Fused-silica (FS) capillaries from Polymicro (Phoenix, AZ, USA) were used. The total length (L_t) was 56 cm and the length to the detector (L_d) was 47.5 cm. The outer diameter was 363 μm and the inner diameter was 50 μm. The pK_a values of the acids were determined by acid–base titration with a Sirius PCA101 instrument (Sirius Analytical Instruments, Forest Row, UK).

2.2. Chemicals

Pilocarpine hydrochloride was obtained from Merck (Darmstadt, Germany). Isopilocarpine nitrate, sodium dodecyl sulfate (SDS) and β-CD were purchased from Sigma (St. Louis, MO, USA). Hydroxypropylmethylcellulose (HPMC), 50 cP, came from Shin Etsu/Syntapharm (Naoetsu, Japan). Purified water was obtained from a Waters Milli-Q system (Watford, Herts, UK). All the chemicals used for the buffers were of analytical grade.

2.3. Procedures

The concentrations of the samples corresponded to 330 μg/ml pilocarpine, 33 μg/ml isopilocarpine and 60 μg/ml of the two acids. The preparation of pilocarpic acid and isopilocarpic acid was done by means of base-catalyzed hydrolysis according to [21]. The ionic strength was kept approximately eight times lower in the samples than in the background electrolyte (BGE) to achieve stacking conditions [22]. Phosphate and borate buffers were prepared by mixing solutions of H_3PO_4/NaOH and H_3BO_3/NaOH, respectively, to give the desired ionic strength (I) and, through adjustment, the desired pH. The ionic strength for the buffers were calculated according to the formula

$$I = \frac{1}{2}\sum_i C_i^* Z_i^2$$

where C_i is the concentration of ion "i" and Z_i is the charge of the ion "i", before the addition of SDS.

Buffers and samples were filtered through a membrane with a pore size of 0.5 μm.

The samples were injected hydrodynamically towards the cathode for 3 s at a pressure of 5 kPa.

Between the runs, the capillary was flushed for 5 min with 0.1 *M* NaOH, 5 min with Milli-Q water and 10 min with BGE.

2.4. Experimental designs and calculations

All experiments were carried out in duplicate in random order.

Partial least squares, PLS [23], in the program Modde 3.0 (Umetri AB, Umeå, Sweden) was used as the multivariate method to establish the quantitative relations between the responses versus the factors. Analysis of variance, ANOVA, was used in the testing for statistically significant factors ($p<0.05$). The test partitions the total source of variation into one part due to the regression model, another due to the residuals, i.e., a lack of fit coefficient, and finally, a pure error coefficient. The predicted measure corresponding to the measure of fit was estimated by the Q^2 value and the percentage of the response explained by the model was estimated by the R^2 value. These values usually lie in the range 0–1, values close to 1 indicating a good model with excellent predictive value.

2.4.1. Screening design

The following six factors which could effect the separation were chosen for the screening design: temperature, voltage, ionic strength, concentration of SDS, concentration of HPMC and concentration of β-CD. The factors were varied at two levels, a high level (+1) and a low level (−1), Table 1. The pH of the phosphate buffer was kept constant (7.0), close to the optimum pH (6.9) according to Baeyens et al. [12]. A neutral hydrophilic polymer, HPMC, which can adsorb to the capillary wall and effect the electroosmotic flow (EOF) was added to the system. Furthermore, SDS and β-CD were added to determine if a selector was needed for the separation. A $2^{(6-2)}$ fractional factorial design, known as a resolution IV design, was chosen. The scheme resulted in 19 experiments, including three replicates of the

Table 1
Experimental design for the screening experiment

Expt. No.	Temperature (°C)	Voltage (kV)	Ionic strength	SDS (m*M*)	HPMC (%, w/v)	β-CD (m*M*)
1	30	15	0.01	82	0.2	9
2	50	15	0.01	82	0.8	9
3	30	25	0.01	82	0.8	16
4	50	25	0.01	82	0.2	16
5	30	15	0.1	82	0.8	16
6	50	15	0.1	82	0.2	16
7	30	25	0.1	82	0.2	9
8	50	25	0.1	82	0.8	9
9	30	15	0.01	160	0.2	16
10	50	15	0.01	160	0.8	16
11	30	25	0.01	160	0.8	9
12	50	25	0.01	160	0.2	9
13	30	15	0.1	160	0.8	9
14	50	15	0.1	160	0.2	9
15	30	25	0.1	160	0.2	16
16	50	25	0.1	160	0.8	16
17	40	20	0.055	121	0.5	12.5
18	40	20	0.055	121	0.5	12.5
19	40	20	0.055	121	0.5	12.5

Six factors varied at two levels and center points replicated three times. Fractional factorial design: $2^{(6-2)}=16$ experiments+3 center-points. All experiments were carried out in duplicate and in random order.

center-point experiment, level (0). Such a design means that the main effects and the two-factor interaction are differentiated, while the two-factor interactions are aliased with other two-factor interactions.

2.4.2. Optimization design

Five factors were varied at two levels, a high level (+1) and a low level (−1): ionic strength, concentration of SDS, pH, temperature and voltage, Table 2. A borate buffer was used to make it possible to vary the pH between 9.5 and 10.5. In this pH range, pilocarpine and isopilocarpine are positively charged, and the corresponding acids have a net zero charge. A $2^{(5-1)}$ fractional factorial design was chosen, known as a resolution V design, which resulted in 16 experiments. In addition, the center-point experiment, level (0), was replicated three times. This means that the main effects and the two-factor interaction are separated, while two-factor interactions are aliased with the three-factor interactions.

2.4.3. The responses

The effects of the separation variables are measured by the responses. These were the same in the two designs, i.e., the symmetry of peak 3, the symmetry of peak 4, the resolution between peaks 1 and 2, the resolution between peaks 3 and 4 and finally the migration time for peak 4. The peak symmetry for peaks 1 and 2 was close to 1.0 in all cases and the resolution between peaks 2 and 3 was always greater than 1.5, and was therefore not included in the responses. The elution order was the same in all the experiments: isopilocarpic acid (peak 1), pilocarpic acid (peak 2), isopilocarpine (peak 3) and pilocarpine (peak 4).

3. Results and discussion

The reported pK_a values for pilocarpine are 7.15 and 12.57. Only one pK_a value is reported in the literature for isopilocarpine. According to [24] the value is 7.17. The pK_a values for pilocarpic acid and

Table 2
Experimental design for the optimization experiment

Expt. No.	Ionic strength	SDS concentration (m*M*)	pH	Temperature (°C)	Voltage (kV)
1	0.01	100	9.5	20	30
2	0.03	100	9.5	20	20
3	0.01	170	9.5	20	20
4	0.03	170	9.5	20	30
5	0.01	100	10.5	20	20
6	0.03	100	10.5	20	30
7	0.01	170	10.5	20	30
8	0.03	170	10.5	20	20
9	0.01	100	9.5	30	20
10	0.03	100	9.5	30	30
11	0.01	170	9.5	30	30
12	0.03	170	9.5	30	20
13	0.01	100	10.5	30	30
14	0.03	100	10.5	30	20
15	0.01	170	10.5	30	20
16	0.03	170	10.5	30	30
17	0.02	135	10	25	25
18	0.02	135	10	25	25
19	0.02	135	10	25	25

Five factors varied at two levels and center points replicated three times. Fractional factorial design: $2^{(5-1)}=16$ experiments+3 center-points. All experiments were carried out in duplicate and in random order.

isopilocarpic acid were found to be 2.38 and 7.17 by acid–base titration.

3.1. Screening study

The factors which significantly affected one or more of the responses at a 95% confidence level were temperature, voltage, ionic strength and concentration of SDS (Fig. 2). The shaded bars show half the effect of each factor on the separate responses, i.e., how much a response will change if the factor is varied from the low level (−1) to the center-point (0), or from the center-point (0) to the high level (+1). The confidence interval is shown as error bars, and an effect is not significant if a bar crosses the *x*-axis. Considering, for example, the migration time coefficients, a change in the voltage from −1 to 0, or from 0 to +1, will reduce the migration time with approximately 3 min. Ionic strength and concentration of SDS showed the largest effects. A low ionic strength resulted in an incomplete resolution between peaks 3 and 4, but a faster migration time and symmetrical peaks. A high SDS concentration resulted in complete resolution between peaks 3 and 4 and symmetrical peaks, but with a longer migration time due to stronger interaction of the analytes with the micelles. Altering the concentration of HPMC or β-CD did not have any significant effect and it was therefore concluded that neither HPMC nor β-CD were needed for the separation.

A mathematical model was created from the results of the screening experiments. The variations of the responses explained by the model, R^2, were 0.5–0.9, and the variations of the responses that could be predicted by the model, Q^2, were 0.2–0.7. The degree of explanation was not increased by log-transformation or by adding a quadratic term to the model.

3.2. Optimization study

The factors and levels chosen for the optimization design were based on the results of the screening design, which showed that MECC should be used, and on the optimal conditions used by Charman et al. [11]. pH had a great effect according to Ref. [11] and was therefore increased and included in the optimization design.

From Fig. 3, it is apparent that all main effects, and the two-factor interactions, ion*pH (B), ion*Te (C), SDS*pH (E), SDS*Te (F), pH*Vo (G) and Te*Vo (H), have a significant effect at a 95% confidence level on one or more of the responses. The voltage and the concentration of SDS caused the largest effects. A short migration time was obtained when the voltage was kept at a high level. This is due to a higher field strength. Good peak symmetries and complete resolution were reached when the concentration of SDS was high.

Initially, when the model included the main effects and all two-interaction effects, the degree of explanation for the responses in the model was low. The model was improved by adding a quadratic term (pH*pH), which indicated curvature in the model, and by removing the two-interaction effects which did not have any significant effect. The two-factor interactions ion*SDS (A) and ion*Vo (D) improved the model and were thus included. The positive pH*pH bars in Fig. 3 means that a change of a response is higher between the center-point (0) and the high level (+1) than between the low level (−1) and the center-point (0). The variations of the responses explained by the model were 0.9–1.0, and the variations of the responses that could be predicted by the model were 0.3–0.8, Fig. 4. The standard deviation of the center-points was lower than the standard deviation of all experiments, which indicates a good model.

3.3. Separation process

From the results it is apparent that a compromise is needed to reach an optimal separation. The resolution between the four peaks was greater than 1.5 in all the experiments and is therefore not a critical response. The most critical responses are found to be the symmetry of the main peak, pilocarpine, and the migration time, and these therefore have the greatest influence on the choice of the optimal separation process. A perspective three-dimensional response surface showing the symmetry for pilocarpine as a function of the most important factors, pH and voltage, can be seen in Fig. 5. The symmetry value is at a minimum at pH 10, exactly in

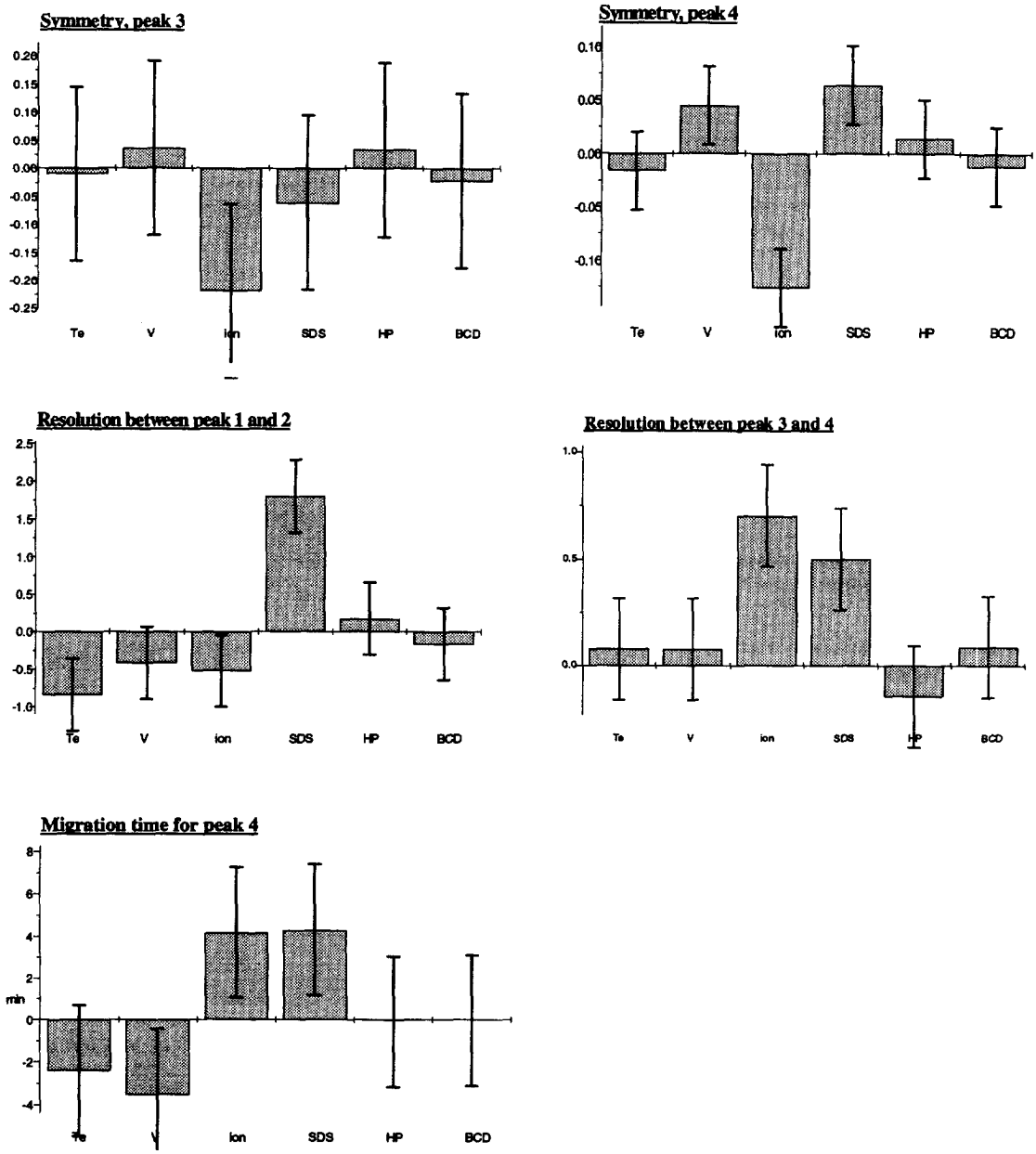

Fig. 2. Coefficient values in the screening design for the responses at a 95% confidence level. The coefficients are divided by the standard deviation of their respective response. For explanation, see Section 2.4.1. Factors: Te = temperature, V = voltage, ion = ionic strength, SDS = concentration of SDS, HP = concentration of HPMC and BCD = concentration of β-CD.

the middle of the two pK_a values of the compound, and improves at lower and higher pH levels.

The choice of the optimal conditions from these studies was due to the fact that the highest symmetry value was reached with the highest pH and voltage, and the fact that a high voltage also results in a short

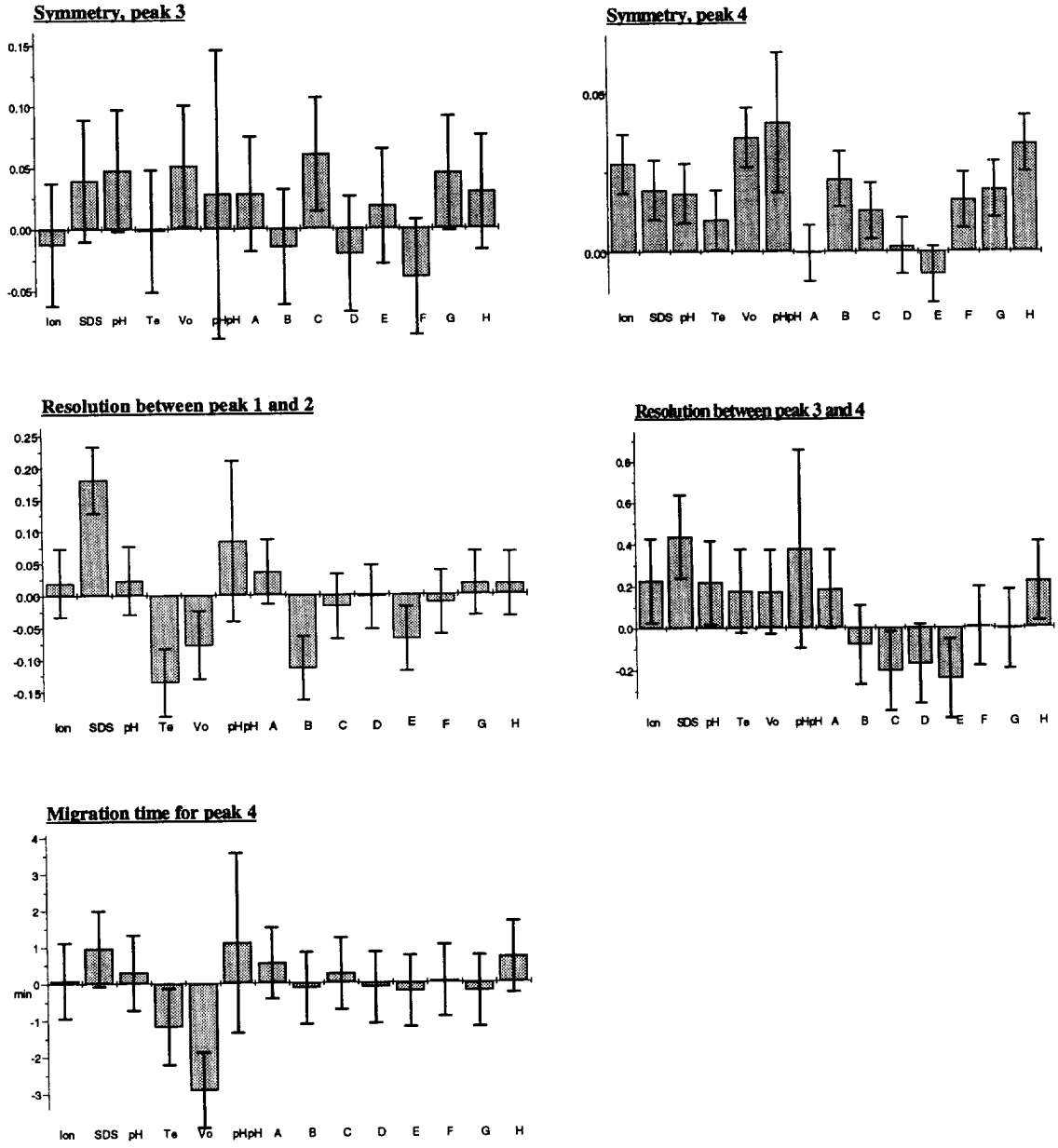

Fig. 3. Coefficient values in the optimization design for the responses at a 95% confidence level. The coefficients are divided by the standard deviation of their respective response. For explanation, see Section 2.4.2. Factors: Ion = ionic strength, SDS = concentration of SDS, pH = pH, Te = temperature, Vo = voltage, pHpH = pH*pH, A = ion*SDS, B = ion*pH, C = ion*Te, D = ion*Vo, E = SDS*pH, F = SDS*Te, G = pH*Vo and H = Te*Vo.

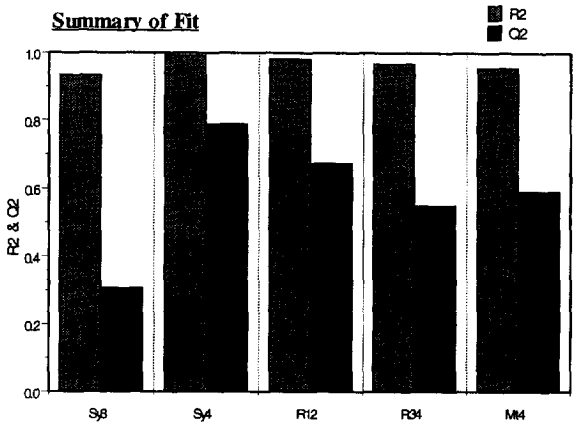

Fig. 4. Variations of the responses explained by the optimization design. The light shaded bars, R^2, denote the percentage of the response explained by the model. These values are between 0.9–1.0 for the responses where 1.0 indicates a good model. The dark shaded bars, Q^2, denote the variations of the responses that can be predicted by the model. These values are between 0.3–0.8 for the responses. A high Q^2 value, e.g., symmetry for peak 4, means that the model can calculate a good predictive value.

migration time. A high SDS concentration and ionic strength were also important not only for the symmetry, but also for a good resolution. This is probably due to a lower EOF and a reduced adsorption to the capillary wall. The temperature was kept low to reach a good resolution between all peaks.

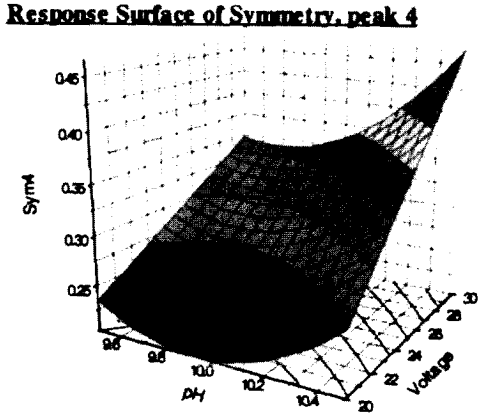

Fig. 5. Three-dimensional response surface showing the symmetry for pilocarpine as a function of pH and voltage. The surface shows how the symmetry value will change when varying pH and voltage and by keeping the other factors stable at: ionic strength 0.03, concentration of SDS 170 mM and temperature 30°C.

Table 3
The predicted results and the experimental results for the optimal separation

Response	Predicted value	Experimental value
Symmetry, peak 3	0.76	0.60
Symmetry, peak 4	0.27	0.24
Resolution between peaks 1 and 2	2.25	2.19
Resolution between peaks 3 and 4	6.45	6.22
Migration time for peak 4	6.81	6.92

The optimal separation is reached with a borate buffer of pH 10.5, ionic strength 0.03, 170 mM SDS, voltage 30 kV and temperature 20°C. Such conditions produce a high current of around 100 μA. The predicted values of the responses are compared with the experimentally obtained values in Table 3, and the results are close. An electropherogram of a separation obtained by using the optimized conditions is shown in Fig. 6.

4. Conclusions

An optimal separation of pilocarpine and its degradation products was reached with a borate buffer of pH 10.5 with an ionic strength of 0.03, the SDS concentration corresponding to 170 mM and the voltage and the temperature set to 30 kV and 20°C, respectively. Using these conditions, a baseline separation between the peaks is reached within 7 min and the symmetry of pilocarpine is at its experimental optimum. The results predicted from the model were shown to correspond well with the experimental results. Hence by using a statistical experimental design it is possible to find the conditions for obtaining improved separation between pilocarpine, its *trans* epimer, isopilocarpine and their hydrolysis products, pilocarpic acid and isopilocarpic acid.

Acknowledgments

The authors wish to thank Ms. Anna Maria Enlund, Ms. Valérie Imbert and Mr. Mats Borén for skillful experimental assistance and Mrs. Valérie Harang for chemometric assistance.

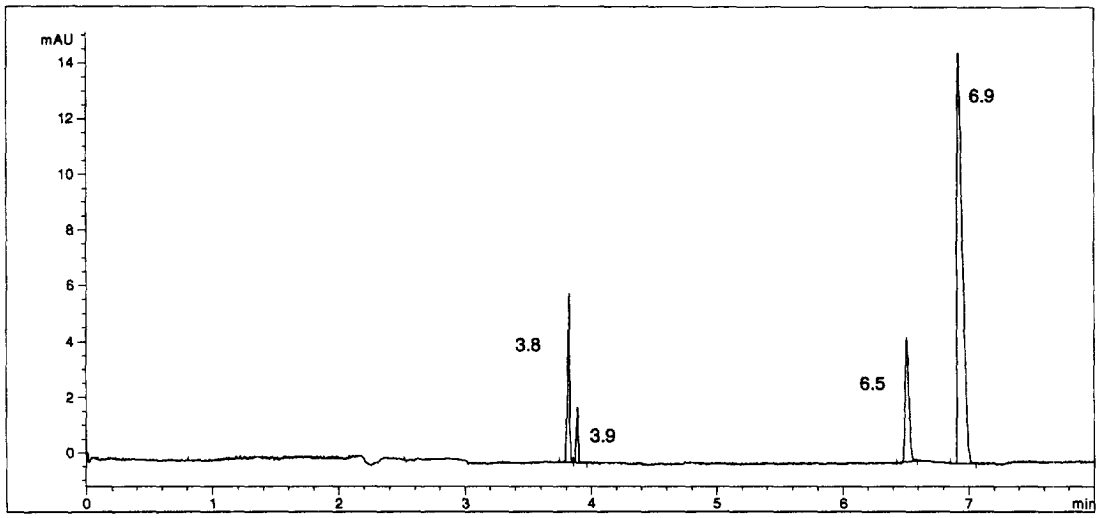

Fig. 6. Optimal separation of pilocarpine and its degradation products. Capillary, 47.5 cm (L_d), 56 cm (L_t), 50 μm I.D., 363 μm O.D. FS; borate buffer pH 10.5 made from H_3BO_3/NaOH to ionic strength 0.03, 170 m*M* SDS; voltage 30 kV; temperature 20°C; wavelength 225 nm; injection: pressure (3 s, 5 kPa); sample concentration, pilocarpine (330 μg/ml), isopilocarpine (33 μg/ml), pilocarpic acid and isopilocarpic acid (60 μg/ml).

References

[1] J.J. O'Donnel, R. Sandman and M.V. Drake, J. Pharm. Sci., 69 (1980) 1096.
[2] A. Noordam, L. Maat and H.C. Beyerman, J. Pharm. Sci., 70 (1981) 96.
[3] A. Noordam, K. Waliszewski, C. Olieman, L. Maat and H.C. Beyerman, J. Chromatogr., 153 (1978) 271.
[4] M.V. Drake, J.J. O'Donnel and R. Sandman, J. Pharm. Sci., 71 (1982) 358.
[5] J.D. Weber, J. Assoc. Off. Anal. Chem., 59 (1976) 1409.
[6] T. Urbátnyi, A. Piedmont, E. Willis and G. Manning, J. Pharm. Sci., 65 (1976) 257.
[7] G.M. McLaughlin, J.A. Nolan, J.L. Lindahl, R.H. Palmieri, K.W. Andersson, S.C. Morris, J.A. Morrison and T.J. Bronzert, J. Liq. Chromatogr., 15 (1992) 961.
[8] T.E. Peterson, J. Chromatogr., 630 (1993) 353.
[9] K.D. Altria, J. Chromatogr. A, 735 (1996) 43.
[10] C.L. Ng, C.P. Ong, H.K. Lee and S.F.Y. Li, J. Chromatogr. A, 680 (1994) 579.
[11] W.N. Charman, A.J. Humberstone and S.A. Charman, Pharm. Res., 9 (1992) 1219.
[12] W. Baeyens, G. Weiss, G. Van Der Weken, W. Van Den Bossche and C. Dewaele, J. Chromatogr., 638 (1993) 319.
[13] K.D. Altria, B.J. Clark, S.D. Filbey, M.A. Kelly and D.R. Rudd, Electrophoresis, 16 (1995) 2143.
[14] S.D. Filbey and K.D. Altria, J. Cap. Electrophor., 3 (1994) 190.
[15] R.C. Plackett and J.P. Burman, Biometrica, 23 (1946) 305.
[16] M.M. Rogan, K.D. Altria and D.M. Goodall, Chromatographia, 38 (1994) 723.
[17] S. Boonkerd, M.R. Detaevernier, Y. Vander Heyden, J. Vindevogel and Y. Michotte, J. Chromatogr. A, 736 (1996) 281.
[18] S.N. Deming and S.L. Morgan, Anal. Chim. Acta, 150 (1983) 183.
[19] G.E.P. Box, W.G. Hunter and J.S. Hunter, Statistics for Experimenters: An Introduction to Design, Data Analysis and Model Building, Wiley-Interscience, New York, 1978, p. 374.
[20] E. Morgan, Chemometrics: Experimental Design, Wiley, Chichester, 1991.
[21] A. Repta and T. Higuchi, J. Pharm. Sci., 60 (1971) 1465.
[22] A. Vinther and H. Soeberg, J. Chromatogr., 559 (1991) 3.
[23] A. Höskuldsson, J. Chemometrics, 2 (1988) 211.
[24] The Merck Index, Merck, Rahway, NJ, 9th ed., 1976.