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Optimisation by experimental design of a capillary electrophoretic method for the separation of several inhibitors of angiotensin-converting enzyme using alkylsulphonates

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Abstract

A statistical experimental design was used to optimise a capillary electrophoretic separation method for eight inhibitors of the angiotensin-converting enzyme: enalapril, lisinopril, quinapril, fosinopril, perindopril, ramipril, benazepril, and cilazapril. Because a free solution capillary electrophoresis system did not achieve a complete separation of these eight compounds in one run, the usefulness of alkylsulphonates as ion-pairing agents was investigated. After preliminary investigations to determine the experimental domain and the most important factors, a three-level full-factorial design was applied to study the impact of the pH and the molarity of the ion-pairing agent on the separation. Improved separations were obtained suggesting a favourable effect of ion-pairing interactions between analytes and the additive; however, it remained impossible to separate them all in one run. A combination of two systems was still necessary for the selective identification of these structurally-related substances.

Keywords: Experimental design; Optimisation; Angiotensine-converting enzyme inhibitors; Enzyme inhibitors; Alkyl sulphonates; Octanesulphonates

1. Introduction

Inhibitors of angiotensin-converting enzyme (ACE inhibitors) are widely used for the treatment of mild to moderate hypertension and heart failure, either alone or in combination with other drugs [2,3]. Until now, high-performance liquid chromatography has been the major technique used for the determination of ACE inhibitors [4–19]. This technique is also applied in the European Pharmacopoeia monographs on enalapril maleate, lisinopril dihydrate and ramipril [20].

Capillary electrophoresis (CE) has been used for the identification and quantification of eight ACE inhibitors [1,21]. Other studies have been limited to the determination and rotamer separation of enalapril

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maleate [22–24], lisinopril [25] and cilazapril [26]. One study reported the determination of fosinopril and its impurities [27]. Another study was limited to the determination of only four ACE inhibitors [28]. As a free solution CE system failed to reach a complete separation of these compounds [1], the usefulness of ion-pairing agents was investigated.

The introduction of micellar electrokinetic capillary chromatography (MEKC) not only has overcome the difficulty of separating neutral analytes in CE, but also has increased selectivity in the separation of charged molecules. Compounds having the same charges and similar structures often migrate at almost the same speed in CE, whereas their differences in distribution constants in the micellar phase lead to baseline separations [29]. Many examples demonstrating improved resolution by MEKC compared with CE have been published [30]. In this paper, we studied the usefulness of alkylsulphonates as ion-pairing agents in the separation of eight ACE inhibitors: enalapril, lisinopril, quinapril, fosinopril, perindopril, ramipril, benazepril, and cilazapril [28,31–38]. A statistical experimental design was used to optimise the method [39,40]. After preliminary investigations to determine the best ion-pairing agent and to adjust the experimental domain under study, a three-level full-factorial design was applied to study the impact of two parameters on the retention of the eight ACE inhibitors [41]. The parameters studied were the pH and the concentration of the ion-pairing agent.

2. Experimental

2.1. Instrumentation and electrophoretic procedure

Experiments were performed on a Crystal CE (Thermo Capillary Electrophoresis, Franklin, USA), equipped with PC 1000 software installed on a Dell computer with an OS/2 operating system. A fused-silica capillary was used, 85 cm in total length (33 cm to the detector) and 50 μm internal diameter (I.D.). The Crystal CE was temperature controlled at 25 °C for the tray and at 30 °C for the capillary. The sample solutions were injected by pressure (50 mbar) for 5 s. A constant voltage of 25 kV was applied and UV absorbance at 214 nm was employed for detection. The detection was by means of a variable-wavelength UV detector (Spectra FOCUS detector, Spectra-Physics, San Jose, CA, USA).

To demonstrate the transferability of the developed method, the experiments were also performed on a Waters Quanta 4000 (Millipore, Milford, USA), equipped with a fused-silica capillary of 60 cm (52.5 cm to the detector)×75 μm I.D. Hydrostatic injections were performed by lifting the sample vial ~10 cm above the height of the buffer vial for 10 s. For detection, the absorbance was measured by means of an on-line fixed-wavelength UV detector with a zinc discharge lamp and a 214-nm filter. The experiments were performed at 8 kV at room temperature (20±2 °C). Data were collected on a Hewlett-Packard Integrator (HP 3396–Series II, Avondale, USA), which was also used for calculating the areas under the peaks.

2.2. Reagents

Sodium dihydrogenphosphate monohydrate and disodium hydrogenphosphate dihydrate (both analytical reagent grade) were obtained from Merck (Darmstadt, Germany). Phosphoric acid (85%, w/w) was obtained from UCB (Leuven, Belgium). Sodium butanesulphonate was obtained from Acros Organics (Geel, Belgium), sodium decanesulphonate from Fluka (Buchs, Switzerland), while sodium hexanesulphonate, sodium heptanesulphonate and sodium octanesulphonate were from Sigma (St. Louis, MO, USA). Lisinopril dihydrate was obtained from MSD (Waterloo, Belgium) and Zeneca (Cheshire, UK), quinapril·HCl from Parke-Davis (Zaventem, Belgium), fosinopril sodium from Bristol-Myers Squibb (Waterloo, Belgium), perindopril from Servier (Orleans, France), ramipril from Hoechst Marion Roussel (Frankfurt am Main, Germany), benazepril from Ciba-Geigy (Basel, Switzerland), and cilazapril from Roche (Basel, Switzerland). Enalapril maleate and lisinopril were also purchased from Sigma.

Reference solutions of fosinopril, perindopril, ramipril, benazepril, and cilazapril for qualitative analysis, were prepared from the commercially available drugs (Fosinil, Coversyl, Tritace, Cibacen, and Inhibace), by mixing the powder with the phosphate buffer (see below). The suspensions were filtered

through a membrane filter (0.45 μm) (Millipore, Bedford, MA, USA).

2.3. Running buffers

During the development of the method, 100 m*M* sodium phosphate buffers of different pH were used. In the pH range 2.0–4.5, a mixture of a phosphoric acid solution and sodium dihydrogenphosphate solution was used, while in the range 4.5–8.0, it was a mixture of a sodium dihydrogenphosphate solution and a disodium hydrogenphosphate solution. These buffers were used as solvent for the preparation of stock, standard and sample solutions. Running buffer solutions were prepared at different concentrations of octanesulfonate (30, 50 and 70 m*M*).

2.4. Reference solutions

Reference solutions of the eight compounds were prepared at 100 μg ml^{-1} in the solvent.

All solutions and buffers were filtered through a Millipore 0.45-μm filter unit.

2.5. Experimental set-up and analysis of results

The set-up of the design and the statistical analysis of the response variables were supported by the statistical graphics software system STATGRAPHICS Plus version 4.1 (STSC, Rockville, MD, USA).

3. Results and discussion

The optimisation of a selective capillary electrophoretic separation of eight ACE inhibitors was described earlier [1]. Separation was performed by means of two methods using phosphate buffers (each 100 m*M*) at pH 7.0 and 6.25, respectively. This combination of methods was necessary for the selective identification of these structurally-related substances because of their similar pK_a values. The addition of organic modifiers had a negative influence on peak symmetry, and selectivity was not improved [1]. Because a free solution CE system failed to reach a complete separation of these compounds, the usefulness of ion-pairing agents was studied.

3.1. Screening phase

Several parameters were considered in order to optimise the separation conditions. From preliminary results it was found that the factors most affecting the responses of migration time and peak width were the pH of the running buffer and the nature of the ion-pairing agent. The pH of the running buffer plays an important role because it influences the separation by affecting the charge of the compounds as well as the electroosmotic flow (EOF). Different alkylsulphonates were tested to enhance separation. The appropriate selection of the experimental domain was made from prior experience and knowledge of the separation system. Other factors such as voltage and molarity of the running buffer were also considered initially, but were found to have less influence and were therefore kept constant. The voltage was fixed at 25 kV. In earlier investigations, the molarity of the sodium phosphate buffers varied from 50 to 125 m*M*. The selectivity of the separation was not influenced by this, and only the migration times increased. Because it has a high buffering capacity and provides acceptable migration times, a sodium phosphate buffer (100 m*M*) was chosen.

The effects of varying pH and concentration of the ion-pairing additive on the migration behaviour were investigated at a constant voltage.

3.1.1. Selection of the pH

Because of the amphoteric character of ACE inhibitors (Fig. 1), their retention is greatly influenced by pH. All ACE inhibitors have an ionisable carboxylic group. With the exception of fosinopril, they also possess a secondary amine in their structure. Depending on the pH of the medium, the inhibitors may be negatively or positively charged. This offers the possibility of using either an acidic or an alkaline running buffer. Most of the ACE inhibitors are esters for which stability problems occur in alkaline medium (above pH 8.5). Therefore, the measurements were performed at three pH levels (pH 2.0, 5.0, and 8.0), covering a large pH range in which no stability problems occur.

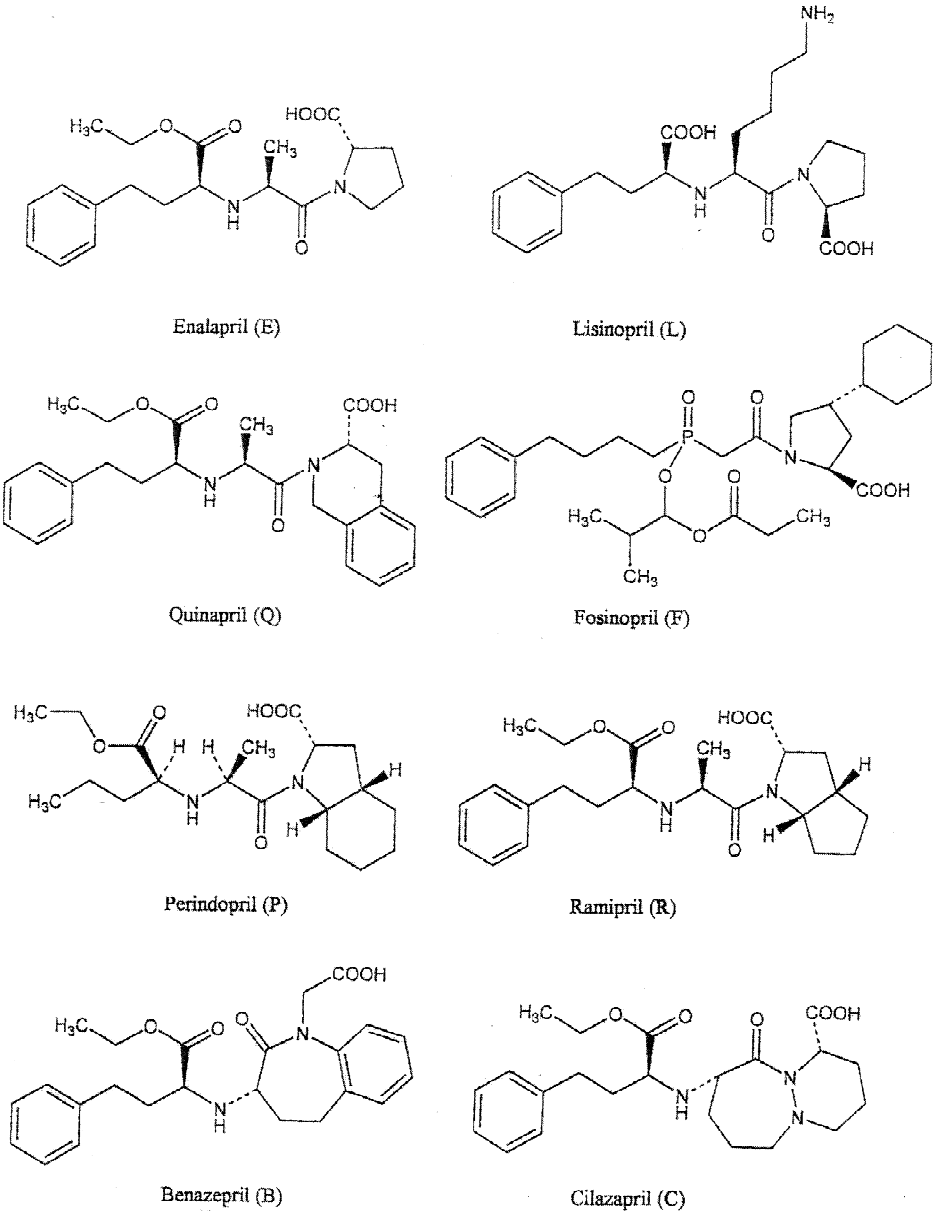

Fig. 1. Chemical structures of the ACE inhibitors.

3.1.2. Choice of sodium octanesulphonate as ion-pairing agent

The effects of different alkylsulphonates on resolution were studied by adding them to a sodium phosphate buffer (pH 2.0, 100 m*M*). If only the buffer was used, co-migration of all ACE inhibitors, with the exceptions of lisinopril (dicarboxylic acid) and fosinopril (uncharged), was observed because they all have a positive charge (Fig. 2). The ACE inhibitors exhibit the best features to interact by

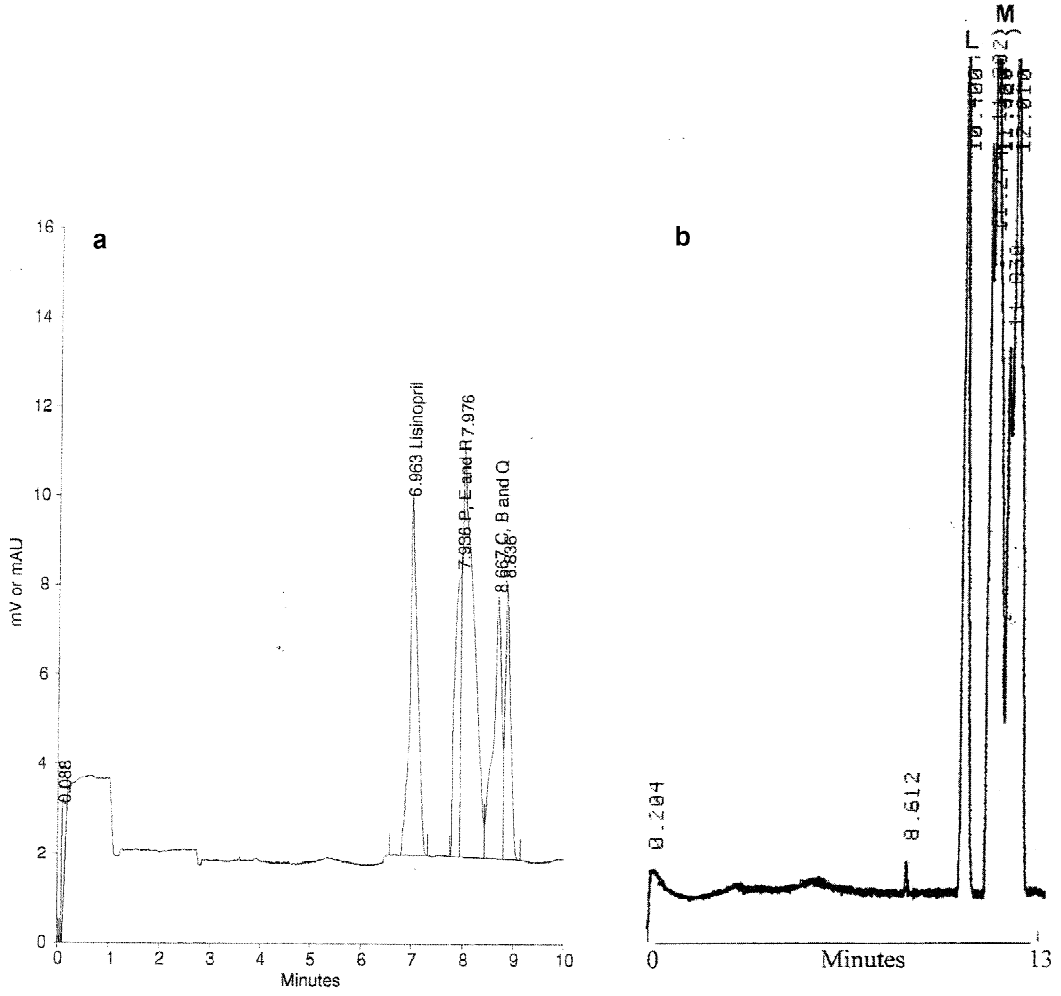

Fig. 2. Electropherogram of a mixture of eight ACE inhibitors. (a) The separation was performed on the Crystal CE instrument using a fused-silica capillary 85 cm (33 cm to the detector)×50 μm I.D., and sodium phosphate buffer (pH 2.0; 100 m*M*) as the running buffer. The applied voltage is 30 kV and detection is at 214 nm. P, E and R, and C, B and Q refer to the co-elution of perindopril, enalapril and ramipril, and cilazapril, benazepril and quinapril, respectively. (b) The separation was performed on the Waters Quanta instrument using a fused-silica capillary 60 cm (52.5 cm to the detector)×75 μm I.D., and sodium phosphate buffer (pH 2.0; 100 m*M*) as the running buffer. The applied voltage is 12 kV and detection is at 214 nm. L and M refer to lisinopril and a mixture of the co-eluting compounds perindopril, enalapril, ramipril, cilazapril, benazepril and quinapril, respectively.

electrostatic forces with alkylsulphonates in acidic solutions. Sodium butanesulphonate gave no improvement in resolution, whereas the addition of sodium hexanesulphonate and sodium heptanesulphonate led to increases in resolution when used in concentrations of 120 and 90 m*M*, respectively. The progressive general increase in resolution led us to evaluate sulphonates with longer chain lengths such as sodium octanesulphonate and decanesulphonate. As the chain length increased, the concentration of the alkylsulphonate that was necessary to give a comparable resolution decreased. Although sodium decanesulphonate had a positive influence on the resolution, this additive led to long migration times

Table 1
Parameter settings in the design

CE parameter	Low value (−1)	Central value (0)	High value (+1)
pH	2	5	8
OS (m*M*)	30	50	70

(above 30 min) and baseline fluctuations. Because it had the lowest concentration required (50 m*M*) and acceptable migration times, sodium octanesulphonate (OS) was used at three concentrations (30, 50, and 70 m*M*) for optimisation.

3.2. Response surface design

To evaluate the influence of the two parameters on the separation, a three-level full-factorial design was applied. This design requires nine runs. The parameter settings in the design are given in Table 1 while the design is reproduced in Table 2. Each compound was individually injected. The individual runs of the design were carried out in a randomised sequence. Randomisation offers some assurance that uncontrolled variation of factors, other than those studied, will not influence the estimations. Replicate measurements ($n=3$) were performed to verify that retention times were stable and the capillary was well equilibrated after changing to new electrophoretic conditions.

Table 2
Two-factor three-level full factorial design

Run	pH	OS (m*M*)
1	−1	−1
2	−1	0
3	−1	1
4	0	−1
5	0	0
6	0	1
7	1	−1
8	1	0
9	1	1

The migration times and peak widths of all ACE inhibitors were measured. In Table 3 the measured migration times (t) for each run of the design are compiled, while in Table 4 the peak widths at baseline (w) are represented. These variables were used to calculate the resolution, according to the equation $R_s = 2(t_2 - t_1)/(w_1 + w_2)$ in which t and w are expressed in the same time units.

In runs 1–6, the exact migration time of fosinopril is missing because it was not detected within 180 min. Fosinopril is the only phosphor-containing ACE inhibitor without a secondary amine group. Fosinopril (pK_a 3.8±0.6) can only exist in two forms (uncharged or anionic), whereas the other ACE inhibitors can exist in three different forms (cation, zwitterion, or anion). Lisinopril is another exception among the ACE inhibitors because it possesses two acidic and two basic groups and thus, in principle, it can exist in five different forms (di-cation, cationic zwitterion, zwitterion, anionic zwitterion, or di-anion) [25]. There is thus no difficulty in separating lisinopril from the other ACE inhibitors [1].

3.3. Results

3.3.1. Regression modelling

From the 3^2 design for each response (migration

Table 3
Measured response variables on the Crystal CE instrument: migration times in minutes (for experimental conditions, see text)

Run	t_L	t_E	t_Q	t_P	t_R	t_B	t_C	t_F
1	6.98	7.96	8.49	7.92	7.98	8.41	8.09	>180
2	7.48	8.83	10.24	8.81	9.06	9.62	9.35	>180
3	7.58	9.27	11.64	9.15	10.16	10.62	10.16	>180
4	10.12	13.08	13.48	11.1	11.51	13.78	10.56	>180
5	10.22	13.89	14.33	11.98	12.72	14.03	11.11	>180
6	9.88	14.33	15.83	12.21	12.93	16.68	12.36	>180
7	4.7	5.13	5.08	5.14	5.1	5.19	5.13	4.83
8	4.72	5.22	5.25	5.22	5.21	5.32	5.28	5.13
9	4.79	5.41	5.49	5.43	5.43	5.62	5.58	5.75

Table 4
Measured response variables on the Crystal CE instrument: peak widths at baseline in seconds (for experimental conditions, see text)

Run	w_L	w_E	w_Q	w_P	w_R	w_B	w_C	w_F
1	24	31	19	40	18	14	14	500[a]
2	23	30	26	34	34	14	14	500[a]
3	20	24	53	26	30	13	12	500[a]
4	17	122	58	148	131	39	19	500[a]
5	18	119	69	88	191	65	40	500[a]
6	18	135	85	196	261	71	19	500[a]
7	9	8	8	7	7	7	7	11
8	8	6	7	7	7	6	7	12
9	8	7	7	7	8	6	7	23

[a] Arbitrarily chosen width (see text).

time and width) the following model was determined:

$$y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2$$

where y is the measured response for each compound, b_0 is the intercept, b_i are the regression coefficients, and X_i are the values of the independent electrophoretic variables (X_1 = pH and X_2 = [OS]). The modelling is performed after scaling the X_1 and X_2 variables in the interval [−1,1].

To obtain good separation of compounds, adequate resolution is needed, and the minimal resolution, i.e. the resolution of the two worst separated peaks ($R_{s,min}$), is especially important. We were therefore interested in the domain(s) where $R_{s,min}$ was maximal. The response $R_{s,min}$ cannot be modelled because it can be determined by different pairs of peaks. Modelling the resolutions of successive peaks ($R_{s,i}$) was also no solution because the migration order of the consecutive compounds can change (e.g. A–B can become B–A) as well as the selectivity (e.g. A–B–C can become C–A–B). Therefore, another approach was necessary.

Firstly, the measured responses (t and w) for each ACE inhibitor were modelled. For fosinopril, 180 min was used as migration time in experiments 1–6. For the peak widths of fosinopril in the corresponding runs, an arbitrary large value (500 s) was taken to correspond with the long migration time.

After modelling, the responses were predicted for all experimentally feasible different conditions in the studied domain. Subsequently, for each of these conditions the migration times of the compounds were sorted, the resolutions of the successive pairs of peaks ($R_{s,i}$) were calculated and $R_{s,min}$ was selected. Finally, all values of $R_{s,min}$ were plotted and the region(s) where $R_{s,min}$ was maximal were investigated.

The three-dimensional plot of $R_{s,min}$ as a function of pH and concentration of sodium octanesulphonate, is shown in Fig. 3, while its contour plot is given in Fig. 3b. Three areas can be distinguished where $R_{s,min}$ was maximal but still low (0.16).

The highest $R_{s,min}$ was found in the pH range 3.2–3.6. In particular, the best point of this domain was at pH 3.2 and a sodium octanesulphonate concentration of 45 mM (A). Applying these conditions led to an inadequate separation because all ACE inhibitors possessed bad peak symmetry while some of them also co-eluted. This inadequate separation is not surprising considering the low predicted value of $R_{s,min}$. The shapes of the peaks for enalapril and perindopril were particularly poor. Fosinopril was uncharged and migrated with the EOF. However, at pH 3.2, it was possible to obtain a baseline separation between lisinopril or ramipril, cilazapril, and benazepril or quinapril. This pH value was therefore only suitable to separate three of the ACE inhibitors.

The second area was also located in acidic medium, specifically at pH 2.0. The best point seemed to be at pH 2.0 and a sodium octanesulphonate concentration of 65 mM (B). At these conditions, the peak symmetry for all ACE inhibitors was acceptable. However, it remained impossible to separate all species because some of them eluted together. It was possible to separate five compounds,

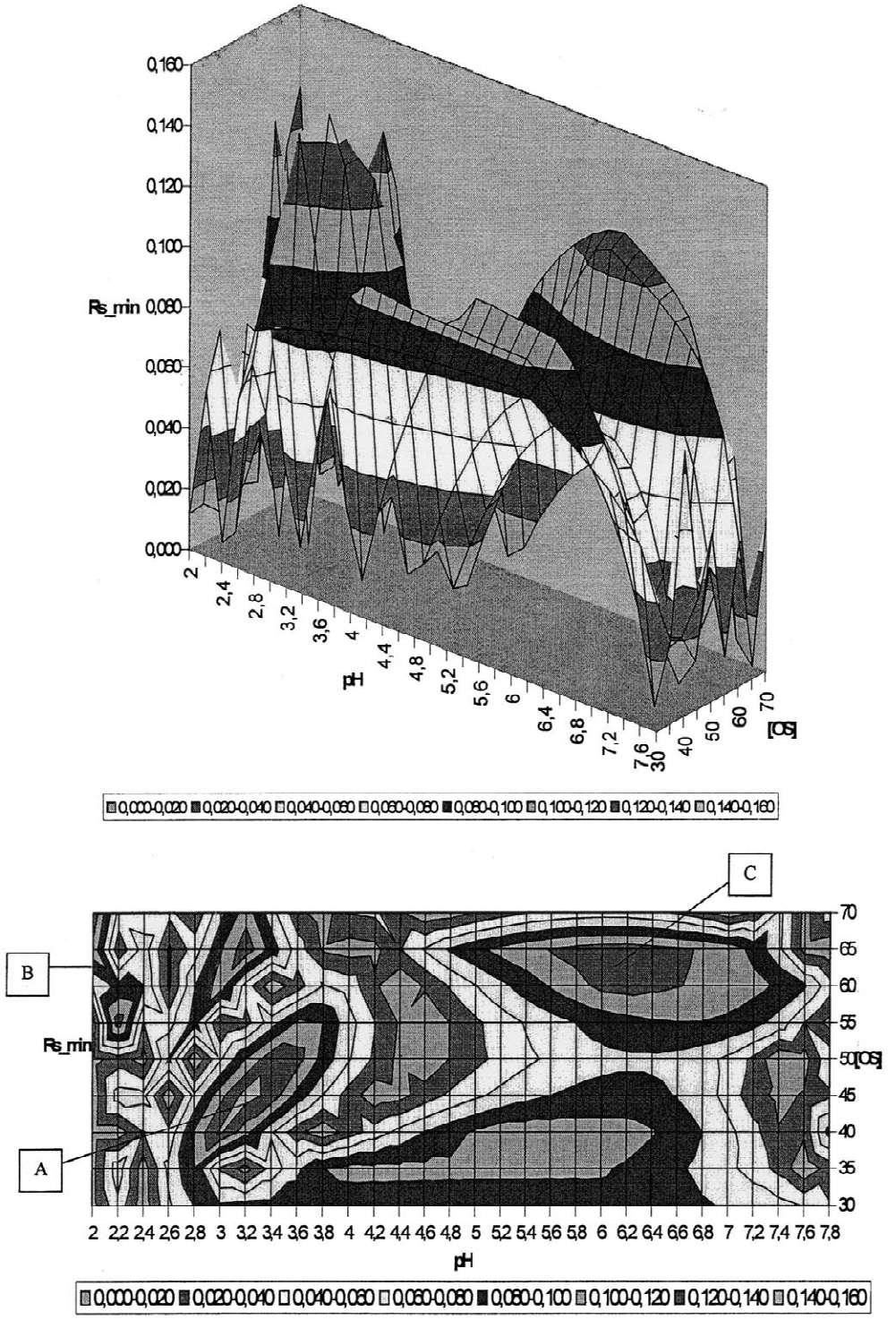

Fig. 3. Minimal resolution as a function of pH and concentration of sodium octanesulfonate (OS). Results obtained on the Crystal CE instrument. (a) Three-dimensional response surface. (b) Contour plot.

namely lisinopril, perindopril or enalapril, ramipril or cilazapril, benazepril, and quinapril. Perindopril and enalapril on one hand, and ramipril and cilazapril on the other, eluted together. Fosinopril was uncharged and migrated with the EOF.

The third area was found in basic medium, in the pH range 6.0–6.4. The optimum point seemed to be at pH 6.2 with a sodium octanesulphonate concentration of 65 m*M* (C). An inadequate separation was obtained because there was little difference in the migration times and, consequently, co-elution occurred. With the exception of ramipril and perindopril, all the ACE inhibitors possessed good peak symmetry. At this pH, it was possible to obtain a baseline separation between lisinopril, cilazapril, benazepril, and one of the following inhibitors: perindopril, ramipril, enalapril, or quinapril. This pH was therefore only suitable for the separation of four of the ACE inhibitors.

As already mentioned, the best conditions for the separation of most of these compounds were found by using a sodium phosphate buffer (pH 2.0; 100 m*M*) containing 65 m*M* sodium octanesulphonate (B). Despite this result, the addition of octanesulphonate to the running buffer improved separation of the eight ACE inhibitors. If using only the buffer, all ACE inhibitors, with the exception of lisinopril and fosinopril (uncharged), co-migrated because they all have a positive charge (Fig. 2a and b). Thus, improved separations were obtained, suggesting a favourable effect of ion-pairing interactions between analytes and additive. Afterwards, fine-tuning was applied to these conditions to shorten the migration times without losing resolution. The voltage was raised to 30 kV. The selectivity of the separation remained while the analysis time decreased to 10 min. A typical electropherogam obtained by applying these optimised conditions is presented in Fig. 4.

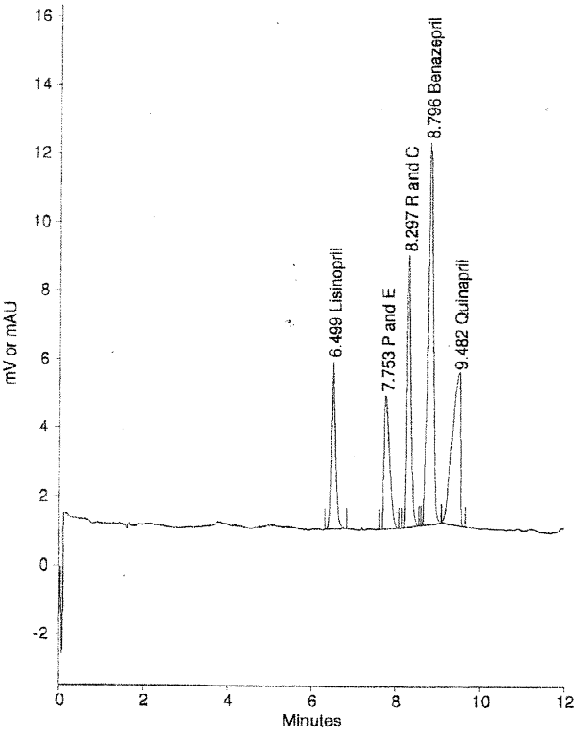

Fig. 4. Electropherogram of a mixture of eight ACE inhibitors using a fused-silica capillary 85 cm (33 cm to the detector)×50 μm I.D., and sodium phosphate buffer (pH 2.0; 100 m*M*) containing 65 m*M* sodium octanesulfonate as the running buffer, performed on the Crystal CE instrument. The applied voltage is 30 kV and detection is at 214 nm. P and E, and R and C refer to the co-elution of perindopril and enalapril, and ramipril and cilazapril, respectively.

3.3.2. Method transferability

The experiments and experimental design were also performed on a Waters Quanta instrument, equipped with a fused-silica capillary of 60 cm (52.5 cm to the detector)×75 μm I.D.

The three-dimensional response surface and the contour plot of $R_{s,min}$ as a function of pH and molarity of sodium octanesulphonate are shown in Fig. 5a and b. The predicted maximal $R_{s,min}$ value was higher on this instrument than on the Crystal CE—0.45 compared with 0.16—but was still low. That means that, for the two ACE inhibitors, determining the $R_{s,min}$, produced a greater difference in migration time and/or a better peak shape. Therefore, separation might be improved. On this instrument two areas with a ''higher'' $R_{s,min}$ can be distinguished. The $R_{s,min}$ was maximal for the highest concentrations of sodium octanesulphonate (65 and 70 m*M*) and at a pH range of 2.0–2.6. In particular, the best point of this domain was at pH 2.0 and a sodium octanesulphonate concentration of 65 m*M* (A). This condition was the same as that for the Crystal CE instrument and separation was similar: five ACE inhibitors, namely lisinopril, perindop-

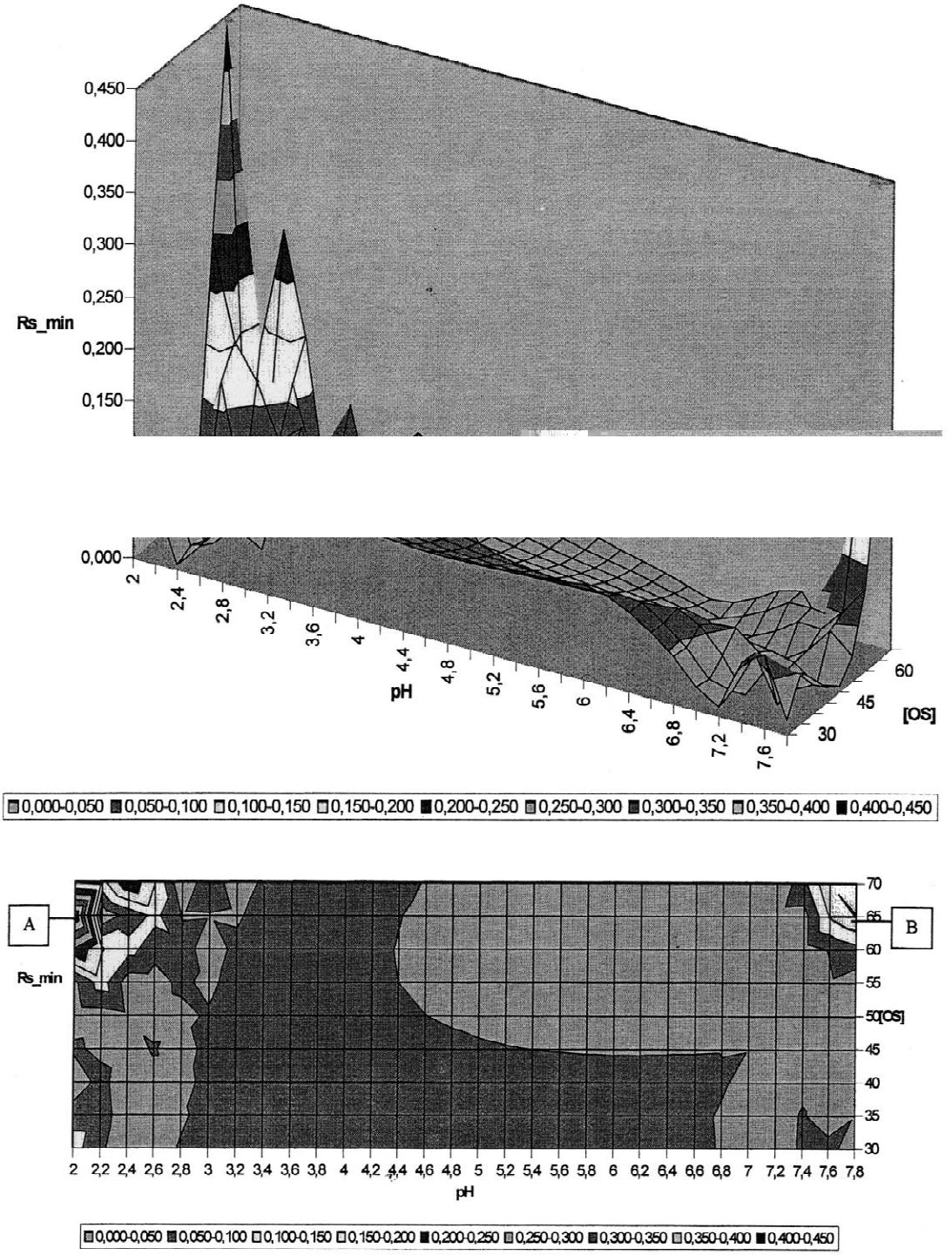

Fig. 5. Minimal resolution as a function of pH and concentration of sodium octanesulfonate (OS). Results obtained on the Waters Quanta instrument. (a) Three-dimensional response surface. (b) Contour plot.

ril or enalapril, ramipril or cilazapril, benazepril, and quinapril can be separated. Perindopril and enalapril on the one hand, and ramipril and cilazapril on the other, could not be separated nor identified because they eluted together. Fosinopril was uncharged and migrated with the EOF. The second area with a higher $R_{s,min}$ value was found in basic medium in the pH range 7.6–7.8. The best point seemed to be at pH 7.8 with a sodium octanesulphonate concentration of 65 m*M* (B). There was no improvement in selectivity if these conditions were applied; only three ACE inhibitors could be separated, namely lisinopril, enalapril or perindopril, and one of cilazapril, ramipril, quinapril or benazepril.

Fine-tuning was applied for the conditions of point A for the same reasons as on the Crystal CE. The voltage was raised to shorten the migration times. At a voltage of 12 kV, the selectivity of the separation remained but the migration times decreased. A typical electropherogam obtained by applying these optimised conditions (pH 2.0, sodium octanesulphonate concentration 65 m*M*) is presented in Fig. 6.

On both instruments, the best separation for the highest number of compounds was found by using a sodium phosphate buffer (pH 2.0, 100 m*M*) containing 65 m*M* sodium octanesulphonate.

Thus, we evaluated the usefulness of alkylsulphonates for the separation of eight ACE inhibitors: enalapril, lisinopril, quinapril, fosinopril, perindopril, ramipril, benazepril, and cilazapril. Improved separations were obtained probably through ion-pairing interactions between analytes and additive. Even when applying the optimised conditions, however, it was impossible to separate all eight ACE inhibitors in one run. Because of the co-migration of perindopril and enalapril on the one hand, and ramipril and cilazapril on the other, this system alone cannot be used for the identification of these structurally related compounds. To identify the eight ACE inhibitors based on their migration times a combination of two systems is therefore necessary. The best conditions found in this study can be used first as a method for identifying five of the ACE inhibitors. The co-migrating compounds then have to be distinguished by another method. Another optimisation of a selective capillary electrophoretic separation of several ACE inhibitors has been reported [1]. In that study, conditions for the identification of the co-migrating

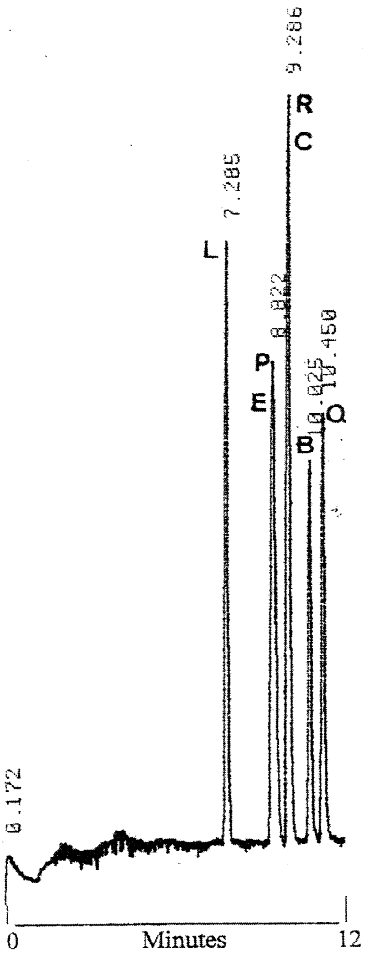

Fig. 6. Electropherogram of a mixture of eight ACE inhibitors using a fused-silica capillary 60 cm (52.5 cm to the detector)×75 μm I.D., and sodium phosphate buffer (pH 2.0; 100 m*M*) containing 65 m*M* sodium octanesulfonate as the running buffer, performed on the Waters Quanta instrument. The applied voltage is 12 kV and detection is at 214 nm. E, L, Q, P, R, B and C refer to the first letters of the corresponding ACE inhibitors.

compounds perindopril/enalapril and ramipril/cilazapril were found. They can be identified when using a sodium phosphate buffer (pH 7.0, 100 m*M*). Fosinopril can also be determined under these conditions. Because it had the shortest analysis time and the best peak shapes, the ion-pairing method is preferred as the first identifying method, and sequential application of both methods allows the identification of all considered substances.

4. Conclusions

A combination of two systems is still necessary for the selective identification of the eight ACE inhibitors. Under the optimised conditions of this study, it was possible to separate lisinopril, perindopril or enalapril, ramipril or cilazapril, benazepril, and quinapril. Fosinopril and the co-eluting compounds perindopril/enalapril and ramipril/cilazapril can then be distinguished using a second system. A combination of the two systems can therefore be applied to identify these compounds pharmaceutically.

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