Experimental Design Optimization of a Capillary Zone Electrophoresis Method for the Screening of Several Diuretics and ACE Inhibitors

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Abstract

Experimental design methodologies are applied to the development of a capillary zone electrophoretic method for the separation of the angiotensin-converting enzyme inhibitor enalapril and its derivative enalaprilat and the diuretics xipamide and hydrochlorothiazide. The effects of pH, buffer concentration, proportion of boric acid in the mixed boric acid–potassium dihydrogen phosphate background electrolyte, temperature, applied voltage, and percentage of organic modifier are studied. Critical factors are identified in a screening design (a 2^{6-2} fractional factorial design), and afterwards, optimal conditions for the separation are reached by means of an optimization design (a $2^2 + 2 \times 2 + k$ central composite design). The studied response is the resolution between peaks. The four studied compounds can be separated in less than 3.5 min using an electrolyte of 20mM boric acid–potassium dihydrogen phosphate (75:25, v/v) with 5% MeOH adjusted to pH 8.0 with KOH, at a potential of 30 kV. The detection wavelength and temperature are 206 nm and 35°C, respectively.

Introduction

According to recent studies (1,2) and the World Health Organization–International Society of Hypertension (WHO–ISH) (3), combined therapies can be successfully applied in cardiovascular treatment, improving actions of the individualized treatment. Thus, adjunctive therapies with different antihypertensive compounds, such as angiotensin-converting enzyme (ACE) inhibitors and diuretics, are being employed. The former have become, in less than 15 years, one of the most important classes of drugs for treating hypertension and chronic heart failure, the latter being one of the most used drug families for the treatment of hypertension.

Because of their safety, efficacy, and ability to reverse some of the structural changes associated with high blood pressure, ACE inhibitors are now recommended as first-line therapy for hypertension, and they are a cornerstone in managing chronic heart failure (4,5). There are different types of ACE inhibitors, some of them being prodrugs that are more easily absorbed than the active compound. Enalapril is the most employed ACE inhibitor in the management of hypertension, and it acts as a prodrug. After hydrolisis (de-esterification), it turns into enalaprilat, which inhibits the ACE and lowers blood pressure.

Among diuretics, hydrochlorothiazide and xipamide have been widely used in cardiovascular monotherapy. Nowadays, they are also being used in adjunctive therapies with ACE inhibitors, their combination having a synergic effect on lowering blood pressure (6).

Formulae of these four compounds are collected in Figure 1. Thus, reliable, simple, and fast analytical methods are needed to separate and quantitate these compounds in samples obtained from hypertensive patients undergoing combined antihypertensive therapy.

Few electrophoretic methods have been reported for the separation or quantitation of ACE inhibitors or hydrochlorothiazide, whereas no reference could be found for xipamide.

Among the developed methods for the former, there are two research works dealing with the separation of several ACE inhibitors (7,8) and a third one also includes hydrochlorothiazide

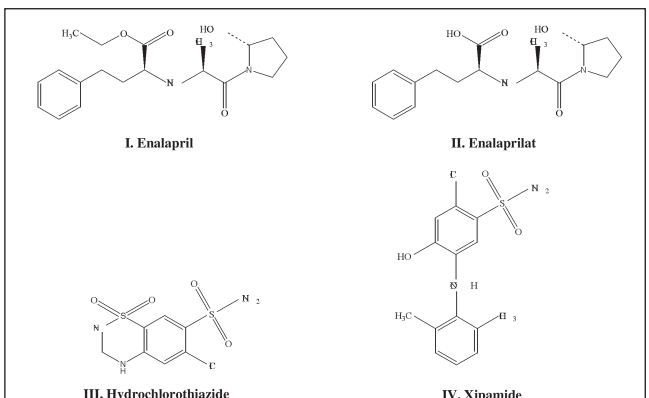

Figure 1. Chemical formulae of enalapril, enalaprilat, hydrochlorothiazide, and xipamide.

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(9). Other studies focused on the determination and chiral separation of enalapril (10) and on the quantitation of this drug in pharmaceuticals (11). All reported research works employed step-by-step optimization of the corresponding method.

Step-by-step methodology has traditionally been employed to develop suitable electrophoretic methods. Nevertheless, this kind of approach involves a long number of independent time-consuming runs and is often limited by the large number of variables that may influence the analysis and size of the experimental domain.

Chemometric methodologies, such as experimental design, have long been important in the development of separation methods, including capillary electrophoresis (CE).

Experimental designs are multivariate approaches based on the variation of several variables at the same time. Analysis of results leads to a response model in which the relationship of each variable (i.e., factor) towards the response, as well as the interactions between factors, is shown.

CE is especially convenient for the use of experimental design, as the experimental conditions can be immediately varied from one experiment to another (no extra time needed, as equilibration time in chromatographic procedures). The suitability of this kind of approach in CE has been thoroughly demonstrated (12–19).

In this work, we develop a simple and fast capillary zone electrophoresis method for the separation of enalapril, enalaprilat, hydrochlorothiazide, and xipamide in less than 3.5 min. Optimization of the developed method was made by means of experimental design methodologies. A screening design was first used to identify critical parameters affecting the separation (fractional factorial design). Afterwards, an optimization design (central composite design) was carried out with regard to the results of the former. The nonlinear correlation between significant factors and the response obtained from these designs led us to the response surface model, from which parameter values to obtain optimal response could be calculated (20,21).

Further effort will be made to develop a suitable cleanup procedure in order to determine these antihypertensive drug levels in samples from patients under combined therapy.

Experimental

Chemicals and solutions

Xipamide was kindly supplied by Lacer S.A. (Barcelona, Spain). Enalapril maleate and hydrochlorothiazide were purchased from Sigma (St. Louis, MO). As it was not possible to purchase enalaprilat from pharmaceutical companies, it was synthesized in our laboratory from enalapril maleate. Methanol was Romil Super Purity Solvent grade (Haverhill, U.K.), and the rest of the chemicals were of analytical grade and were supplied by Merck (Darmstadt, Germany). Water was obtained from Milli-RO and Milli-Q systems (Millipore, Bedford, MA).

Stock solutions of enalapril, enalaprilat, hydrochlorothiazide, and xipamide (1000 µg/mL) were prepared in methanol and kept in amber glass volumetric flasks. These stock solutions were stored in the dark under refrigeration to avoid possible decomposition. Working solutions were also prepared in amber glass volumetric flasks by appropriate dilution with water just before use. Running electrolytes were prepared by mixing the appropriate volumes of solutions of 0.5M boric acid, 0.5M potassium dihydrogen phosphate, and methanol to give the desired pH value and organic modifier percentage. The pH was adjusted by adding drops of KOH concentrate.

Apparatus and electrophoretic conditions

This work was performed on a Hewlett-Packard HP ^{3D}CE Capillary Electrophoretic system (Waldbronn, Germany) equipped with a diode array detector. The sample tray was refrigerated at 20ºC with a Selecta Frigiterm-10 external bath (Barcelona, Spain). The fused-silica capillaries were 58.5-cm × 50-mm i.d., 375-µm external diameter, obtained from Composite Metal Services (Worcester, U.K.), with the detection window at 50 cm. The samples were introduced hydrodynamically for 22 s at 50 mbar injection pressure, and the capillary temperature was set at 35 ± 0.1ºC. The running electrolyte consisted of a 20mM boric acid–potassium dihydrogen phosphate (75:25, v/v) mixture with a 5% MeOH adjusted to pH 8.0 with KOH. The applied potential was 30 kV, and the detection wavelength was 206 nm.

The pH of the solutions was measured with a Radiometer Copenhagen PHM84 pH meter (Bargsvaerd, Denmark) using a Crison glass-combined electrode model 5209 (Barcelona, Spain) equipped with a reference system Ag/AgCl and electrolyte KCl 3M saturated AgCl.

Capillary conditioning

The capillary was conditioned every day with an initial wash cycle consisting of 1M NaOH for 15 min, deionized water for 10 min, and running electrolyte for 5 min. Between injections, the capillary was washed with 0.1M NaOH for 2 min, deionized water for 1 min, and running electrolyte for 3 min. The separation buffer was refreshed after a few runs. Daily, after finishing the experiments, the capillary was washed with 1M NaOH for 10 min and deionized water for 10 min and purged with air for 3 min.

Results and Discussion

Experimental designs and calculations

Every experiment was carried out in duplicate in random order in order to avoid bias. The statistical analysis of results was performed by means of the Nonlinear Regression (NLREG, Nashville, TN) nonlinear analysis program (22). Microsoft Excel was employed to draw response surfaces.

Screening design: fractional factorial design

In order to evaluate which of the considered factors had an influence on the studied response, a fractional factorial design was run. This kind of design is a fraction of a full factorial design that confounds some main effects with interactions or interactions among themselves, resulting in a smaller set of experiments, and, nevertheless, it is able to identify the influence of each parameter, as well as first-order interactions between factors.

The number of experiments in a fractional factorial design is 2^{k-p}, where k is the number of factors studied and p is an arbitrary number smaller than k that accounts for the degree of frac-

tionality of the fractional factorial design ($p < k$).

In this experimental design, up to six variables were studied, either chemical or instrumental parameters: pH (x_1), percentage of boric acid (x_2), applied potential (x_3), total concentration of the running electrolyte (x_4), temperature (x_5), and percentage of methanol as organic modifier (x_6) (Tables I and II).

The background electrolyte was a mixed boric acid–potassium dihydrogen phosphate buffer, which showed high buffer capacity over the studied pH range (7.0–9.5). The use of mixed electrolytes to cover wide pH ranges has been previously reported elsewhere (23). The nature of the buffer was also considered in this screening design, and the percentage of borate buffer in the running electrolyte was included as another factor, its proportion varying between 25–75%. The methanol percentage was varied between 5% and 20%, the aim of including an organic modifier being to improve resolution and avoid peak overlapping. The buffer concentration was varied between typical levels for inorganic buffer solutions such as borate and phosphate (10–40mM). Finally, temperature and applied potential ranged 20–35ºC and 20–30 kV, respectively.

Table I. The 2^{6-2} Fractional Factorial Design

	Experimental factors					
Trial	x_1	x_2	x_3	x_4	x_5	x_6
1	–1	–1	–1	–1	–1	–1
2	+1	–1	–1	–1	+1	+1
3	–1	+1	–1	–1	+1	–1
4	+1	+1	–1	–1	–1	+1
5	–1	–1	+1	–1	+1	+1
6	+1	–1	+1	–1	–1	–1
7	–1	+1	+1	–1	–1	+1
8	+1	+1	+1	–1	+1	–1
9	–1	–1	–1	+1	–1	+1
10	+1	–1	–1	+1	+1	–1
11	–1	+1	–1	+1	+1	+1
12	+1	+1	–1	+1	–1	–1
13	–1	–1	+1	+1	+1	–1
14	+1	–1	+1	+1	–1	+1
15	–1	+1	+1	+1	–1	–1
16	+1	+1	+1	+1	+1	+1
17	0	0	0	0	0	0
18	0	0	0	0	0	0
19	0	0	0	0	0	0
20	0	0	0	0	0	0

Table II. Level Codification for the 2^{6-2} Fractional Factorial Design

Factor		–1	0	+1
x_1	pH	7	8.25	9.5
x_2	Percentage of boric acid (%)	75	50	25
x_3	Voltage (kV)	20	25	30
x_4	Total concentration (mM)	10	25	40
x_5	Temperature (°C)	20	27.5	35
x_6	Percentage of methanol (%)	5	12.5	20

In this screening study, a 2^{6-2} fractional factorial design was run (Table I), associating two of the studied factors to two third-order interacting columns of the matrix. Four replicates at the center point were added in order to determine experimental variance (21,24). Factors and levels of the fractional factorial design are summarized in Table II. The temperature and percentage of methanol were the factors defined as a combination of three of the other variables, as stated in the following equations:

$$\text{Temperature} = \text{pH} \times \text{boric acid (\%)} \times \text{voltage} \qquad \text{Eq. 1}$$

$$\text{Methanol (\%)} = \text{pH} \times \text{voltage} \times \text{buffer concentration} \qquad \text{Eq. 2}$$

The value of these parameters in the experiments was fixed according to the product in coded variables of the other three variables (Table I). When the result of the product was –1, the parameter was fixed at its low level, whereas the opposite occurred when the result of the product was +1.

The analytical response studied was resolution between peaks (Rs) as dealing with a separation, and it was calculated according to the following equation:

$$\text{Rs} = 1.177 \times \frac{(t_j - t_i)}{(w_{(0.5)_j} + w_{(0.5)_i})} \qquad \text{Eq. 3}$$

where t_j and t_i and $w_{(0.5)j}$ and $w_{(0.5)i}$ are the migration time and peak widths at half height of two successive peaks, peak i being the first one and peak j being the last one.

Migration time was not set as response, but it was thoroughly considered in order to minimize analysis time. Analysis of the data was carried out by means of the NLREG nonlinear regression program. Responses (Y) were defined as a function of the considered variables using polynomials at different degrees, depending on the experimental design followed. The most general polynomial function for response and variables in a fractional factorial design is:

$$Y = \beta_0 + \sum_i b_i x_i + \sum_{ij} b_{ij} x_i x_j \qquad \text{Eq. 4}$$

where b_0, b_I, and b_{ij} represent the numerical parameters to be calculated. The final estimation of the parameters is achieved when square the sum of errors (U) is minimized:

$$U = \sum_i^n (Y_{\text{exp}} - Y_{\text{calc}})^2 \qquad \text{Eq. 5}$$

where n is the number of experiments, Y_{exp} is the resolution calculated by means of experimental data (migration times and peak widths), and Y_{calc} is the response given by NLREG following the proposed regression model.

The analysis of the output was made with regard to the prob(t) parameter associated to each b_i parameter. Prob(t) indicates the probability of the associated b_i being zero. Those calculated parameters with linked prob(t) > 0.1 (the probability of the factor being zero is higher than 10%) were systematically eliminated. Every time a parameter was eliminated, the new regression model, together with the experimental data, was re-evaluated by NLREG. Iteration continued until no b_i parameter had an associated prob(t) value higher than 0.1. When this happened, the last proposed model was accepted.

The most general function for the fractional factorial design of the studied compounds, considering the confusions introduced for the fractionality, was:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_5 + b_6x_6 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{26}x_2x_6 + b_{34}x_3x_4 \quad \text{Eq. 6}$$

where Y represents resolution between peaks, x_i the experimental factors in coded variables, b_{ij} the coefficients for each factor, and b_0 the intercept. This model results from a more general equation that includes every possible two-factor interaction, except quadratic terms, which are not considered in the fractional factorial design. Replacing x_5 and x_6 for $x_1x_2x_3$ and $x_1x_3x_4$, respectively, and considering that every $x_i^2 = 1$, the initial complex model results in equation 6.

The optimal regression models for each resolution calculated by NLREG from equation 6 and experimental data are shown in the following equations:

$$Rs_{12} = 440.0 - 50.8x_1 - 17.0x_3 - 9.0x_4 + 1.5x_6 + 1.8x_1x_3 + 0.7x_1x_4 + 0.2x_3x_4 \quad \text{Eq. 7}$$

$$Rs_{23} = 11.71 - 0.04x_2 - 0.28x_3 - 0.28x_4 + 0.09x_6 + 0.01x_3x_4 \quad \text{Eq. 8}$$

$$Rs_{34} = -2.9x_3 + 0.4x_1x_3 \quad \text{Eq. 9}$$

The choice criterion for these models among the different ones tried was the best fit to the regression (percentage of variance explained).

From these equations, it could be concluded that temperature was the only parameter that had no influence on resolution. Because an increase in temperature usually leads to shorter migration times and higher solubility of the solutes in the buffer, it was fixed at 35ºC for the optimization runs.

The remaining five factors studied in the screening design resulted in significant design factors, as they caused either main or interaction effects in the monitored resolutions. Applied voltage was the only variable that was significant as a main factor in all the three studied responses. pH, proportion of boric acid in the mixed buffer, concentration of the running electrolyte, and percentage of methanol appeared as main factors either in one or two of the calculated equations for resolutions. Regarding the second-order interactions, the interaction between voltage and pH was the most important one attending to the value of the corresponding b_i parameter, the ones including concentration of the background electrolyte being far less important. However, it was needed to run a more complex experimental design to exhaustively set the influence of these factors on the experimental response.

In order not to overload the optimization design with a high number of experiments resulting from the five significant factors, it was decided to fix the values of the less relevant factors.

Several criteria were considered when fixing these factors. On the one hand, general electrophoretic theory was considered; on the other, the influence of these factors on analysis time was evaluated. Even if the last one was not the monitored response, it was a secondary quality criterion, affecting separation by capillary zone electrophoresis.

The methanol percentage was set at a 5% value. This amount of methanol provided suitable selectivity without increasing much analysis time, which was negatively affected by an increase in the proportion of the organic modifier.

The negative parameter for the percentage of boric acid in the equation for Rs_{12} suggests that it should be kept at its lowest level, corresponding to a 75% boric acid in the background electrolyte.

Concentration of the running electrolyte appeared both as a main factor and in second-order interactions. Considering the experimental values of the other factor in the interaction and the numerical parameters associated to these terms, the positive and negative terms were nearly balanced, and a new criterion had to be employed. As increasing buffer concentration when working at high potentials may lead to lower Rs values (25), it was decided to set it at an intermediate concentration (20mM).

Further optimization of pH and applied voltage was carried out by means of an optimization design.

Optimization design

The previously described not-so-relevant variables were fixed at a certain value (temperature, 35ºC; percentage of boric acid, 75%; percentage of methanol, 5%; and buffer concentration, 20mM), and an optimization design was carried out considering the two remaining factors (pH and applied voltage).

The optimization study consisted of a central composite design. This model consists of a full factorial design plus a star design, thus resulting in $2^k + 2k + p$ experiments, where k is the number of factors studied (2) and p the replicates of the center point (3). The star points are located at $+\alpha$ or $-\alpha$ from the center of the experimental domain, its value depending upon the design criterion. In this optimization study, $+\alpha$ and $-\alpha$ were set at +2 and –2 from the center point of the design. Thus, the optimization design involved 11 runs.

Experimental limits for the applied potential remained the same as in the screening study (20–30 kV) considering instrumental limitations and migration time values, but limits for pH were varied towards higher values (8–10). The reason for this change was that the determination of hydrochlorothiazide at pH values below 8.0 was difficult because the uncharged form is pre-

Table III. Central Composite Design of Two Factors

	Experimental factors		Experimental values	
Trial	x_1	x_2	pH	Voltage (kV)
1	–1	–1	8.5	22.5
2	+1	–1	9.5	22.5
3	–1	+1	8.5	27.5
4	+1	+1	9.5	27.5
5	–2	0	8	25
6	+2	0	10	25
7	0	–2	9	20
8	0	+2	9	30
9	0	0	9	25
10	0	0	9	25
11	0	0	9	25

dominant (26) and it partially comigrates with the injection peak.

Table III shows the assayed optimization study, the design matrix for the central composite design, and values for each factor.

A second-order polynomial function was postulated to obtain a precise and accurate response model for the resolution of these antihypertensive drugs:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x^2_1 + b_{22}x^2_2 \quad \text{Eq. 10}$$

where Y is the studied response (Rs_{ij}) and x_1 and x_2 are pH and the applied potential, respectively.

NLREG computed suitable regression models for each of the studied responses, as stated in the following expressions:

$$Rs_{12} = 672.2 - 111.0x_1 - 11.7x_2 + 5.8x^2_1 + 0.2x^2_2 \quad \text{Eq. 11}$$

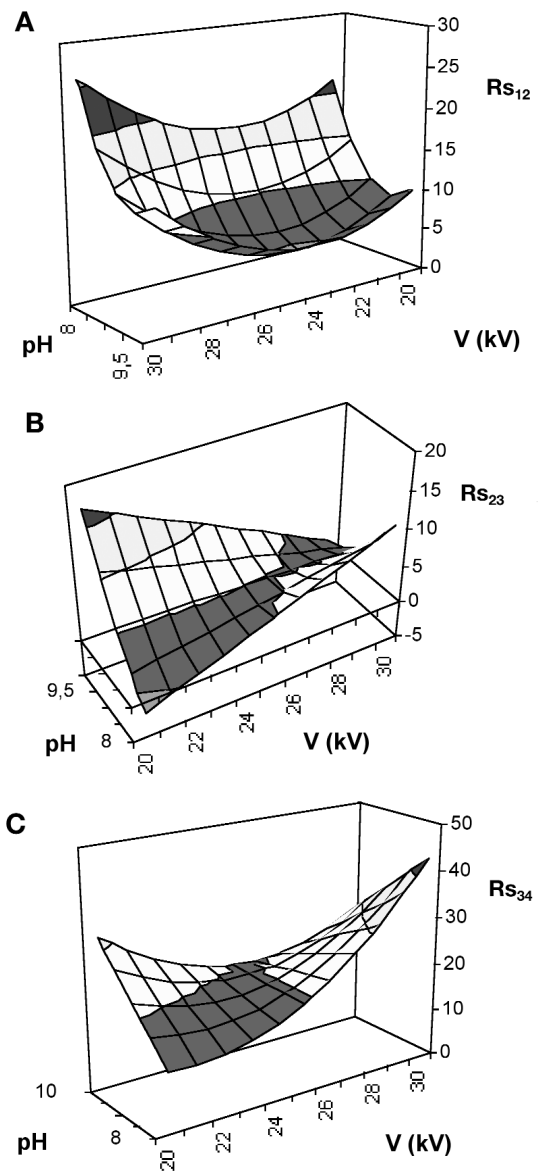

Figure 2. Response surfaces for resolution as a function of pH and applied potential: (A) Rs_{12}, (B) Rs_{23}, and (C) Rs_{34}.

$$Rs_{23} = -158.2 + 12.4x_2 - 1.4x_1x_2 + 2.0x^2_1 \quad \text{Eq. 12}$$

$$Rs_{34} = -209.2 + 47.0x_1 - 1.9x_1x_2 + 0.4x^2_2 \quad \text{Eq. 13}$$

The regression models found for the three studied responses showed no general trend. Each of the monitored resolutions had a different dependence on the experimental parameters. This could be because of the fact that each of the antihypertensive drugs to be separated belongs to a different chemical family, having very different physical-chemical properties and behavior when changing experimental conditions.

The dependence of resolution on pH and voltage is not a simple one because quadratic terms and second-order interactions have to be considered. Drawing the three-dimensional response surfaces for each resolution as a function of pH and applied potential will give us a more accurate view of the behavior of the analytical response as a function of these two factors. These response surfaces are shown in Figure 2. Optimal resolution values for Rs_{12} and Rs_{34} were found at the highest level of applied voltage (30 kV) and at the lowest value of the considered pH domain (pH 8).

Response surfaces for Rs_{23} and Rs_{34} showed two maximum areas, located either at the highest pH – lowest voltage area or at the lowest pH – highest voltage area. The absolute maximum for Rs_{23} was located at pH 10 and 20 kV, whereas for Rs_{34} it was located at pH 8.0 and 30 kV. In both cases, the secondary relative maximum was located at the experimental conditions of the absolute maximum of the other response, values of the absolute and relative maximums being not very different.

Response surface for Rs_{12} clearly showed that its optimal resolution values were always reached at pH 8.0 whatever the voltage, and resolution values decreased drastically when pH increased. Thus, a compromise was needed. The final analysis conditions were those that gave maximum resolution values for Rs_{12} and Rs_{34} and relative maximum value for Rs_{23}, thus pH 8.0 and 30 kV.

Separation of the four studied compounds was optimal when a 20mM boric acid–potassium dihydrogen phosphate (75:25, v/v) buffer with a 5% methanol percentage at pH 8.0 was used at an applied voltage of 30 kV. Figure 3 shows an electropherogram of a standard solution of the four studied compounds at the optimal conditions.

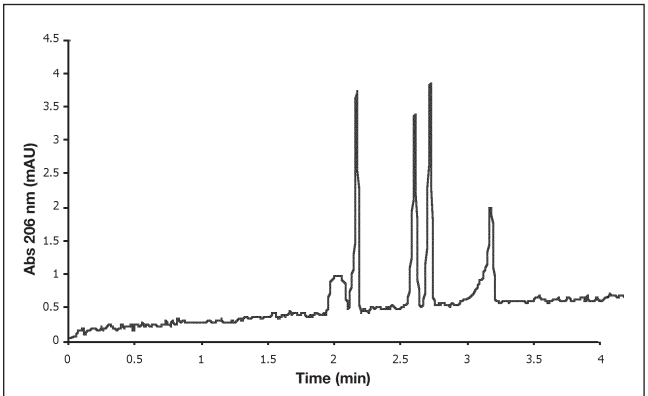

Figure 3. Electropherogram of a standard solution of the four studied compounds in the optimal conditions (5 µg/mL each): (I) hydrochlorothiazide, (II) enalapril, (III) xipamide, and (IV) enalaprilat. See the Optimization Design section for experimental conditions.

Conclusion

Experimental design methodologies proved suitable to develop a simple, fast, and non-time-consuming capillary zone electrophoretic method for the separation of four antihypertensive drugs, enalapril, enalaprilat, hydrochlorothiazide, and xipamide.

The fractional factorial design applied in the preliminary screening study considered six chemical and instrumental factors, pH, percentage of boric acid in the mixed electrolyte, applied potential, total concentration of the running buffer, temperature, and percentage of methanol as organic modifier. The monitored responses were the resolution values between peaks.

After selecting the critical factors on response, an optimization study was carried out by means of a central composite design. Analysis of experimental data and proposed regression models led to the three-dimensional response surfaces for each of the considered resolutions.

The four studied compounds were optimally separated with a mixed 20mM borate–dihydrogenphosphate (75:25, v/v) buffer with 5% methanol as organic modifier at pH 8.0. Applied voltage and temperature were 30 kV and 35ºC, respectively. The fused-silica capillary was 58.5-cm long (50-cm effective length) and 50-µm i.d. Complete separation of the four compounds was achieved within 3.5 min.

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