Improvement of the Chromatographic Separation of Several 1,4-Dihydropyridines Calcium Channel Antagonist Drugs by Experimental Design

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Abstract

A high-performance liquid chromatographic method with diode array detection has been developed and optimized for the separation of five calcium channel blockers belonging to the 1,4-dihydropyridine subgroup (nifedipine and related drugs). The possibility of the simultaneous drug analysis allows a decrease of time during the assay as well as a saving of reagents and solvents. In this work, the effect of four experimental parameters (organic modifier percentage, pH value, concentration of the buffer in the mobile phase, and column temperature) on the chromatographic resolution are investigated by experimental design in order to optimize the chromatographic separation of five 1,4-dihydropyridines (amlodipine, nitrendipine, felodipine, lacidipine, and lercanidipine). Fractional factorial design, central composite design, and finally the Multisimplex program are used to establish the optimal conditions in terms of resolution and minimum analysis time. Optimal separation of the five compounds under study is achieved in less than 12 min using a Sulpecosil LC-ABZ+Plus C18 column, a composition of mobile phase of acetonitrile–10mM acetic acid acetate buffer pH 5 (72:28, v/v) at a flow rate of 1 mL/min, a column temperature of 30°C ± 0.1°C, and a detection wavelength of 238 nm.

Introduction

The dihydropyridines (nifedipine and related drugs) are calcium channel blockers (CCAs) used most frequently as antihypertensive drugs. Apart from the lowering of blood pressure, the dihydropyridines (DHPs) may lead to other, theoretically beneficial effect such as regression of the left ventricular activity (1).

The characteristic skeleton of the most important group among the CCAs is the 1,4-dihydropyridine structure exhibiting phenyl substitution in position 4. The vascular selectivity of 1,4-DHPs is apparently coupled to the chemistry of the substituents in the 2-position and phenyl substituents in the 4-position of the dihydropyridine ring as well as the kind of substituents (2,3).

Amlodipine, lacidipine, and lercanidipine are considered representatives of third-generation calcium channel antagonists (4–6). Because the compounds of the third generation are actually the most potent and safe antihypertensive drugs, these three compounds have been used for the presented assay. Taking into account that compounds of the second generation are still widely used in hypertension therapy, nitrendipine and felodipine have been also considered as calcium blocking agents to be studied in this work (2,7). Figure 1 shows the structure of the compounds studied in this work.

Determination of 1,4-DHPs in raw material and pharmaceutical dosage forms has been carried out by UV–vis spectrophotometry (8,9), voltammetry (10–13), or high-performance liquid chromatography (HPLC) with different kinds of detection (14–18). For their quantitation in biological fluids, gas chromatography (GC)–electron capture, GC–nitrogen phosphorus, GC–mass spectrometry (MS), HPLC–UV, HPLC–amperometric detection, and liquid chromatography (LC)–MS–MS have been used (19–27).

The experimental design has shown utility in pharmaceutical development. Multivariate methods are based on the design of an

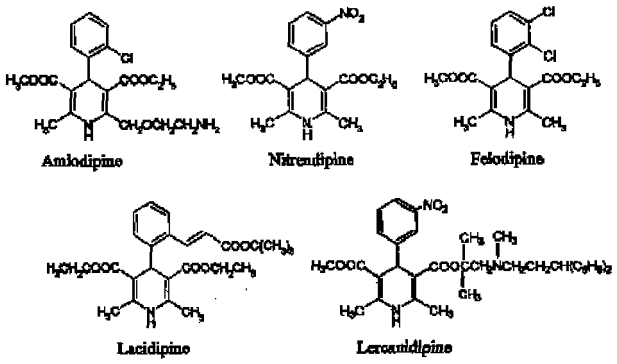

Figure 1. Structures of the studied compounds.

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experimental plan (i.e., a series of experiments in each of which the values for several parameters are changed at the same time). The results of these experiments are then evaluated using simple statistical methods like analysis of variance and regression analysis. The main experimental designs that are carried out include screening and optimization designs. With the first one it is possible to determine the parameters that have an effect, interaction effects among these parameters, influence of this interaction (positive or negative), and their significance. The most commonly used are fractional and full factorial design at two levels for each studied parameter. In order to find the optimum, optimization designs such as Box-Behnken design or central composite designs are used. This kind of methodology, together with other chemometric tools, has been used in chromatography for the prediction of the retention as well as to optimize the separation of compounds (28–31).

In a previous work, a fractional factorial design has been used for the study of the chromatographic behavior of five compounds belonging to the group of 1,4-DHPs (amlodipine, nitrendipine, felodipine, lacidipine, and lercanidipine). This fractional factorial design was based on variables, which can influence the elution of the compound from the chromatographic column. In order to achieve the maximum sensitivity of the system, a global response in terms of chromatographic peak area, retention time, and band width was considered (18). The best conditions were used to determine each compound separately, and the maximum sensitivity was achieved. These results were useful in the development of bioanalytical methods for the support of pharmacokinetic and toxicological studies for which good sensitivity is required. This is because the plasma concentrations of these five compounds are very low (ng/mL).

However, there are processes for which high sensitivity is not required and other factors such as the saving of solvents, reagents, and analysis time are considered. For example, these processes could include the development of a method to control the active components in pharmaceutical formulations or for quantitation of these drugs in biological fluids. In the case of bioanalysis, it should be noted that, prior to the sample introduction in a chromatographic system, the sample clean-up is needed, which requires an optimized extraction procedure. It also has to be considered that after the development of a chromatographic method, validation is required to assure reliable results in the quantitation of the drugs. All of the mentioned processes, development and validation of methods, and optimization of extraction procedures are very time consuming. However, the possibility of the simultaneous analysis gives the opportunity of saving time as well as reagents and solvents.

With the purpose of performing these kinds of analysis in the future, the major goal of this investigation was to obtain a quality separation of the five studied compounds in a reasonable analysis time by adjusting acceptable chromatographic factors. Fractional factorial design, central composite design, and a Multisimplex program were used in order to consider the effect on the resolution between peaks of the experimental parameters: organic modifier percentage, pH, and concentration of aqueous buffer in the mobile phase as well as the column temperature.

Experimental

Chemicals and solutions

The 1,4-DHPs amlodipine and nitrendipine were obtained from Bayer (Barcelona, Spain), felodipine was from Astra (Barcelona, Spain), lacidipine was from Glaxo (Madrid, Spain), and lercanidipine was from Recordatti (Madrid, Spain).

Methanol and acetonitrile (ACN) were Lab-Scan HPLC grade (Dublin, Ireland). All reagents used were from Merck p.a. (Darmstadt, Germany). The water used in all the experiments was obtained from a Milli-RO and Milli-Q (Waters, Milford, MA) system.

The buffer solutions were H_3PO_4–KH_2PO_4 (pH 2), CH_3COOH–CH_3COONa (pH 4), and KH_2PO_4–K_2HPO_4 (pH 6). All were in 1M concentrations. In order to achieve the desired pH value, volumes of 1M HCl and NaOH solutions were added.

For each compound, standard stock solutions (1000 µg/mL) were prepared in methanol, stored at 4°C and protected from light. Working solutions were prepared by appropriate dilution in mobile phase just before use, using amber glass volumetric flasks in order to avoid photodegradation (32).

Apparatus

The HPLC system used consisted of a Waters 510 pump and Rheodyne (Coati, CA) Model 7125 injector fitted with a 20-µL loop. For the quantitation of the compounds, photometric detection was performed using a Waters 996 photodiode array detector set to 238 nm. Millennium 3.2 software was used for the acquisition and processing of chromatographic data (Waters, Milford, MA). Chromatography was achieved using a Supelcosil LC-ABZ+Plus analytical column (250- × 4.6-mm i.d., 5-µm particle size) (Supelco, Barcelona, Spain) with a µBondapack C18 precolumn module (Waters). The column was stored at constant temperature using a Waters TMC temperature control system.

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

Final chromatographic conditions

Separation was carried out using an ACN–water (72:28, v/v) mobile phase containing 10mM acetate buffer (pH 5) at a flow rate of 1 mL/min. The temperature of the column was set at 30°C ± 0.1°C. The mobile phase was filtered through a 0.45-µm membrane, and dissolved air was removed by flushing helium through the solution.

Results and Discussion

Definition of the system to study

Several instrumental variables can be considered in the optimization of the chromatographic separation of a group of compounds. Before setting the experiments to be performed, the knowledge of the assay system, as well as previous experience

in the analysis of this family of compounds, makes it easier to define the variables of study.

Because of some previous experience in the analysis of these drugs with HPLC, some chromatographic parameters that could affect the elution of the compounds were fixed and some were studied by screening design.

On one hand, the column type, flow rate, and kind of organic modifier were fixed as follows. The chromatography was performed on a Supelcosil LC-ABZ+Plus C18 column because, after some trials with other kinds of columns, this one provided the best symmetry of the chromatographic peaks. The flow rate was set at 1 mL/min as a normal working value on the LC.

These compounds are quite apolar, and long analysis time was not desired. For this reason, ACN was chosen as organic modifier instead of methanol. The pH was also an important factor to be considered because amlodipine and lercanidipine have pK_a values of 9 and 7, respectively, because of the presence of an amino group in their structure (33,34).

On the other hand, the method was based on the optimization of four other factors attending to: (*i*) the composition of the mobile phase in terms of percentage of organic modifier (%ACN), pH, and buffer concentration; and (*ii*) the temperature of the column.

The response to be studied is defined by the aim of this work. Because the separation is of fundamental importance to any analyst using HPLC, resolution (Rs) is a quantitative description of the separation that is obtained between two peaks. This term is defined by the following relationship, which describes how good the separation is:

$$Rs = 2 \cdot \frac{(t_2 - t_1)}{(w_1 + w_2)} \qquad \text{Eq. 1}$$

where t_1 and t_2 represent retention times of peaks 1 and 2 and w_1 and w_2 represent widths of peaks 1 and 2, respectively. The separation between two peaks that are assumed to be Gaussian is taken as the distance between the band centers divided by the average peak widths. Thus, the greater the separation in retention times and the narrower the peaks, the higher the resolution. In general, for quantitative work, the aim would be a minimum resolution of 1, whereas a smaller resolution could be tolerated for qualitative work. As five compounds were studied, four responses should be defined as Rs_{12}, Rs_{23}, Rs_{34}, and Rs_{45} for the separation between peaks 1 and 2, 2 and 3, 3 and 4, and 4 and 5, respectively. In each experiment, the numbering of the peaks was considered as they eluted from the column.

To achieve the chromatographic resolution of the studied compounds, the optimization was set in two steps (including a full factorial design) to evaluate the variables that were significant factors and a central composite design in order to obtain the response surface from which the factors that give optimal responses can be deduced.

All experiments were performed in random order to avoid systematic errors. In both designs, two more trials were run corresponding to the center of the experimental domain to test the model linearity and to obtain an estimation of experimental variance. The analysis of the results was performed using the nonlinear regression analysis (NLREG) (35). Terms that were not significant were excluded, and a new model was made. Among the different regressions assayed, the choice criterion was the best fit to the regression (percentage of variance explained).

This group of compounds has some degree of configuration or functional groups that absorb UV–vis radiation. As the UV absorbance detection shows some advantages such as being easy to use, reliable, and relatively inexpensive, it was chosen for the detection of these compounds. For the quantitation of the data, the UV diode array was set at 238 nm because the group of 1,4-DHPs present a common absorption maximum at this wavelength. The resolution for every two peaks was calculated from the obtained data after the injection of the compounds at a concentration of 10 µg/mL in mobile phase.

Screening design

Considering the four factors mentioned previously for a full two-level factorial design (2^4), 16 experiments should be carried out. In order to perform a lower number of experiments, one of the variables was defined as a combination of the other

Table I. Level Codification for the 2^{4-1} Fractional Factorial Design

Factor	–1	0	+1
x_1 (%ACN)	50	60	70
x_2 (pH)	2	4	6
x_3 temperature (°C)	25	30	35
x_4 buffer concentration (mM)	1	10	20

Table II. Responses Obtained in the 2^{4-1} Fractional Factorial Design for Four Factors; Experimental Design and Experimental Set-Up Derived from the Theoretical Design

	Experimental design				Experimental set-up							
Trial	x_1	x_2	x_3	x_4	%ACN	pH	T (°C)	Conc. (mM)	Rs_{12}	Rs_{23}	Rs_{34}	Rs_{45}
1	+1	+1	+1	–1	50	6	25	1	0.99	1.93	3.62	5.55
2	–1	+1	+1	+1	70	6	25	20	0.65	3.35	2.36	2.24
3	+1	–1	+1	+1	50	2	25	20	2.29	1.92	2.87	3.19
4	–1	–1	+1	–1	70	2	25	1	1.68	0.18	1.08	2.84
5	+1	+1	–1	+1	50	6	35	20	1.77	3.69	3.69	6.50
6	–1	+1	–1	–1	70	6	35	1	0.11	2.36	2.50	1.43
7	+1	–1	–1	–1	50	2	35	1	0.17	0.98	2.83	3.83
8	–1	–1	–1	+1	70	2	35	20	0.91	1.98	2.24	3.67
9	0	0	0	0	60	4	30	10	3.92	0.96	1.22	2.79
10	0	0	0	0	60	4	30	10	4.41	0.47	1.77	2.85

three variables as shown in the following equation:

Buffer concentration =
percentage of ACN × pH × temperature Eq. 2

The experimental domain in which every factor was evaluated as well as the codification for each factor appears in Table I. Percentages higher than 50% were chosen to avoid long chromatograms and pH values lower than 6 were chosen to work with the compounds amlodipine and lercanidipine in their ionic species. Resolution between peaks was set as the response, and it was calculated according to equation 1. The responses, as well as the set up of the experiments, are summarized in Table II.

The most general polynomial function for response and variable is:

$$Y = \beta_0 + \sum_i \beta_i x_i + \sum_{ii} \beta_{ij} x_i x_j \quad \text{Eq. 3}$$

where Y is the studied response; x_i and x_j are the variables considered in the study; and β_0, β_i, and β_{ij} are the numerical parameters to be calculated. The final estimation of the parameters is achieved when the square sum of errors (U) is minimized:

$$U = \sum_i^n (Y_{exp} - Y_{calc})^2 \quad \text{Eq. 4}$$

where n is the number of experiments, Y_{exp} is the response calculated by means of experimental data (area, retention time, and band width), and Y_{calc} is the response given by the program following the proposed regression model.

The analysis of the output was based on the evaluation of the prob(t), which indicates the probability of β_0, β_i, and β_{ij} of being zero. Those parameters whose probability of being zero was greater than 10% (i.e. prob(t) > 0.1) were systematically eliminated.

In this case, a second-order polynomial function was postulated to obtain a precise and accurate response model for the resolution of these calcium channel antagonists.

$$Rs = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_4 x_4 + \beta_{12} x_1 x_2 + \beta_{23} x_2 x_3 + \beta_{22} x_2^2 \quad \text{Eq. 5}$$

where Rs represents the studied response, x_i is the experimental factors in coded variables, β_{ij} is the coefficients for each factor, and β_0 is the intercept. Table III shows the values of the coefficients calculated with NLREG.

From these results it could be concluded that the temperature of the column and the buffer concentration in the mobile phase have less influence than the other two variables in the peak resolution.

Prior to the optimization of the system by means of central composite design, the temperature of the column and the

Table III. Final Parameters for Regression Models Obtained from Fractional Factorial Design

	Resolution			
Parameter	Rs_{12}	Rs_{23}	Rs_{34}	Rs_{45}
β_0	–8.55	6.74	10.51	22.49
β_1	0	0	–0.07	–0.31
β_2	6.89	–2.98	–2.19	–3.08
β_4	0	0.07	0	0
β_{12}	0	0	0	0.05
β_{23}	–0.02	0	0	0
β_{22}	–0.77	0.32	0.26	0

Table IV. Level Codification for the Central Composite Design

Factor	–2	–1	0	+1	+2
x_1 (%ACN)	50	57	65	72	80
x_2 (pH)	3.5	4	4.5	5	5.5

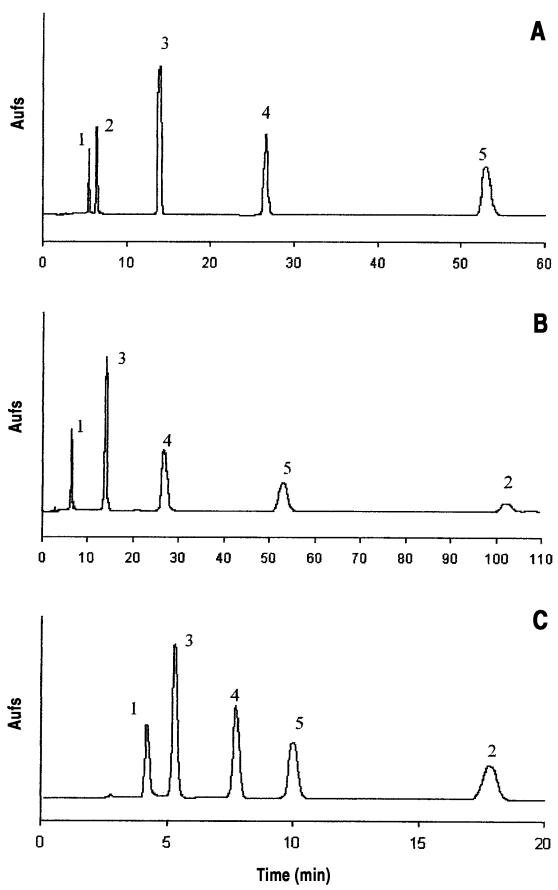

Figure 2. UV chromatograms corresponding to sample solution level at concentration 10 µg/mL for each compound in different chromatographic conditions: (A) ACN–H_2O (50:50, v/v), 0.1mM acetate buffer (pH 2), 25°C; (B) ACN–H_2O (50:50, v/v), 20mM acetate buffer (pH 6), 25°C; and (C) ACN–H_2O (70:30, v/v), 20mM acetate buffer (pH 6), 35°C. Peaks: amlodipine (1), lercanidipine (2), nitrendipine (3), felodipine (4), and lacidipine (5).

buffer concentration in the mobile phase were set at 30°C ± 0.1°C and 10mM, respectively. These values correspond to the central point of the experimental domain.

It has already been explained that the resolution between peaks depends on retention times and on the width of the peaks. However, the width is predetermined by the time in which the compound elutes from the column. It was observed that lower values of retention time have resulting narrow peaks, whereas a late elution of the compounds gives an enlargement of the band width. Thus, not only the values in the resolution but also the retention time values of the compounds helped in the establishment of a new experimental domain to study.

Taking these data into account, it was also observed that when low percentages of ACN (i.e., 50% or 60%) were used, the neutral compounds (nitrendipine, felodipine, and lacidipine) eluted at high retention times. Apart from the influence of the percentage of ACN, the pH value was the main affecting factor in the case of the basic compounds (amlodipine and lercanidipine). Thus, a pH value of 3 showed that amlodipine appears with the injection peak, and at pH values of 6, the retention time of lercanidipine is too high to obtain a short analysis time. These two factors seemed to be significant for the retention time of the compounds as well as for the resolution of the peaks as it was discussed. Considering the obtained results, a new experimental domain was considered for the optimization of the system by means of central composite design.

Optimization design

As an increase in the percentage of ACN showed shorter retention times, the new domain ranged from 50% to 80%. Limits for pH were varied towards the central point because of the obtained results. Thus the new domain ranged from 3.5 to 4.5. The new domain as well as the new codification for the variables are collected in Table IV.

In the data collected in the fractional factorial design, it was also observed that more than one possibility in terms of composition of the mobile phase gave a complete resolution of the peaks (Figure 2). To define the analysis time in which the separation should be achieved, two other responses were included in the optimization design. The new responses to be considered were defined as minimum and maximum analysis time.

The minimum analysis time (t_{min}) was defined as the retention time in which the first compound elutes from the column, and the maximum retention time (t_{max}) was defined as the retention time in which the last compound elutes from the column.

Thus, in order to get the equations for these two new responses, all of the retention times from all the first and last chromatographic peaks from the experiments run in the central composite design were taken into account.

For the optimization design, Table V shows the set up of the experiments and the responses calculated with experimental data.

For the resolution function the response surface was approximately by a second order polynomial function. The equation model obtained was:

$$Rs = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^{2+} \beta_{12} x_1 x_2 + \beta_{112} x_1^2 x_2 + \beta_{112} x_1 x_2^2 \quad \text{Eq. 6}$$

where Rs represents the studied response, x_i the experimental factors in coded variables, β_{ij} the coefficients for each factor, and β_0 the intercept. The values of each parameter calculated with NLREG are collected in Table VI.

The variance for every case: Rs_{12} (98.06%), Rs_{23} (81.88%), Rs_{34} (95.35), and Rs_{45} (94.21%) indicated that this model could explain the experimental data in a proper way.

The proposed model for the responses related with the time analysis are showed by equations 7 and 8:

$$t_{min} = (16.94) + (7.70)x_2 + (-0.0021)x_1^2 + (1.57)x_2^2 + (0.0010)x_1^2 x_2 + (-0.017)x_1 x_2^2 \quad \text{Eq. 7}$$

$$t_{max} = (-1167.80) + (916.58)x_2 + (0.30)x_1^2 + (-125.92)x_2^2 + (-11.42)x_1 x_2 + (-0.05)x_1^2 x_2 + (1.27)x_1 x_2^2 \quad \text{Eq. 8}$$

Table V. Level Quantitation, Experimental Set-Up, and Results for Central Composite Design

	Experimental design		Experimental set up		Responses					
Trial	x_1	x_2	%ACN	pH	Rs_{12}	Rs_{23}	Rs_{45}	Rs_{56}	t_{min} (min)	t_{max} (min)
1	–1	–1	57	4	6.21	1.09	2.28	3.90	2.88	24.37
2	+1	–1	72	4	3.45	0.52	0.64	2.40	2.95	9.24
3	–1	+1	57	5	4.10	2.69	5.60	0.48	4.14	24.26
4	+1	+1	72	5	1.28	1.97	2.31	1.62	3.99	10.86
5	–2	0	50	4.5	7.38	0.92	2.03	5.38	3.79	48.18
6	+2	0	80	4.5	0.10	1.57	0.19	1.15	4.12	7.11
7	0	–2	65	3.5	1.94	2.26	6.67	2.73	2.50	15.46
8	0	+2	65	5.5	1.24	2.23	1.0	2.79	4.84	21.94
9	0	0	65	4.5	3.95	2.83	1.89	2.40	3.36	12.96
10	0	0	65	4.5	3.44	2.41	1.89	2.57	3.37	13.08

Table VI. Final Parameters for Regression Models Obtained from Central Composite Design

	Resolution			
Parameter	Rs_{12}	Rs_{23}	Rs_{34}	Rs_{45}
β_0	327.96	–287.88	–874.35	64.55
β_1	–15.18	12.92	40.92	–11.68
β_2	0	0	0	148.74
β_{11}	0.15	–0.13	–0.41	0.17
β_{22}	–15.33	13.16	42.89	–34.78
β_{12}	2.43	–1.79	–6.20	0
β_{112}	–0.03	0.03	–0.09	–0.04
β_{122}	0.20	–0.20	–0.63	0.54

where t_{min} is the retention time of the first peak of the chromatogram, t_{max} is the retention time of the last peak of the chromatogram, and x_1 and x_2 are the two studied variables: percentage of ACN in the mobile phase and the pH value.

In this case, the proportion of the explained variance was 98.83% and 99.87%, respectively. Response surfaces as a function of percentage of ACN and pH value in the mobile phase were drawn with Microsoft Excel (Redmond, WA) for each resolution and they are shown in Figure 3.

At the sight of the group of responses, it can be deduced that it is difficult to determine visually the optimal conditions for which the chromatographic separation is achieved. Because of this, other chemometrical tools had to be used.

Optimization by means of the Multisimplex program

The Multisimplex program was used for the study of all the responses simultaneously (35). Once the range of each variable and responses were defined, the program suggested a $k + 1$ number of experiments, where k is the number of variables to be studied. After the responses were calculated by equations 6–8, the answers were introduced and the program suggested a new experiment. The same process was repeated until the optimal conditions were achieved. Multisimplex makes use of the "membership value", which ranges from 0 to 1 and takes into account the responses considered in the optimization. Optimal conditions are achieved when the membership value is close to 1.

The target values of every response were defined as follows: for resolution, the value of 1 was given, and the t_{min} value was fixed on 4 min in order to avoid the elution of the first compound very near to the injection peak. It was also taken into account that this method could be the basis for a future method for the screening of these compounds in biological samples. Although it was seen that lower retention times could be achieved, more polar compounds present in this kind of matrix would elute at retention times lower than 4 min. This avoids the interferences for the quantitation of the compounds of interest. For the t_{max} variable, a value near 12 min was decided upon. Thus, not only a fast chromatogram could be run, but also there is enough time to achieve a total resolution of the peaks.

Table VII shows the experiments proposed by the Multisimplex program as well as the calculated value for each response with the equations 6–8 and the global response calculated by the program under the term "current membership" (order in terms of increasing response).

Because of the results, the resolution for the five 1,4-DHPs seemed to get the optimum response when values near 70% ACN and a pH value of 5 were used in the composition of the mobile phase. The last values of Table VII for the composition of the mobile phase (ACN–acetate buffer, pH 5, 72:28, v/v) were used to verify the results. Thus, the compounds were injected in the chromatographic system under these conditions. With this composition of mobile phase, the capacity factors (k') for each compound were 0.39 for amlodipine, 1.38 for nitrendipine, 2.54 for felodipine, 3.72 for lacidipine, and 4.61 for lercanidipine in 12 min of analysis time. Robustness of the chromatographic system was tested for this composition of mobile phase for several days, with a freshly prepared mobile phase. Variations in the

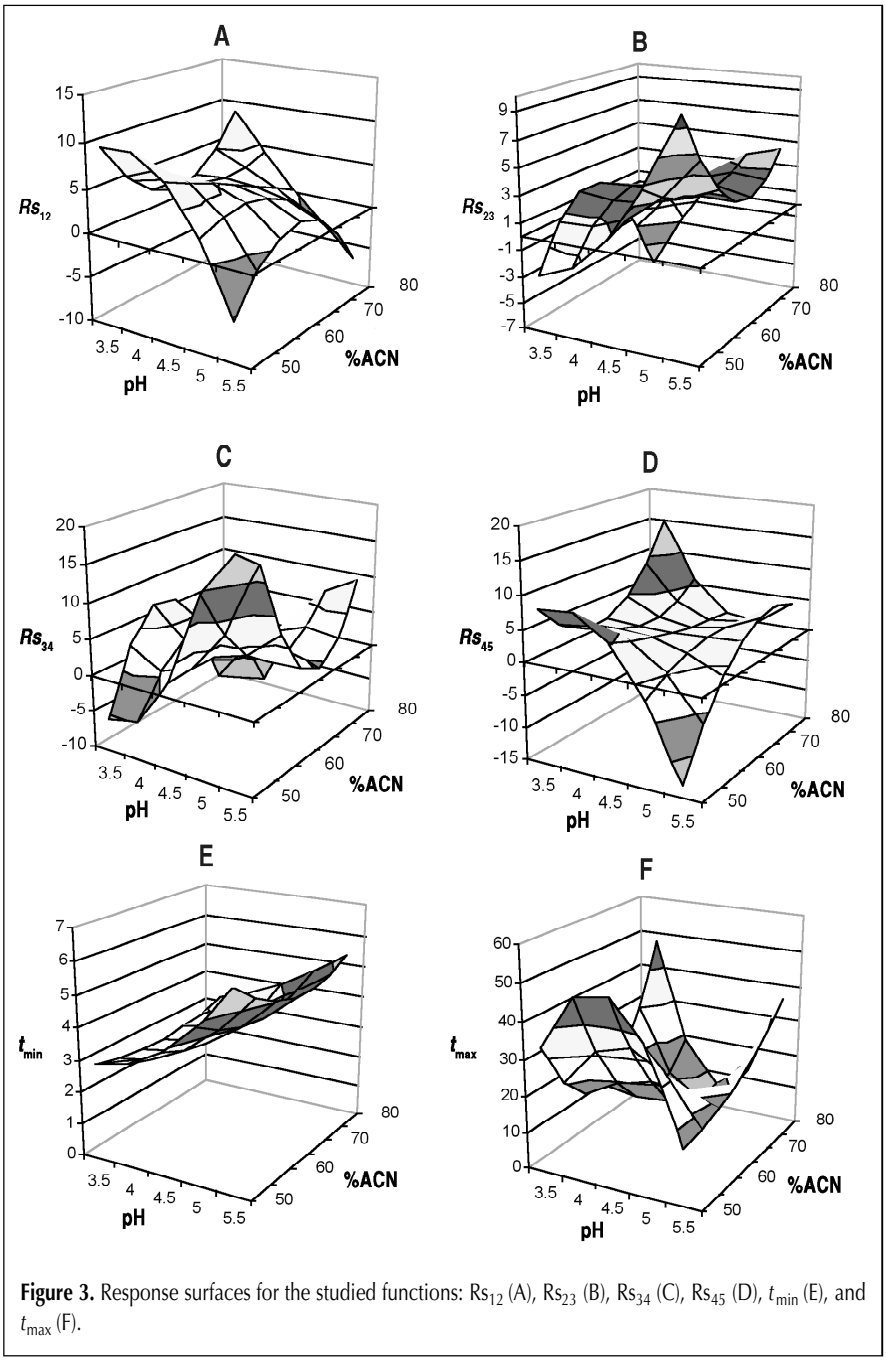

Figure 3. Response surfaces for the studied functions: Rs_{12} (A), Rs_{23} (B), Rs_{34} (C), Rs_{45} (D), t_{min} (E), and t_{max} (F).

retention times lower than 0.05 min were observed for all the peaks. Figure 4 shows the complete separation of the chromatographic peak under these conditions.

Conclusion

This experimental design, coupled with knowledge of the aspects of chemical properties of the compounds and the chromatographic chemical system, turned out to be an efficient and fast tool for the optimization of compound separation in the chromatographic system.

In a previous work (18), the use of fractionated factorial and central composite design allowed for the definition of the best chromatographic conditions in which the maximum sensitivity in the measurement for each compound was achieved. In that case, the chromatographic conditions did not allow a simultaneous quantitation of the five compounds.

However, the use of the Multisimplex program in this work allowed the simultaneous study of the responses for all of the compounds as well as the considered factors, achieving a total chromatographic separation in a short period of time.

The obtained simultaneous chromatographic method has been applied to the optimization of several clean-up procedures involving liquid–liquid extraction and solid-phase extraction (37) for extraction of these compounds in plasma samples, which have been used for further experiments in bionalysis (38).

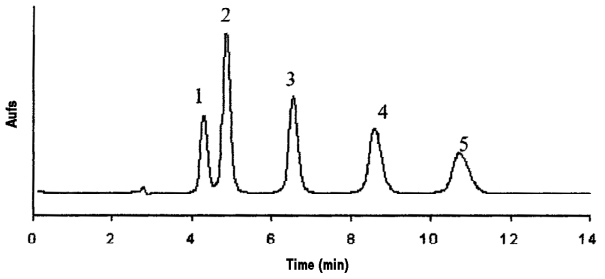

Figure 4. UV chromatogram corresponding to sample solution level at concentration 10 µg/mL for each compound under final optimized chromatographic conditions: ACN–H_2O (72:28, v/v), 10mM acetate buffer (pH 5), 30°C. Peaks: amlodipine (1), nitrendipine (2), felodipine (3), lacidipine (4), and lercanidipine (5).

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Table VII. Values of Each Response and Global Response Calculated by Multisimplex Program for Each of the Experiments Proposed

	Variables		Responses						
Trial no.	%ACN	pH	Rs_{12}	Rs_{23}	Rs_{34}	Rs_{45}	t_{min} (min)	t_{max} (min)	Current membership
1	60	4.0	5	1.9	3.4	3.3	2.9	51	0.000
2	70	4.3	3	2	1.8	1.6	2.9	20.9	0.000
3	63	5.0	3.6	2.4	1.7	2	4	12.3	0.000
4	73	5.0	0.7	2.5	1.7	2	4.1	11.4	0.000
5	80	4.3	1.9	0	–2.8	2.4	3.8	9.4	0.000
13	76	4.2	2.6	0.6	–1.4	2.4	3.4	7.9	0.000
9	72	5.0	1.1	2.4	1.3	2.1	2.9	11.6	0.744
17	70	5.0	1.9	2.3	0.8	2.3	4	12.3	0.782
15	76	4.6	0.9	2.1	1.8	0.9	3.8	5.4	0.821
8	73	4.5	2.1	2.1	1.4	1.2	3.5	5.5	0.827
21	72	4.7	1.9	2.3	1.5	1.4	3.7	7	0.830
16	71	4.8	2	2.3	1.3	1.7	3.8	8.7	0.834
18	73	4.8	1.3	2.4	1.7	1.4	3.8	7.8	0.844
22	73	4.7	1.5	2.3	1.7	1.3	3.7	6.6	0.846
11	74	4.7	1.2	2.3	1.9	1.1	3.8	6.3	0.850
23	72	5.0	1.1	2.4	1.3	2.1	4.1	11.6	0.859

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