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Micellar electrokinetic chromatography as a fast screening method for the determination of 1,4-dihydropyridine calcium antagonists

V. Martínez, J.A. López, R.M. Alonso, R.M. Jiménez*

Departamento de Química Analítica, Facultad de Ciencias, Universidad del País Vasco UPV/EHU, Apdo. 644, 48080 Bilbao, Spain

Abstract

A micellar electrokinetic chromatographic method (MEKC) was optimized for the separation of six calcium antagonists. The effects of the buffer (concentration and pH), concentration of sodium dodecyl sulphate (SDS), the organic modifier, the injection time, and the voltage applied were studied. A final appropriate electrolyte of 50 m*M* borate buffer, pH 8.2, containing 20 m*M* SDS and 15% (v/v) acetonitrile was found to provide the optimum separation with respect to resolution and migration time. The samples were introduced hydrostatically for 4 s at 50 mbar injection pressure and the applied voltage was +25 kV. The screening of the six compounds was achieved in less than 15 min: nifedipine (migration time, t_m=6.9 min), nimodipine (t_m=10.1 min), felodipine (t_m=12.2 min), nicardipine hydrochloride (t_m=12.7 min), lacidipine (t_m=13.5 min) and amlodipine besylate (t_m=14.1 min, t_m=8 min). The method developed showed to be linear at least up to 70 μg/ml with a detection limit of about 5 μg/ml for each compound. The within-day and inter-day area reproducibility (R.S.D.) were, respectively, lower than 4.8 and 8.6% for six replicate samples.

Keywords: Pharmaceutical analysis; Dihydropyridines; Calcium antagonists

1. Introduction

Calcium antagonists have become one of the most important advances in the treatment of hypertension since their introduction over 20 years ago. The increase in the number of available calcium antagonists (as new formulations of pre-existing drugs or new chemical entities) over recent years has contributed to an ever-changing scenario regarding their appropriate use compared with other antihypertensive agents [1].

Since the discovery of the vasodilating activity of nifedipine (dimethyl-1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate), many analogues of 1,4-dihydropyridines have been synthesised with somewhat different pharmacological and therapeutic profiles. This homologous series of 4-aryl-1,4-dihydropyridine belongs to the pharmacological parent drug known as calcium antagonists. The common action mechanism of these compounds is to block the slow calcium channels, and to inhibit the transmembrane influx of calcium ions into cardiac and vascular smooth muscles. These drugs are clinically used mainly in the basic treatment of essential arterial hypertension [2].

Chemically, this family contains as pattern structure a substituted benzene included in position 4 of 1,4-dihydropyridine (1,4-DHP) ring tetrasubstituted. The 1,4-DHP moiety is essential for their pharmacological activity on the cardiovascular system [3].

*Corresponding author.

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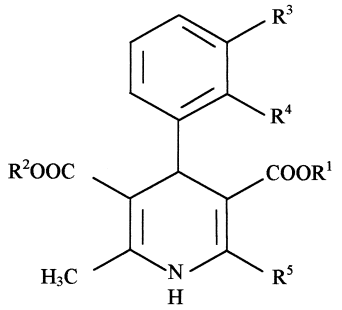
The calcium antagonists are extensively metabolised in the liver, prior to excretion; the main metabolic pathway is oxidation of the dihydropyridine ring to the pyridine analogue. None of the metabolites is pharmacologically active [4,5].

In literature, a reversed-phase HPLC method [6] and a gas chromatographic method [7] are described for the screening of these calcium antagonists. Moreover, a capillary electrophoretic method has been reported for only three compounds of this family (nifedipine, nicardipine hydrochloride and amlodipine besylate) [8].

Micellar electrokinetic chromatography (MEKC) is an adaptation of capillary electrophoresis. It was initially conceived for the electrokinetic analysis of neutral compounds but it is also effective for the separation of ionic compounds that have similar electrophoretic mobilities [9].

The aim of this work is the development of a micellar electrokinetic chromatographic (MEKC) method for the screening of calcium antagonists. An optimization study of the technique variables: pH and buffer concentration (borate buffer), surfactant concentration (SDS), organic modifier, injection time and applied voltage, was carried out.

The compounds under study were: nifedipine (dimethyl-1, 4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate), nimodipine (isopropyl-2-methoxyethyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate), nicardipine hydrochloride (1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)3,5-pyridine dicarboxylic acid, methyl-2-[methyl(phenylmethyl)amino]-ethyl ester monohydrochloride), felodipine (ethyl methyl 4-(2,3-dichlorophenyl)1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate), lacidipine ((*E*)-4-{2-[3-(1,1-dimethylethoxy)-3-oxo-1-propenyl]phenyl}-1, 4-dihydro-2, 6-dimethyl-3,5-pyridine dicarboxylic acid diethyl ester) and amlodipine besylate (2-[(2-aminoethoxy)methyl] - 4 - (2 - chlorophenyl) - 3 - ethoxycarbonyl - 5 - methoxy carbonyl-6-methyl-1,4-dihydropyridine benzene sulphonate) (Fig. 1).



DRUG	R^1	R^2	R^3	R^4	R^5
Nifedipine	$-CH_3$	$-CH_3$	-H	$-NO_2$	$-CH_3$
Nimodipine	$-CH(CH_3)_2$	$-CH_2CH_2OCH_3$	$-NO_2$	-H	$-CH_3$
Felodipine	$-CH_3$	$-CH_2CH_3$	-Cl	-Cl	$-CH_3$
Nicardipine	$-CH_3$	$-CH_2N(CH_3)CH_2C_6H_5$	$-NO_2$	-H	$-CH_3$
Lacidipine	$-CH_2CH_3$	$-CH_2CH_3$	-H	$CH=CH-COOC(CH_3)_3$	$-CH_3$
Amlodipine	$-CH_2CH_3$	$-CH_3$	-H	-Cl	$-CH_2O(CH_2)_2NH_2$

Fig. 1. Structures of the studied calcium antagonists.

2. Experimental

2.1. Reagents

Nifedipine and nimodipine were supplied by Bayer (Barcelona, Spain), nicardipine hydrochloride by Roger (Barcelona, Spain), felodipine by Astra (Bilbao, Spain), amlodipine besylate by Pfizer (Madrid, Spain) and lacidipine by Glaxo (Madrid, Spain).

Sodium dodecyl sulphate (SDS) was purchased from Sigma (Bilbao, Spain). Sodium tetraborate and boric acid, supplied by Merck (Bilbao, Spain), were suprapur quality. Solvents (acetonitrile and acetone) were Lab-Scan HPLC grade (Dublin, Ireland). The water was obtained from Milli-RO and Milli-Q Waters system (Barcelona, Spain).

2.2. Apparatus and electrophoretic conditions

MEKC was performed on a HP ^{3D}CE capillary electrophoretic system equipped with a Hewlett-Packard (Bilbao, Spain) diode array UV detector. A Frigiterm-10 external water bath (Selecta, Barcelona, Spain) was used for tray cooling.

Samples were injected by pressure (4 s, 50 mbar) onto a fused-silica capillary (Composite Metal Services, Worcester, England) of 58.5 cm×75 μm I.D.× 375 μm O.D. with the detection window at 50 cm. The capillary temperature was kept constant at 23.00±0.01°C and a voltage of +25 kV (current, 30 μA) was applied. The UV detector was set at 200 or 236 nm. Tray temperature was maintained at 25.0±0.1°C.

Prior to the first analysis the capillary was rinsed for 15 min with 1 *M* sodium hydroxide, 10 min with deionized water and 5 min with the running electrolyte. After each run the column was flash for 2 min with 0.1 *M* sodium hydroxide. The separation electrolyte buffer was refreshed after six runs.

A personal computer workstation with the HP^{3D} software was used for instrumental control and data analysis.

2.3. Standard solutions

Stock solutions of each individual cardiovascular drug were prepared by dissolving the compounds in acetonitrile–water (50:50, v/v) at the concentration of 1000 μg/ml. Dilutions of the stock solutions were performed in 50 m*M* borate buffer, pH 8.2, containing 20 m*M* SDS, to give a final appropriate analyte concentration.

All the solutions were prepared in amber glass volumetric flasks due to the easy photodegradation of these compounds, and were stored under refrigeration.

2.4. Buffer preparation

The different separation buffers were prepared by mixing the appropriate volumes of stock solutions of 0.1 *M* boric acid, 0.1 *M* sodium tetraborate, 0.2 *M* SDS and acetonitrile, to give the desired pH, surfactant concentration and organic modifier percentage. The pH of the buffers was controlled by the borax/boric acid ratio in the mixture.

The final appropriate running buffer consisted of 50 m*M* borate buffer, pH 8.2, containing 20 m*M* SDS and 15% (v/v) acetonitrile.

3. Results and discussion

The neutral nature of some of the calcium antagonists studied makes it necessary the development of a micellar electrokinetic chromatographic method for their separation.

MEKC requires the addition of charged surfactants, forming micelles, to the background electrolyte at a level greater than its critical micelle concentration. The micelles formed have their own electrophoretic mobility that is different from the surrounding aqueous phase. Analytes may differentially partition themselves between the micellar and the aqueous phases depending on their polarity thus promoting selectivity. Hence, the migration order can be a way to predict the polarity of the compounds. The hydrophobic core of the micelles provides sites of interaction that greatly enhance the solubility of insoluble non-polar compounds in aqueous media [10]. This is the case of nifedipine, nimodipine, felodipine and lacidipine, which are insoluble in aqueous media due to their neutral nature in the whole pH range.

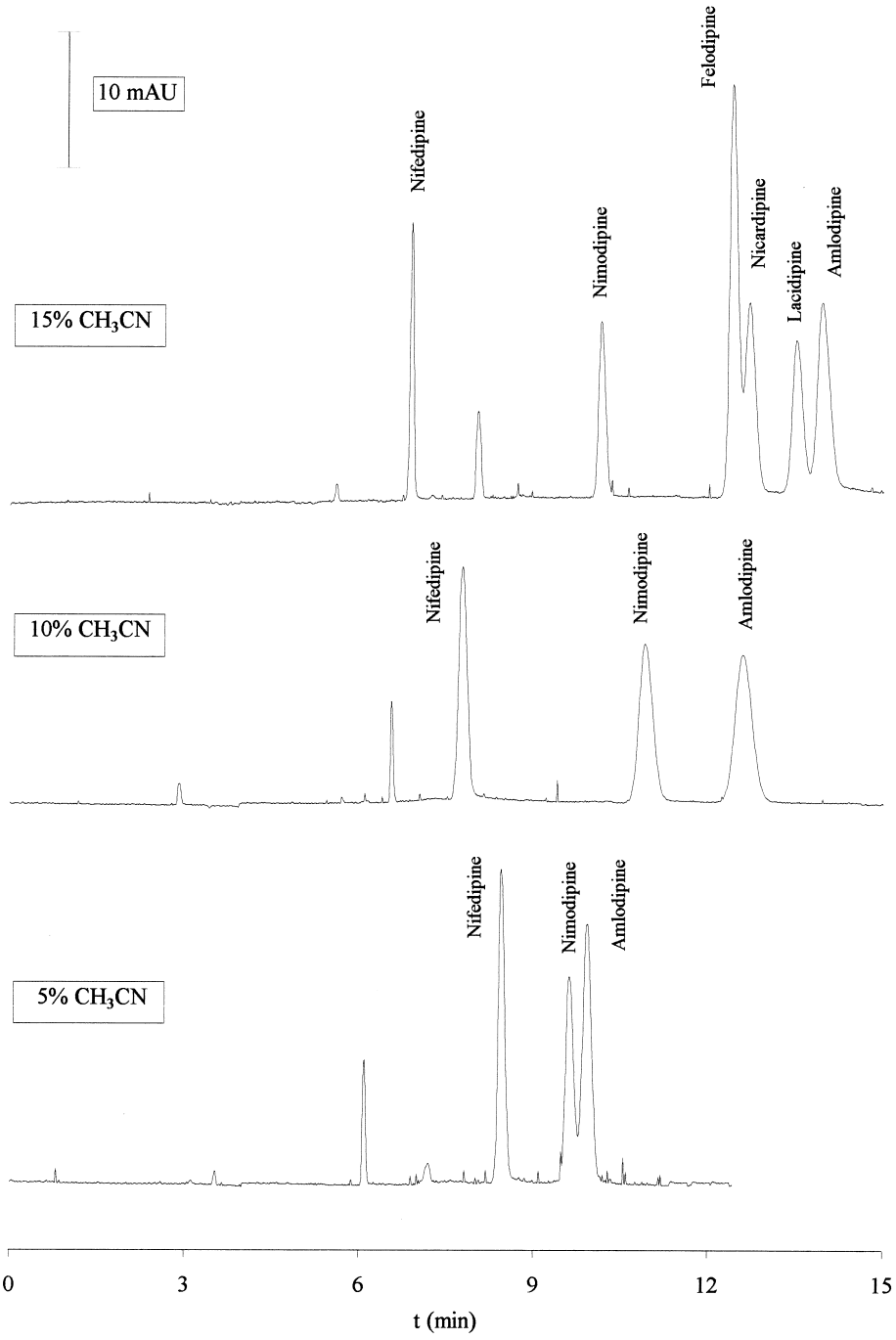


Fig. 2. Effect of the acetonitrile percentage on the screening of a standard solution of 1,4-DHP (40 μg/ml): 20 m*M* SDS, 50 m*M* H_3BO_3–$Na_2B_4O_7$, pH 8.2; V=25 kV; t_{inj}=4 s; λ=236 nm.

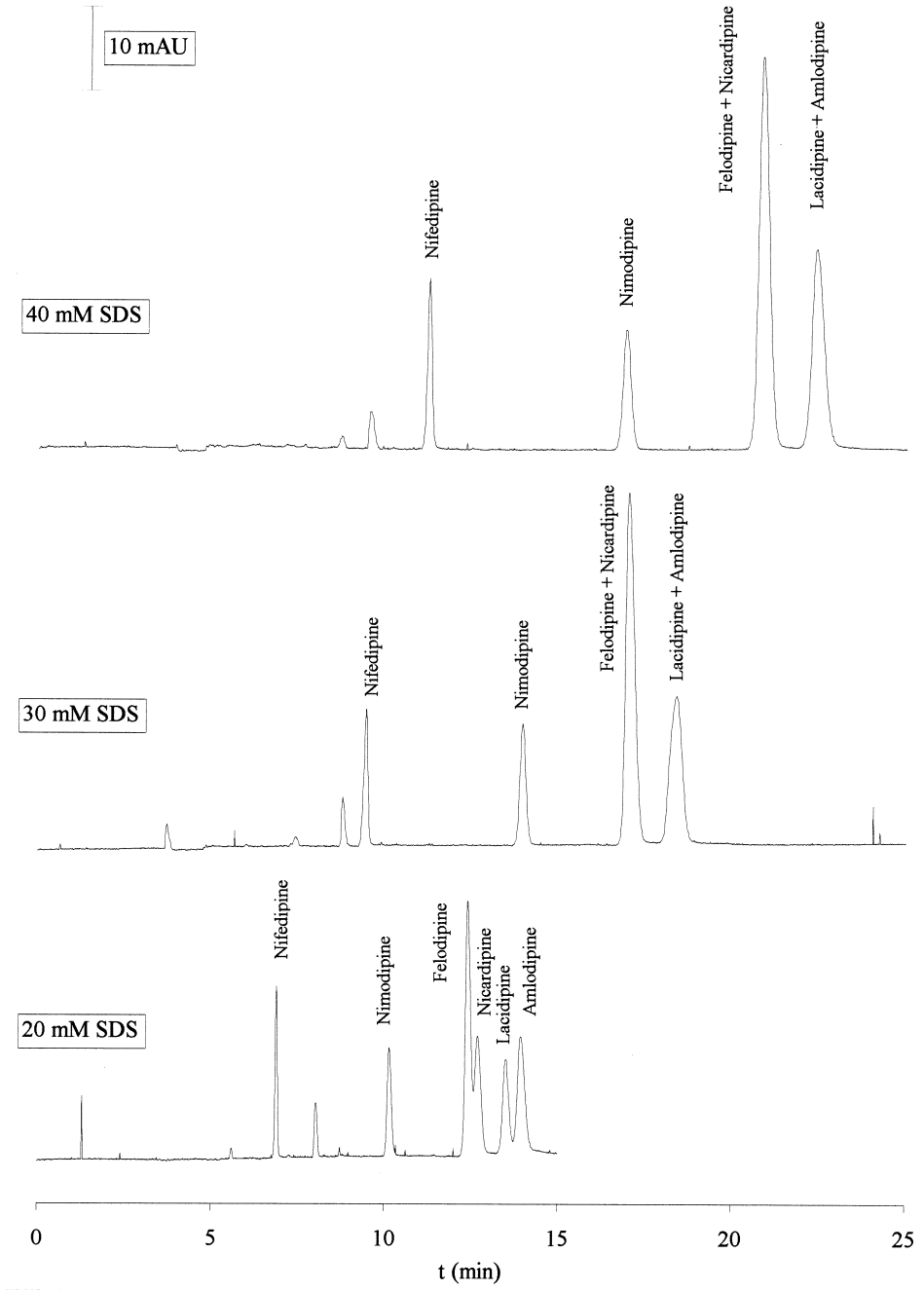


Fig. 3. Influence of the SDS concentration on the screening of a standard solution of 1,4-DHP (40 μg/ml): 50 m*M* H_3BO_3–$Na_2B_4O_7$, pH 8.2, 15% CH_3CN; V=25 kV; t_{inj}=4 s; λ=236 nm.

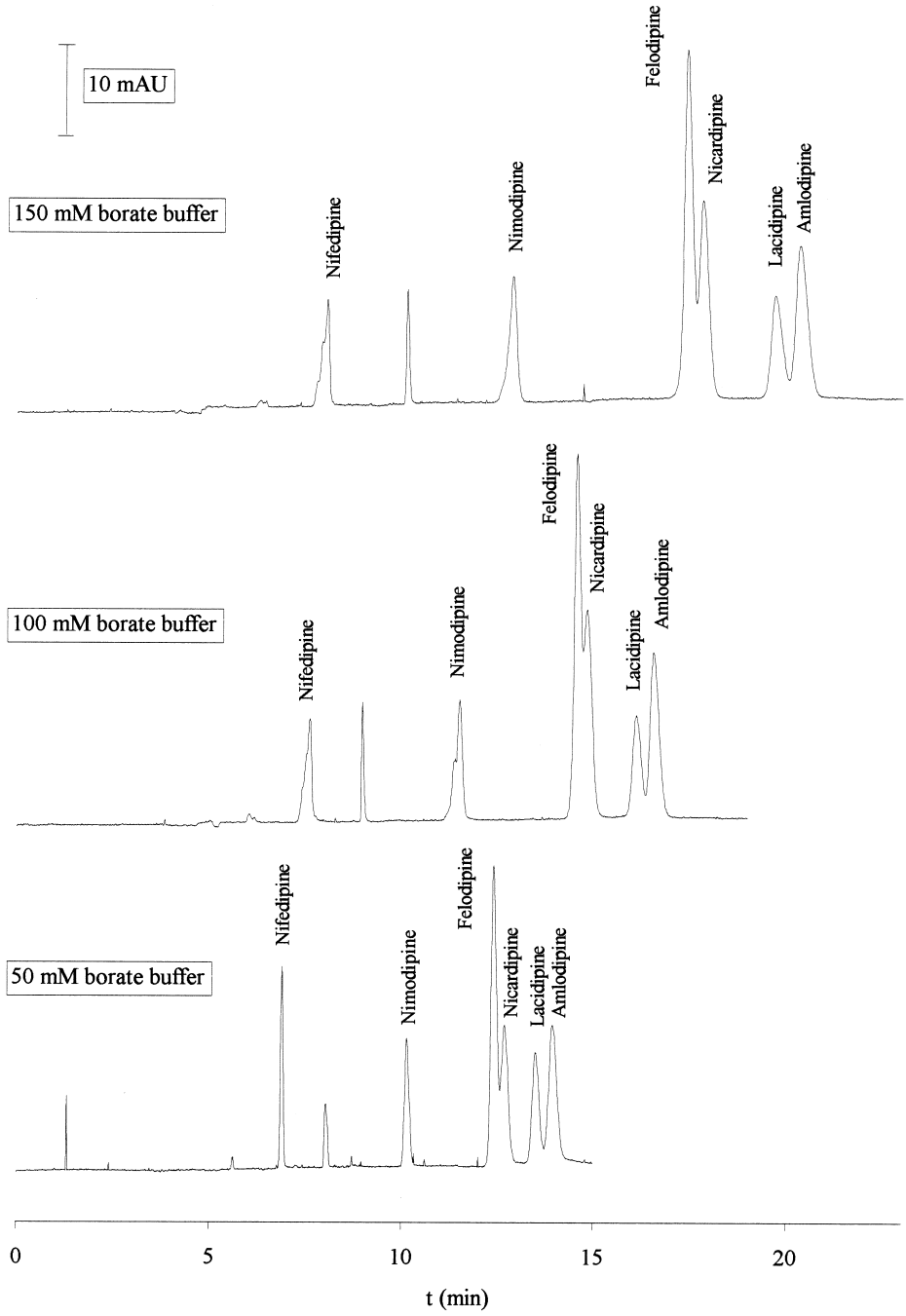


Fig. 4. Influence of the borate buffer concentration on the screening of a standard solution of 1,4-DHP (40 μg/ml): 20 m*M* SDS, H_3BO_3–$Na_2B_4O_7$, pH 8.2, 15% CH_3CN; V=25 kV; t_{inj}=4 s; λ=236 nm.

The selectivity of the separation can be further manipulated by the addition of an organic modifier such as acetonitrile or acetone that cause a reduction in electroosmotic flow and subsequently an extension of the elution window. Adding an organic solvent actually facilitates micelle formation at low con-

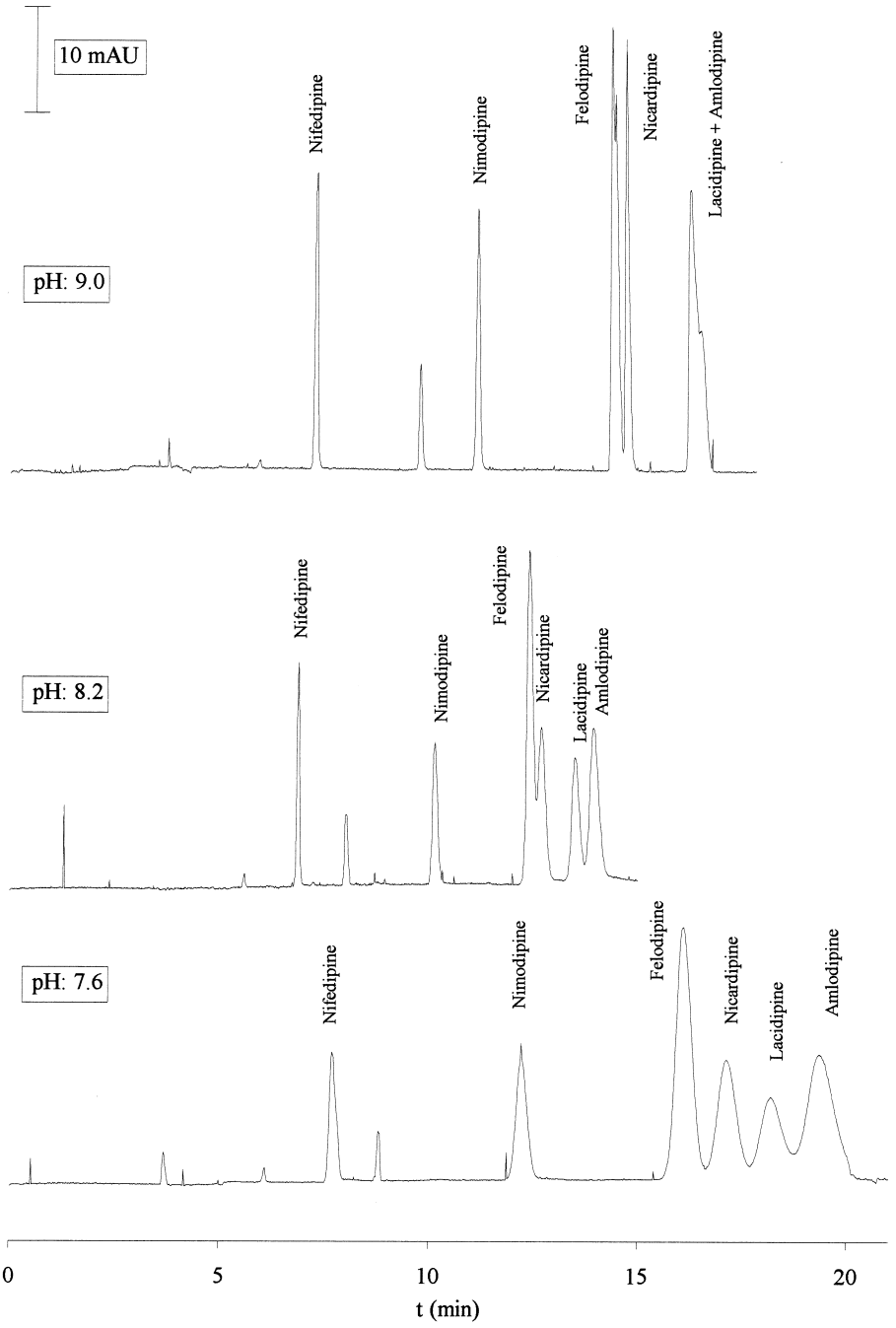


Fig. 5. Effect of the pH of the borate buffer on the screening of a standard solution of 1,4-DHP (40 μg/ml): 20 m*M* SDS, 50 m*M* H_3BO_3–$Na_2B_4O_7$, 15% CH_3CN; V=25 kV; t_{inj}=4 s; λ=236 nm.

centrations since the electrostatic repulsions between the head groups in an ionic micelle are reduced. At high concentrations, however, the hydrophobic interactions between the hydrocarbon chains, which are the main driving force in micelle formation, decrease and formation of micellar aggregates becomes more difficult [10].

Taking into account these facts it is crucial to study the effects of the organic modifier percentage in the electrolyte and in the injected sample on the 1,4-DHP separation.

3.1. Optimization of the electrophoretic conditions

Spectra of the six calcium antagonists studied were recorded in the following conditions: 25 m*M* SDS and 50 m*M* borate buffer, pH 9, and a drug concentration of 50 μg/ml. Two absorption peaks in the range of 188–200 and 224–240 nm were obtained for all the compounds, with the exception of lacidipine which shows another absorption peak at 284 nm. The wavelengths selected for the detection of the six 1,4-DHP were 200 and 236 nm. The maximum absorption was obtained for all the compounds at 200 nm except for nimodipine.

Initially, the conditions used for the optimization study were chosen upon the basis of the ones reported by Bretnall and Clarke [8] for the screening of hypertensive agents by MEKC, with some modifications: 50 m*M* borate buffer (pH 8.2) containing 25 m*M* SDS. In these experimental conditions the 1,4-DHP studied co-migrated at a migration time of 9.1 min.

In an attempt to achieve resolution of these drugs,

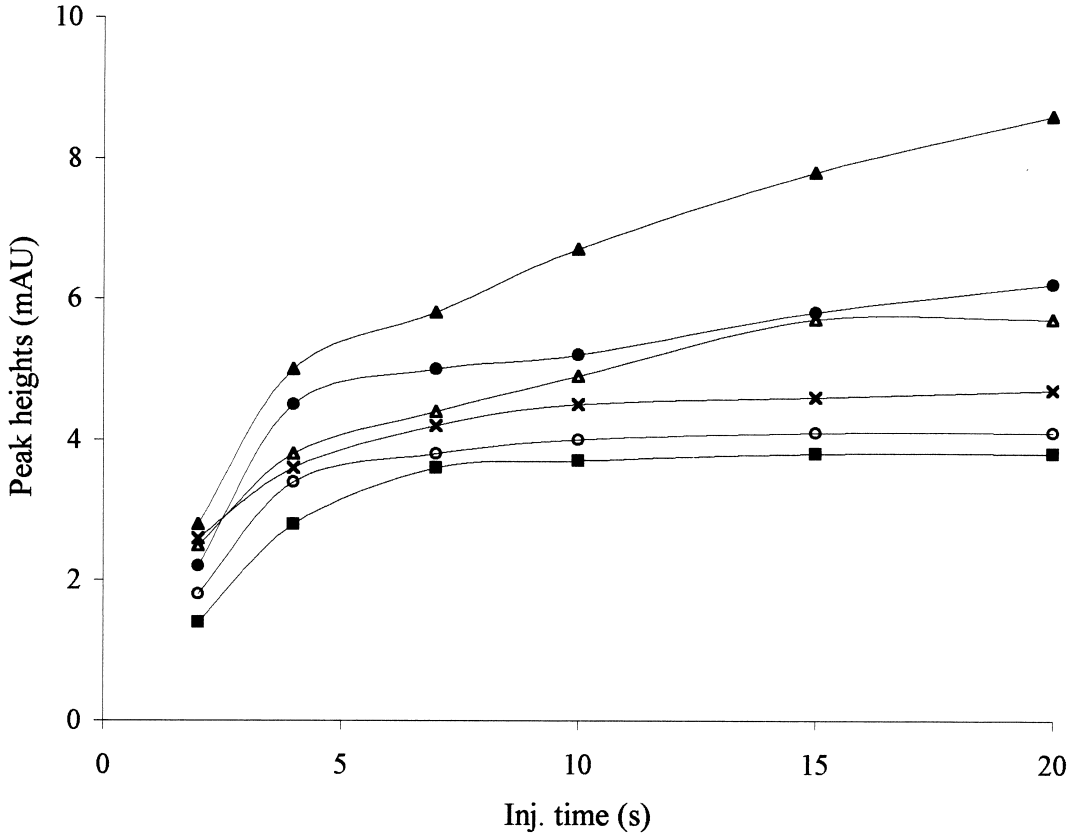


Fig. 6. Effect of the injection time on the peak heights of the 1,4-DHP (10 μg/ml): 20 m*M* SDS, 50 m*M* H_3BO_3–$Na_2B_4O_7$, pH 8.2, 15% CH_3CN; V=25 kV; t_{inj}=4 s; λ=236 nm. (△) Nifedipine, (×) nimodipine, (■) nicardipine, (▲) felodipine, (○) lacidipine and (·) amlodipine besylate.

the effect of the addition of an organic modifier was studied using an initial electrolyte consisted of 50 m*M* borate buffer, pH 8.2, and 20 m*M* SDS. The use of acetone at a concentration of 5% (v/v), only allowed the resolution of three of the compounds (nifedipine, nimodipine and amlodipine) and the electropherogram showed a high noise level. This noise problem was resolved using acetonitrile as the organic modifier.

Different percentages of acetonitrile were studied in order to get the resolution of all the compounds. As can be seen in Fig. 2 a percentage of 15% is adequate for the separation of the six drugs, whereas at lower percentages, felodipine, nicardipine and lacidipine co-migrate with amlodipine. In this work, it has been observed that there is a critical concentration of acetonitrile in the capillary above which the micelle formation is avoided. This fact has been proved by the injection of samples with different percentages of acetonitrile. Higher values than 50% (v/v) in the samples cannot be used.

The influence of the SDS concentration on the migration times and resolution can be observed in Fig. 3. The increment of SDS concentration gives rise to an overlapping of felodipine and nicardipine, and lacidipine and amlodipine, due to the increase of the electroosmotic flow. A SDS concentration of 20 m*M* was chosen as optimum.

The effect of borate buffer concentration is shown in Fig. 4. An increase of the borate buffer concentration produces an increase of the migration times without improving the resolution of the electrophoretic peaks; 50 m*M* borate buffer was used throughout this work.

The study of the pH effect was made using 50 m*M* borate buffer in 20 m*M* SDS and 15% (v/v) acetonitrile at three different pH values: 7.6, 8.2 and 9.0. In Fig. 5, electropherograms at the different pH values

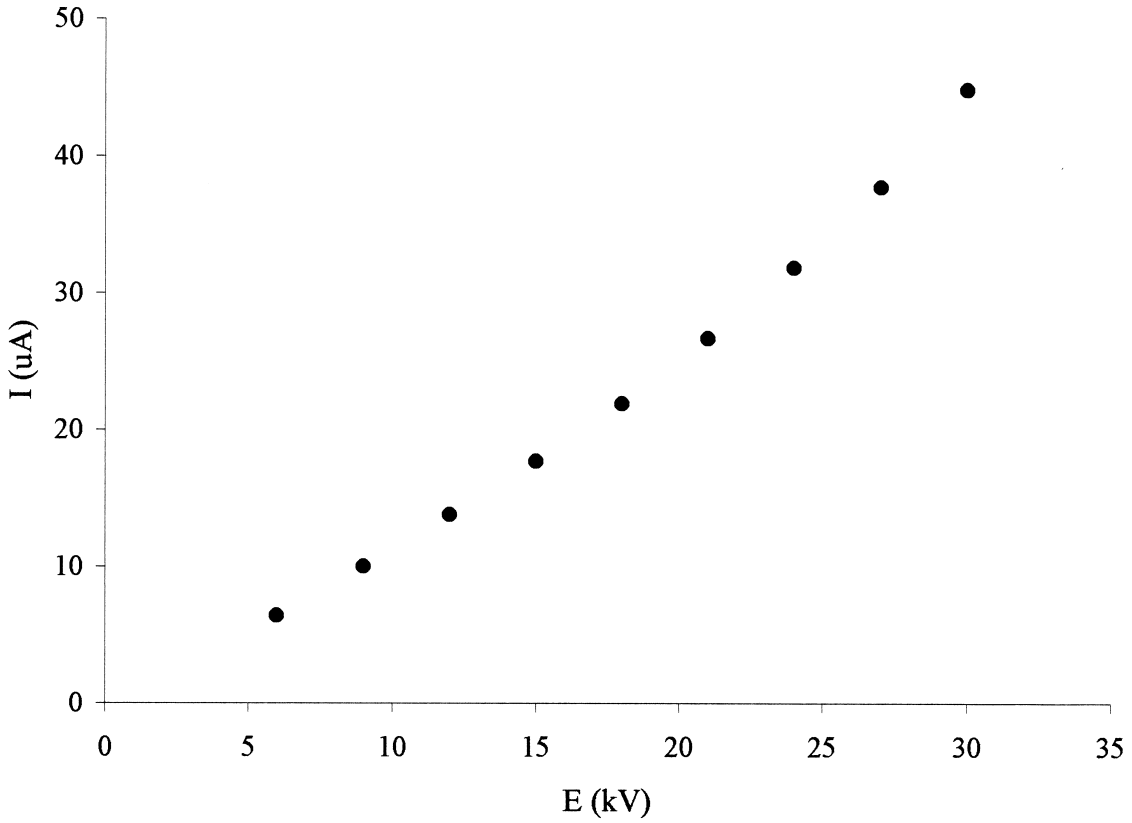


Fig. 7. Ohm's plot: electrolyte, 20 m*M* SDS, 50 m*M* H_3BO_3–$Na_2B_4O_7$, pH 8.2, 15% CH_3CN; V=25 kV; t_{inj}=4 s; λ=236 nm.

studied are shown. pH 8.2 was considered as the most adequate with respect to resolution and migration time.

In Fig. 6 the influence of the injection time on the peak heights is shown. An optimal injection time of 4 s was selected since peak-height saturation occurs above this value for all the six calcium antagonists.

In order to determine the optimal voltage to be applied, an Ohm's plot was made (Fig. 7). Values over 25 kV induced an increase on the current intensity, thus that voltage was selected as the optimum.

In the optimal experimental conditions (50 m*M* borate buffer, pH 8.2, 20 m*M* SDS, 15% acetonitrile), the separation of the calcium antagonists undergoes in less than 15 min: nifedipine (6.9 min), nimodipine (10.1 min), felodipine (12.2 min), nicardipine (12.7 min), lacidipine (13.5 min) and amlodipine (14.1 min, besylate: 8 min) (Fig. 8).

In order to show the relation between area of electrophoretic peak and concentration, calibration graphs were made on standard solutions, containing all the calcium antagonists at different concentrations, under the optimized conditions. A linear relationship between peak area and concentration of each drug at least up to 70 μg/ml was obtained. The within-day and inter-day area reproducibility were determined by injecting six replicate samples of the compounds at 50 μg/ml level. It is expressed as the relative standard deviation (R.S.D.), and calculated by the formula R.S.D. (%)=(standard deviation/mean of the peak areas)×100. The detection limit of the method, considered as the quantity of compound required for a signal-to-noise ratio of 3, is about 5

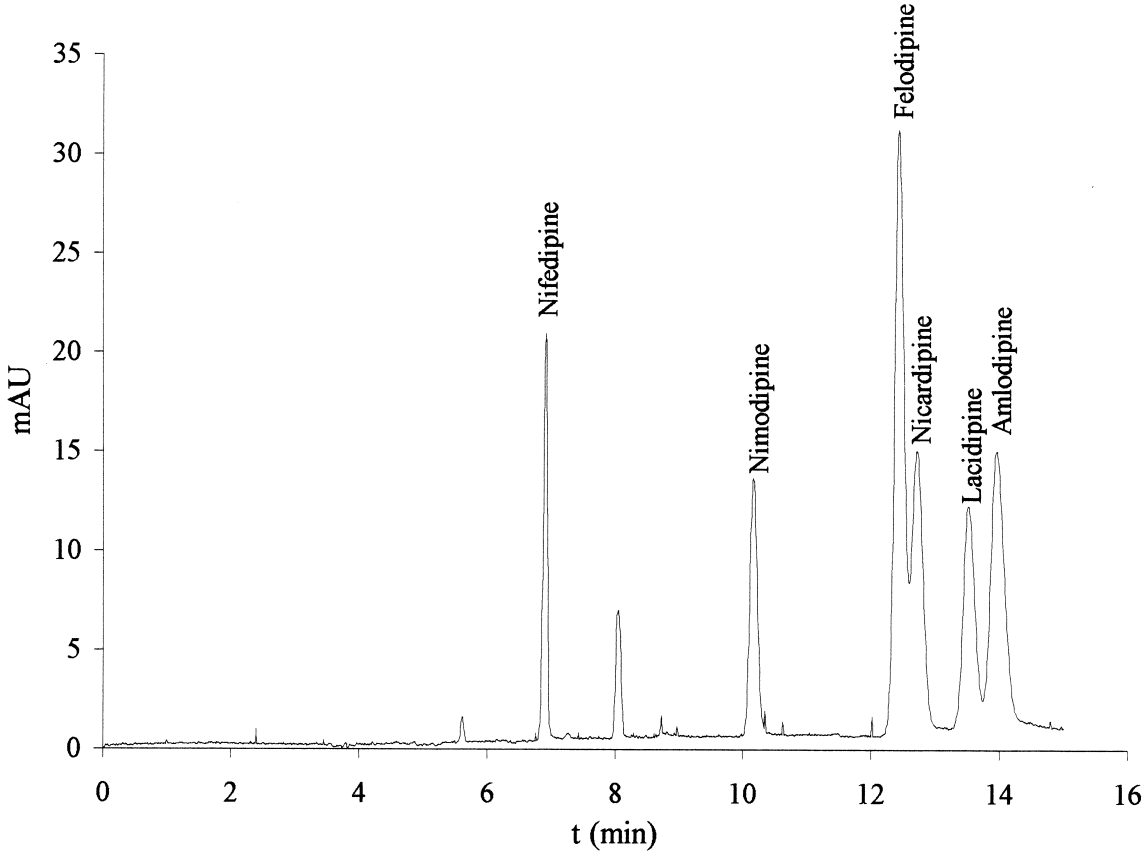


Fig. 8. Electropherogram of a standard solution of 1,4-DHP (40 μg/ml): 20 m*M* SDS, 50 m*M* H_3BO_3–$Na_2B_4O_7$, pH 8.2, 15% CH_3CN; V=25 kV; t_{inj}=4 s; λ=200 nm.

Table 1
Regression data and LOD for the assayed drugs

Calcium antagonists	Nifedipine	Nimodipine	Felodipine	Nicardipine	Lacidipine	Amlodipine
Detection limit (μg/ml)	5.1	6.7	6.4	6.0	4.4	3.1
Slope (area/μg per ml)	2.34±0.67	3.56±1.37	9.10±1.33	5.63±0.50	3.80±0.70	5.39±0.19
Intercept (area)	−1.77±1.03	9.57±13.75	−22.65±20.58	−14.86±11.77	−1.41±2.95	−9.95±8.19
Regression coefficient (r^2)	0.997	0.996	0.997	0.999	0.995	0.999
Within-day reproducibility (R.S.D., %)	4.8	1.8	3.9	4.2	3.4	3.6
Inter-day reproducibility (R.S.D., %)	6.7	3.4	7.4	8.6	6.9	6.6

Electrolyte: 20 m*M* SDS, 50 m*M* H_3BO_3–$Na_2B_4O_7$, pH 8.2, 15% CH_3CN; V=25 kV; t_{inj}=4 s; λ=200 nm.

μg/ml for each drug. Table 1 collects the quantitative results obtained for each compound in presence of the rest of 1,4-dihydropyridines studied.

4. Conclusions

The micellar electrokinetic chromatographic method developed has proved to be adequate for the screening of the six analytes (nifedipine, nimodipine, nicardipine, felodipine, lacidipine and amlodipine besylate) in less than 15 min.

This work is the basis for the development of electrophoretic methods for the determination of each calcium antagonists in their pharmacological formulations and plasma or serum. The low plasma levels of 2–100 ng/ml of these 1,4-DHP reached after the intake of their conventional formulations, makes necessary the use of a clean-up procedure previous to their determination in plasma samples in order to preconcentrate them (detection limit about 5 μg/ml) and eliminate some of the interferences from the endogenous compounds of the matrix [11–16].

MEKC has shown to be a potent technique for a quick separation of calcium antagonists with good reproducibility and could be considered as an alternative approach to high-performance liquid chromatography.

Acknowledgements

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