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High-performance liquid chromatography with amperometric detection applied to the screening of 1,4-dihydropyridines in human plasma

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Abstract

A high-performance liquid chromatographic method with electrochemical detection has been developed for the determination of six 1,4-dihydropyridines: nifedipine, nimodipine, nisoldipine, nicardipine, felodipine and lacidipine. The chromatographic separation was performed using a Supelcosil LC-ABZ+Plus C_{18} column. A mobile phase of methanol–water (70:30), containing 2 m*M* CH_3COOH–CH_3COONa at a flow-rate of 1 ml/min and a pH of 5.0, was used. The temperature was optimized at 30±0.2°C. The amperometric detector, equipped with a glassy carbon electrode, was operated at 1000 mV versus Ag/AgCl in the direct current mode. The method was applied to the determination of these compounds at ng/ml concentrations, obtaining intra-day reproducibilities of lower than 5.0% in terms of relative standard deviations and detection limits ranging from 16 to 44 ng/ml. The method was applied to the screening of 1,4-dihydropyridines in spiked plasma samples, with a total elution time of lower than 18 min, obtaining the best recoveries for nimodipine and felodipine (91 and 88%, respectively). These recoveries together with the low detection limits achieved allow its application to the analysis of these drugs in human plasma.

Keywords: Dihydropyridines

1. Introduction

The utilization of antihypertensive drugs in Spain has seen a significant increase during the last decade. Angiotensin-converting enzyme inhibitors (ACEIs) and calcium channel blockers (CCBs) are mainly responsible for this increase [1].

The calcium channel blockers are a diverse group of drugs from the chemical and pharmacological point of view. The World Health Organization divides CCBs into three different classes, the dihydropyridines (nifedipine-like drugs) being one of these classes.

The dihydropyridine calcium channel blockers are widely used in the treatment of arterial hypertension, angina pectoris and in the therapy of cerebrovascular spasm of various origins [2].

Chemically, this family contains as their basic structure a substituted benzene at position 4 of the tetrasubstituted 1,4-dihydropyridine (1,4-DHP) ring. The 1,4-DHP moiety is essential for their pharmacological activity on the cardiovascular system. Most of these compounds undergo a photodegradation process generated by daylight and UV light [3].

Evidence of their clinical efficiency in the long-term therapy of hypertensive patients is very limited and in the last few years several reports have been published indicating that the use of short-acting 1,4-

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DHPs produces an increased risk of cardiovascular complications, especially myocard heart attack [1]. This fact has been thoroughly discussed, making the determination of the plasma levels of these drugs important.

These drugs are well absorbed after oral administration, but there are distinctions in oral bioavailability related to the differences in their presystemic (first-pass) metabolism that may result in wide variations in plasma levels (always at μg/l levels) and marked differences between the oral and intravenous doses required to produce similar physiologic effects. All these drugs have high protein-binding percentages (>90%) [4,5]. Dihydropyridine calcium channel blocking agents are usually enzymatically oxidized to pyridine metabolites after oral administration [6], and these are not pharmacologically active [7]. Their therapeutic dose is from 5 to 60 mg depending on the compound, and plasma concentrations reach 20–300 ng/ml. Therefore, the determination of the level of unchanged drug in plasma requires an analytical method with high sensitivity.

The determination of dihydropyridines in biological fluids has mainly been carried out using gas chromatographic (GC) methods with electron-capture [8,9], N–P ionization [10] and mass spectrometric (MS) detection [11–13], and high-performance liquid chromatography (HPLC) with UV [14–20] and electrochemical detection [21,22].

On the other hand, reports related to the screening of these drugs are scarce. The techniques used for this purpose include GC–MS [23], HPLC–UV [24,25] and micellar electrokinetic chromatography (MEKC) [26]. None of these reported methods have been applied to biological fluids.

The aim of this work was the development of a HPLC method with amperometric detection, based on the oxidation of the DHP ring to pyridine, for the separation and determination of six 1,4-dihydropyridines. This method was applied to plasma samples for a unique cleanup procedure for the 1,4-DHPs studied: 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], nisoldipine [3-isobutyl-5 - methyl - 1,4 - dihydro - 2,6 - dimethyl - 4 - (2 -nitrophenyl)pyridine-3,5-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] and 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] (Fig. 1).

This work attempted to provide a useful analytical tool for the monitoring of these compounds in plasma.

2. Experimental

2.1. Apparatus and column

The HPLC system consisted of a Model 2150-LKB (Pharmacia, Barcelona, Spain) HPLC pump and Rheodyne Model 7125 (Pharmacia) injector fitted with a 20 μl injection loop.

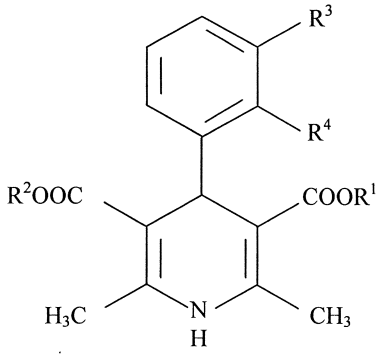
The electrochemical detector (PAR Model 400) equipped with a glassy carbon cell (EG&G Princeton Applied Research, Madrid, Spain) was operated in the d.c. mode at 1000 mV vs. a Ag/AgCl/NaCl 3 *M* reference electrode and a platinum rod as auxiliary electrode, with a 5 s low pass filter time constant and a current range of 20 nA. Chromatograms were recorded with the help of a computer and data were treated with the Millennium 2010 Chromatography Manager software from Waters (Barcelona, Spain).

A Supelcosil ABZ+Plus, 25 cm×4.6 mm I.D., 5 μm (Supelco, Barcelona, Spain) HPLC column with a μBondapak C_{18} precolumn module (Waters) were used. The column was kept at constant temperature using a Waters TMC temperature control system.

Solid-phase extraction was performed using Bond Elut 1 ml/100 mg C_{18} cartridges (Varian, Barcelona, Spain) which were placed on a vacuum manifold system (Supelco). The extracted plasma samples were evaporated to dryness under a nitrogen stream using a Zymark Turbovap LV evaporator (Varian).

2.2. Reagents and solutions

The 1,4-dihydropyridines, nifedipine, nimodipine



DRUG	R^1	R^2	R^3	R^4
Nifedipine	$-CH_3$	$-CH_3$	-H	$-NO_2$
Nimodipine	$-CH(CH_3)_2$	$-CH_2CH_2OCH_3$	$-NO_2$	-H
Nisoldipine	$-CH_3$	$-CH_2CH(CH_3)_2$	-H	$-NO_2$
Nicardipine	$-CH_3$	$-CH_2N(CH_3)CH_2C_6H_5$	$-NO_2$	-H
Felodipine	$-CH_3$	$-CH_2CH_3$	-Cl	-Cl
Lacidipine	$-CH_2CH_3$	$-CH_2CH_3$	-H	$CH=CH-COOC(CH_3)_3$

Fig. 1. Molecular structures of 1,4-dihydropyridine drugs.

and nisoldipine were supplied by Bayer (Barcelona, Spain), nicardipine hydrochloride by Roger (Barcelona, Spain), felodipine by Astra (Barcelona, Spain) and lacidipine by Glaxo (Madrid, Spain). Solvents were Lab-Scan HPLC grade (Dublin, Ireland), and all reagents were of analytical reagent quality and supplied by Merck (Bilbao, Spain). Water was obtained from a Milli-RO and Milli-Q Waters system.

Standard stock solutions of drugs (1000 μg/ml) were prepared in methanol, stored at 4°C and protected from light by covering them with aluminium foil. Working solutions were prepared by appropriate dilution in the mobile phase just before use, using amber glass volumetric flasks in order to avoid photodegradation.

2.3. Chromatographic conditions

Separation was performed using a methanol–water (70:30) mobile phase containing 2 m*M* acetate buffer as supporting electrolyte. The pH of the aqueous phase was adjusted to 5.0 using adequate volumes of acetic acid (0.1 *M*) and sodium acetate (0.1 *M*) solutions. The mobile phase was filtered through a 0.45 μm membrane, and dissolved air was removed by bubbling helium through the solution.

The Supelcosil column head pressure was 110 bar at a flow-rate of 1 ml/min. The injection volume was 20 μl. The column temperature was kept constant at 30±0.2°C.

2.4. Electrode maintenance

The electrode was cleaned electrochemically at the end of each working day by keeping it at −600 mV for 5 min and then at +1600 mV for 10 min. This operation was carried out using the mobile phase at a flow-rate of 1 ml/min. When the baseline noise increased and the signal-to-noise ratio decreased, the

glassy carbon electrode was cleaned with a tissue wet with methanol to remove possible adsorbed compounds, and rinsed with deionized water to dissolve precipitated salts.

2.5. Plasma collection

Blood was collected by syringe from healthy volunteers and immediately transferred into lithium heparin tubes and gently mixed. Samples were immediately centrifuged at 3000 rpm at a temperature of 4°C. The drug-free plasma obtained was transferred to 5 ml tubes and stored at −20°C until analysis.

2.6. Sample cleanup procedure

Deep-frozen human plasma was thawed at room temperature. A volume (1 ml) of plasma was doped with 1,4-DHP drugs and alkalinized with 50 μl of 0.1 *M* NaOH solution. A Bond Elut C_{18} cartridge was conditioned with 1 ml of methanol and washed with 1 ml of water to remove trapped methanol traces from the bed volume. The cartridge was not allowed to dry before the application of the sample, which was slowly drawn through the column at very low vacuum. The column was washed with 1 ml of water, drying at full vacuum (67 kPa) for 10 min. The analytes were eluted with 1 ml of diethyl ether. The eluate was then evaporated to dryness at 50°C under a gentle stream of nitrogen. The remaining residue was dissolved in 1 ml of mobile phase and injected into the chromatographic system under the optimized chromatographic conditions. The same procedure was applied to blank plasma samples.

2.7. Calibration curves

A stock solution of 40 μg/ml concentration of each 1,4-DHP studied was prepared from aliquots of the 1000 μg/ml standard stock solutions in methanol.

Calibration standards covering the range 50–1000 ng/ml were prepared from the stock solution in mobile phase. The calibration curves were obtained by plotting peak area versus the nominal concentration of each compound. The slope and intercept of the calibration lines were determined by unweighed least-squares regression analysis.

2.8. Reproducibility and extraction efficiency

The reproducibility and efficiency of the extraction procedure were determined by extracting replicate ($n = 5$) spiked plasma samples. The samples were spiked with aliquots of stock solution to achieve a final concentration of 500 ng/ml of each 1,4-DHP drug.

The extraction efficiency of the 1,4-DHPs studied was estimated by measuring the peak areas of non-extracted standard solutions and comparing them with the peak areas obtained from extracting spiked plasma samples of the same concentration.

The reproducibility of the extraction was expressed as relative standard deviation [%R.S.D. = (standard deviation/mean of the recoveries)·100].

2.9. Repeatability, accuracy and detection limit

The intra-day and inter-day repeatabilities were determined by injecting replicate samples ($n = 5$) of each 1,4-dihydropyridine, and are expressed as R.S.D.

The accuracy of the method was determined by the analysis of five control plasma samples spiked with 200 and 500 ng/ml of each 1,4-dihydropyridine. Accuracy is defined as mean (found concentration/actual concentration)·100%.

The detection limit of the method was defined as the quantity of compound required for a signal-to-noise ratio of 3.

3. Results

Under static conditions, 1,4-DHPs proved to be electroactive over the whole pH range studied (2–12). The oxidation peak potential shifts to less positive potentials as the pH increases. All 1,4-dihydropyridines show similar anodic electrochemical behaviour, with a relatively low oxidation potential, from 400 to 850 mV. The anodic process can be attributed to the oxidation of the dihydropyridine ring to the pyridine derivative compound [27].

On the basis of this oxidative behaviour, a chro-

matographic system with amperometric detection can be developed for the determination of these compounds.

3.1. Stability study

As it is known that most of the nifedipine-like drugs are very sensitive to the light, undergoing oxidation to pyridine analogues [28–30], a study of the photodegradation of these compounds was carried out. A solution of 400 ng/ml of each drug was exposed to room light, and aliquots of this solution were collected at different times and injected into the chromatographic system. The decrease of the chromatographic peak area of nifedipine and nisoldipine with time showed the instability of these two compounds, while nimodipine, nicardipine, felodipine and lacidipine were stable for up to 100 min. All the solutions were protected from light throughout this work.

3.2. Optimisation of the chromatographic system

Initially a μBondapak C_{18} column was tested for the separation of 1,4-DHPs. Some of these compounds, especially nicardipine, showed long retention times (>15 min), bad peak symmetry and resolution. Generally, the way to solve this problem is to use an ion-pairing or suppressing agent, such as alkylamine salts, but these agents cause a high background level when electrochemical detection is utilized at oxidation potentials higher than +0.9 V [31]; therefore, Supelcosil ABZ+Plus was used and good peak symmetry and resolution were obtained for the six compounds studied.

Hydrodynamic voltammograms of each compound were obtained in order to select the optimum potential for their detection. For this purpose a potential scan in the range 700–1200 mV was made by applying different potentials to standard solutions of 2 μg/ml of each compound. Three replicates of each injection were made and the mean value of the peak areas obtained was plotted versus potential (Fig. 2). An oxidative potential of 1000 mV was chosen as working potential for the analysis of these compounds. Although this potential was not in the plateau of the hydrodynamic voltammograms for all the 1,4-DHPs, it induced a lower baseline noise and provided a higher selectivity because a lower amount of endogenous plasma compounds could be oxidized.

The pH of the mobile phase was considered as one of the parameters for the optimization of the retention times and peak symmetry. Nicardipine is the only one of the 1,4-DHPs studied with a reported pK_a value of 7.2 [3] due to the presence of an amino group in its structure. Thus it was expected that its retention time was affected by the pH of the mobile phase. The variation of retention time with pH of the mobile phase was tested for the appropriate separation of all these drugs. For this purpose acetate and phosphate buffers were used. The retention times and peak symmetry of non-ionic drugs were not influenced by pH (nifedipine, nimodipine, nisoldipine, felodipine and lacidipine), while the retention time of nicardipine increased with the pH. At pH >6, the chromatographic peak of nicardipine lost its symmetry due to band broadening. At pH <4, this compound eluted along with plasma endogenous compounds. A pH value of 5.0 was chosen as optimum for the chromatographic separation and resolution of the six drugs. No differences were observed when phosphate or acetate buffer was used, so acetate buffer, pH 5.0, was chosen and also utilized as supporting electrolyte, necessary for the electrochemical detection.

The effect of the electrolyte concentration on the signal-to-noise ratio was also studied. Concentrations ranging from 1 to 10 m*M* were assayed and an increase in the background signal was observed when the electrolyte concentration exceeded 5 m*M*. A decrease of the retention time of the compounds when the concentration increased was also observed. The optimal electrolyte concentration of the mobile phase was set at 2 m*M*.

Different proportions of methanol–water and acetonitrile–water containing 2 m*M* acetate buffer (pH 5.0) were tested as mobile phase. Fig. 3 shows the variation of retention time with the percentages of acetonitrile and methanol. The use of methanol–water as mobile phase provides a better resolution of the compounds and a lower background signal than the acetonitrile–water mixtures. The 70:30 (methanol–water) ratio was chosen as the most adequate, since good resolution in terms of k' (nifedipine 0.86, nimodipine 1.79, nisoldipine 2.27, nicardipine 2.91, felodipine 3.55, lacidipine 5.36) and an elution time

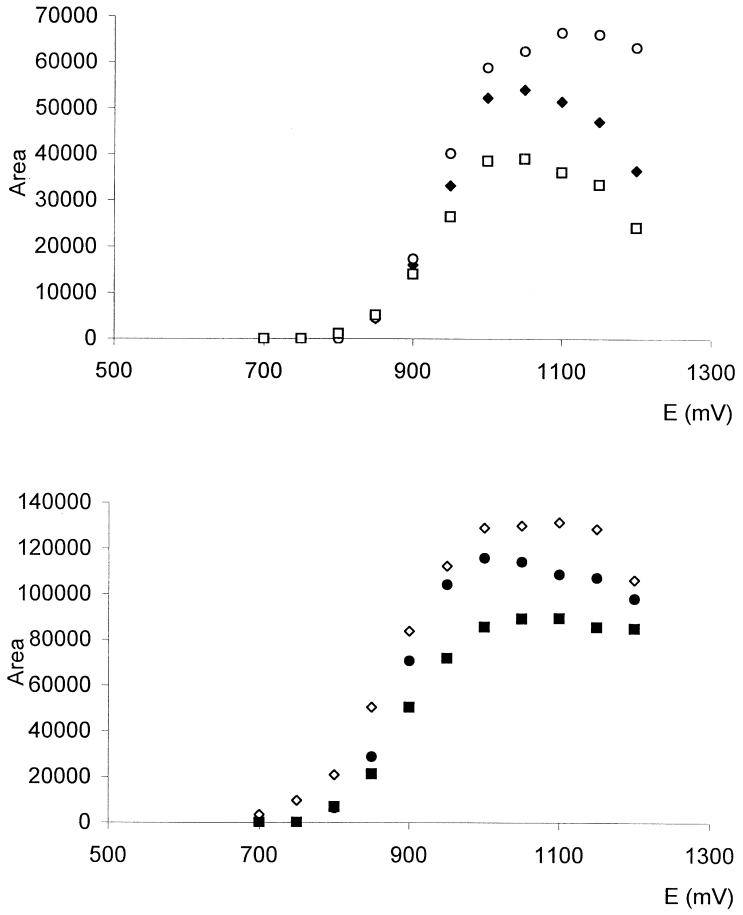


Fig. 2. Hydrodynamic voltammograms of (□) nifedipine, (○) nimodipine, (♦) nisoldipine, (◇) nicardipine, (●) felodipine and (■) lacidipine. Amount of drug injected: 40 ng. For chromatographic conditions, see Section 2.3.

of lower than 18 min were achieved for the six 1,4-dihydropyridines studied (Fig. 4).

A study of the influence of the flow-rate on the chromatographic separation was carried out. As expected, the peak area decreased with an increase in flow-rate, while the effect on k' was practically negligible. A value of 1.0 ml/min was used.

An increase of the temperature caused lower retention times, decreasing the selectivity of the process without affecting the sensitivity. A temperature of 30±0.2°C was used throughout the work.

When optimum chromatographic conditions were established, a quantitative method for the simultaneous determination of nifedipine, nimodipine, nisoldipine, nicardipine, felodipine, and lacidipine was developed at ng/ml levels (Table 1), taking into account the usual dose administered and the excretion percentages of these 1,4-DHPs in plasma.

3.3. Optimization of the extraction

Different cartridges (C_2, C_8, C_{18}) were tested for the solid-phase extraction of 1,4-DHPs from plasma samples; C_{18} cartridges were chosen as the most adequate, since these cartridges gave rise to higher recovery values for most of the compounds (Table 2).

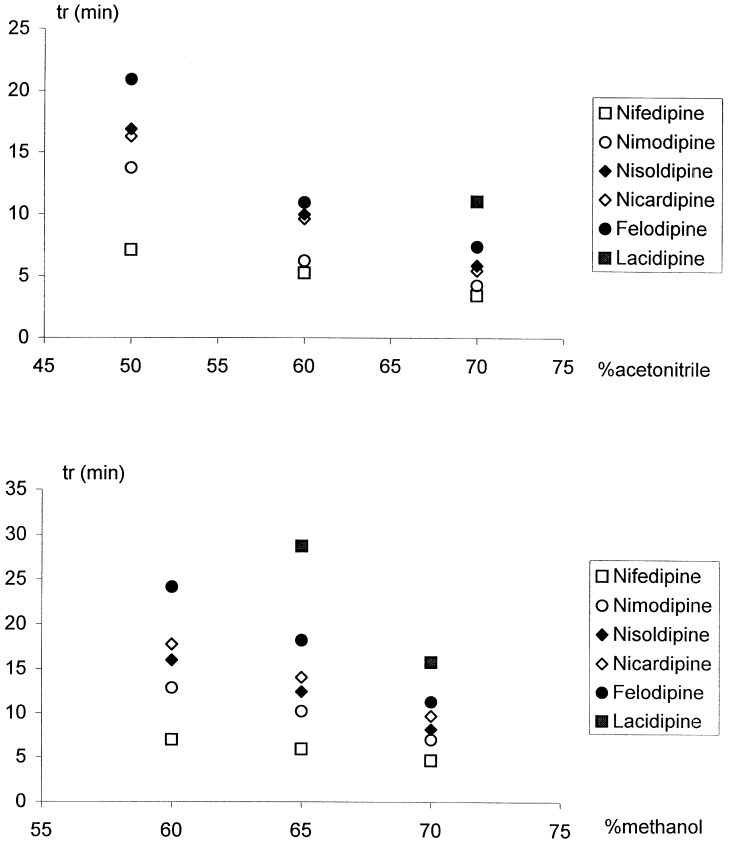


Fig. 3. Variation of retention time with the percentage of acetonitrile and methanol in the mobile phase, containing 2 m*M* acetate buffer (pH 5.0). Electrochemical detection at a working potential of 1000 mV vs. Ag/AgCl.

Once the cartridges were conditioned with 1 ml of methanol and 1 ml of water, the effect of the pH of the plasma sample was studied. The addition of 50 μl of 1 *M* NaOH to 1 ml of plasma gave rise to the cleanest extracts. The washing step of the extraction procedure consisted of 1 ml of water, since the use of basic buffer solution did not eliminate a greater amount of endogenous compounds. On the other hand, several solvents (methanol, acetonitrile, dichloromethane and diethyl ether) were tested as elution solvents. Diethyl ether was chosen as the most adequate, because a good cleanup of the plasma samples and adequate recovery values were obtained. In order to improve the recoveries of the compounds, different volumes of diethyl ether were tested. A volume of 1 ml of diethyl ether was chosen as the most adequate, because an increase of the elution volume produced the elution of a greater amount of interfering endogenous compounds.

Quantitative recoveries calculated for each 1,4-DHP are higher than 60%, being the maximum recoveries obtained for nimodipine and lacidipine (Table 3).

3.4. Linearity, repeatability and accuracy

The relative standard deviation of the retention times was $<1\%$, thus indicating high stability of the system. Linearity occurred at least from 50 to 1000 ng/ml.

The intra-day and inter-day repeatabilities of the

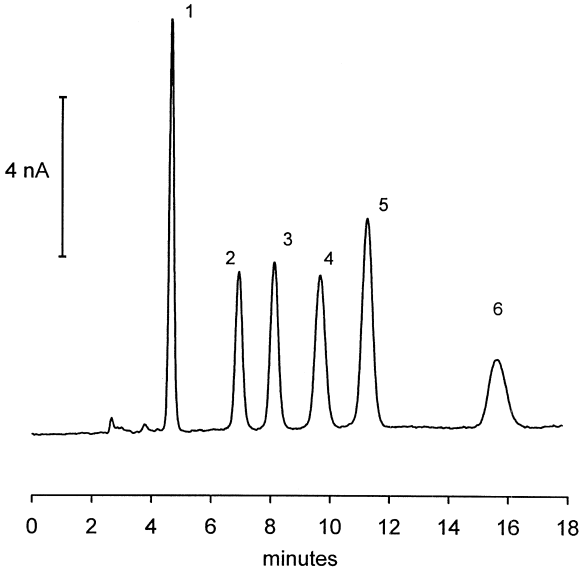


Fig. 4. Chromatogram obtained for a 800 ng/ml standard solution of (1) nifedipine, (2) nimodipine, (3) nisoldipine, (4) nicardipine, (5) felodipine and (6) lacidipine. For chromatographic conditions, see Section 2.3.

analytical method are <5% and <15%, respectively (Table 1).

The accuracies of the assay obtained for each compounds were: nifedipine 101.4±3.7%, nimodipine 101.6±4.4%, nisoldipine 98.8±6.6%, nicardipine 100.1±4.6%, felodipine 101.4±3.1%, and lacidipine 101.2±4.8%.

Table 2
Recovery of 1,4-dihydropyridine drugs from human plasma samples using different cartridges

1,4-DHP	Recovery (%)		
	C_{18}	C_8	C_2
Nifedipine	66	65	35
Nimodipine	91	76	63
Nisoldipine	63	68	40
Nicardipine	76	63	60
Felodipine	88	81	65
Lacidipine	73	87	63

Table 3
Recovery of 1,4-dihydropyridine drugs from human plasma samples spiked with 500 ng/ml of each compound (550 ng/ml for felodipine) ($n = 5$)

	Recovery (%)	R.S.D. (%)
Nifedipine	66	3.6
Nimodipine	91	4.3
Nisoldipine	63	6.7
Nicardipine	76	4.6
Felodipine	88	3.1
Lacidipine	73	4.7

Table 1
Quantitative determination of nifedipine, nimodipine, nisoldipine, nicardipine, felodipine, and lacidipine at ng/ml levels[a]

1,4-DHP	Retention time (min) ±SD	Linear range (ng/ml)	Slope[b]	Intercept (area)	r^2	Repeatability [R.S.D. (%)]		Detection limit (ng/ml) ($S/N = 3$)
						Intra-day	Inter-day	
Nifedipine	4.66±0.02	50–1000	33.2±0.6	444±295	0.9986	3.52[c] 3.48[d]	7.5[c] 10.9[d]	44
Nimodipine	6.98±0.01	50–1000	38.4±0.3	234±175	0.9996	2.11[c] 1.76[d]	8.5[c] 9.4[d]	22
Nisoldipine	8.17±0.02	50–1000	21.8±0.3	−137±165	0.9998	3.94[c] 4.86[d]	14.0[c] 14.9[d]	37
Nicardipine	9.68±0.03	50–1000	49.6±0.3	−588±166	0.9998	2.46[c] 3.90[d]	10.2[c] 11.5[d]	16
Felodipine	11.28±0.06	55–1100	72.6±0.6	−1489±386	0.9997	3.22[c] 3.62[d]	14.3[c] 13.6[d]	19
Lacidipine	15.75±0.13	50–1000	39.3±0.6	−1831±358	0.9990	3.45[c] 4.25[d]	5.4[c] 8.6[d]	37

[a] For chromatographic conditions, see the Experimental section.
[b] (area/ng/ml).
[c] Five determinations at the 500 ng/ml level (550 ng/ml for felodipine).
[d] Five determinations at the 200 ng/ml level (220 ng/ml for felodipine).

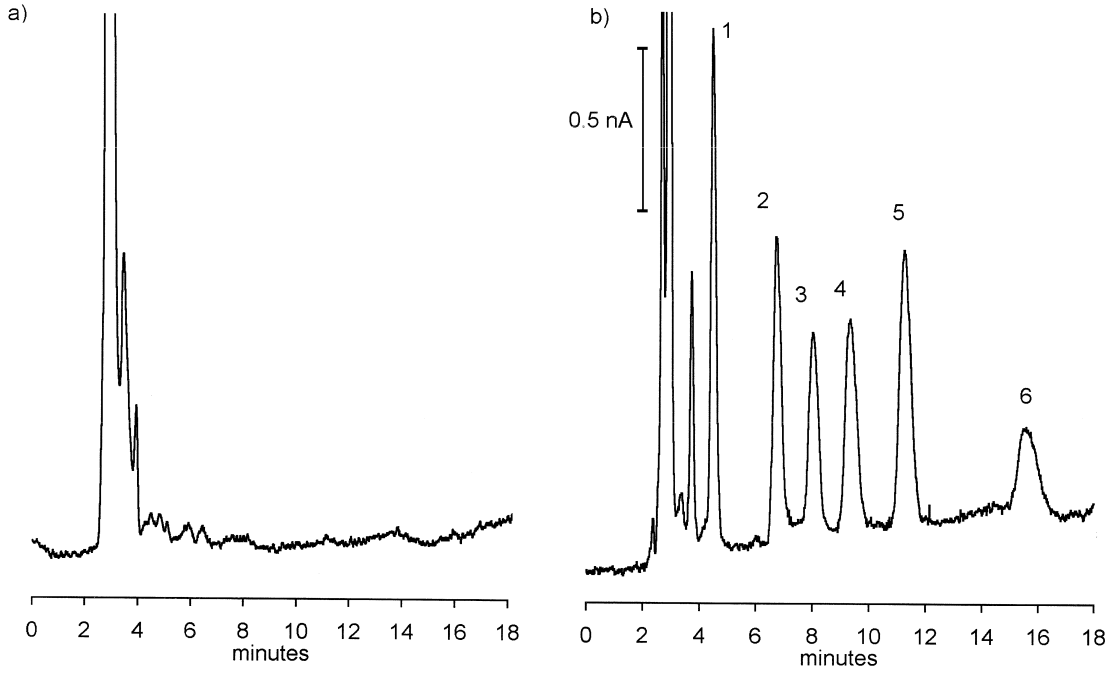


Fig. 5. Chromatograms obtained from an extract of (a) blank plasma sample and (b) plasma sample spiked with 200 ng/ml of nifedipine, nimodipine, nisoldipine, nicardipine, felodipine and lacidipine. For chromatographic conditions, see Section 2.3.

The detection limit ranged from 16 to 44 ng/ml, depending on the compound.

3.5. Analytical applications

The method developed was applied to plasma samples obtained from healthy volunteers, using the solid–liquid extraction procedure described in the Experimental section. The absence of interference from the plasma matrix in the chromatograms obtained for drug-free and spiked plasma samples can be seen in Fig. 5.

4. Discussion

The chromatographic method described in this paper allows a rapid, sensitive and selective analysis of all considered molecules in human plasma samples, extending the application of electrochemical detection to the simultaneous determination of 1,4-DHPs in biological fluids. The validation assays were adequate in terms of reproducibility, linearity and accuracy (Table 1).

Reversed-phase chromatography with electrochemical detection has proved to be adequate for the screening and simultaneous determination of 1,4-dihydropyridines in plasma samples. Electrochemical detection allows the quantitation of the unchanged 1,4-DHPs without interference from their degradation products, since they are not electroactive under the experimental conditions used.

New reversed-phase methods with electrochemical detection are being developed in our laboratory for the determination of each 1,4-dihydropyridine in real plasma samples obtained from hypertensive patients under treatment with these drugs.

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