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Talanta 67 (2005) 933–941
Talanta
www.elsevier.com/locate/talanta

Development of a liquid–liquid extraction procedure for five 1,4-dihydropyridines calcium channel antagonists from human plasma using experimental design

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Received 15 November 2004; received in revised form 7 March 2005; accepted 7 April 2005
Available online 31 May 2005

Abstract

A liquid–liquid extraction method using diethyl ether as organic solvent was optimized simultaneously for five 1,4-dihydropyridines (amlodipine, nitrendipine, felodipine, lacidipine and lercanidipine) belonging to the group of calcium channel blockers. Some experimental tools such as a full factorial design, a central composite design and the Multisimplex program were used to optimise the concentration of NaOH, volume of organic solvent and shaking time as main factors that influence the liquid–liquid extraction procedure. Following the extraction, the quantitation of the 1,4-dihydropyridines concentrations were performed by high-performance liquid chromatography with diode-array detector. Therefore, the studied compounds were separated quantitatively on a Supelcosil ABZ+Plus, 25 cm × 4.6 mm i.d., 5 μm column which was set at 30 °C, using as mobile phase, a mixture of acetonitrile–water (70:30, v/v) containing 10 mM acetate buffer (pH 5) and setting the detector at a wavelength value of 360 nm. It was concluded that the main factors that influence in the extraction process were the volume of organic solvent and the shaking time. The Multisimplex program suggested as optimal conditions an average of 6 ml of organic solvent and 23 min of shaking time. For these values, the optimised liquid–liquid extraction method showed good values of recoveries (80% for amlodipine and higher than 90% for the rest of the compounds) and low values of R.S.D. (<10%) in the reproducibility of the extraction what makes it reliable for the quantification of all the studied compounds in human plasma.

Keywords: 1,4-Dihydropyiridines; Calcium channel antagonists; LLE; Plasma and method optimization

1. Introduction

Calcium channel antagonists (CCA) and angiotensin enzyme inhibitors (ACE) are used today as first-line therapy for hypertension. The 1,4-dihydropyridines (1,4-DHPs) are the largest class of CCAs and are typified by the drug nifedipine. The characteristic skeleton of this important group among the CCAs is the 1,4-dihydropyridinic structure exhibiting phenyl substitution in position 4. Structural variations of this prototype concern primarily the ester functions and variations in phenyl substitution as well as changes in position 2 (Fig. 1).

The 1,4-dihydropyridines studied in this work are: amlodipine, nitrendipine, felodipine, lacidipine and lercanidipine. They act upon the L-type channel, which has a specific dihydropyridines site on its extracellular surface and bind more selectively to vascular calcium channel to those in the myocardium. Thus, the principal effect of the 1,4-DHPs is to reduce blood pressure, with minimal change in heart rate or cardiac output [1–6].

The determination of some of the above-mentioned compounds in plasma, serum or urine has been mainly carried out by liquid chromatographic methods with photometric, electrochemical or mass spectrometric detection [7–15]. Gas chromatography with different kinds of detection has also been widely used for this purpose [16–26].

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doi:10.1016/j.talanta.2005.04.028

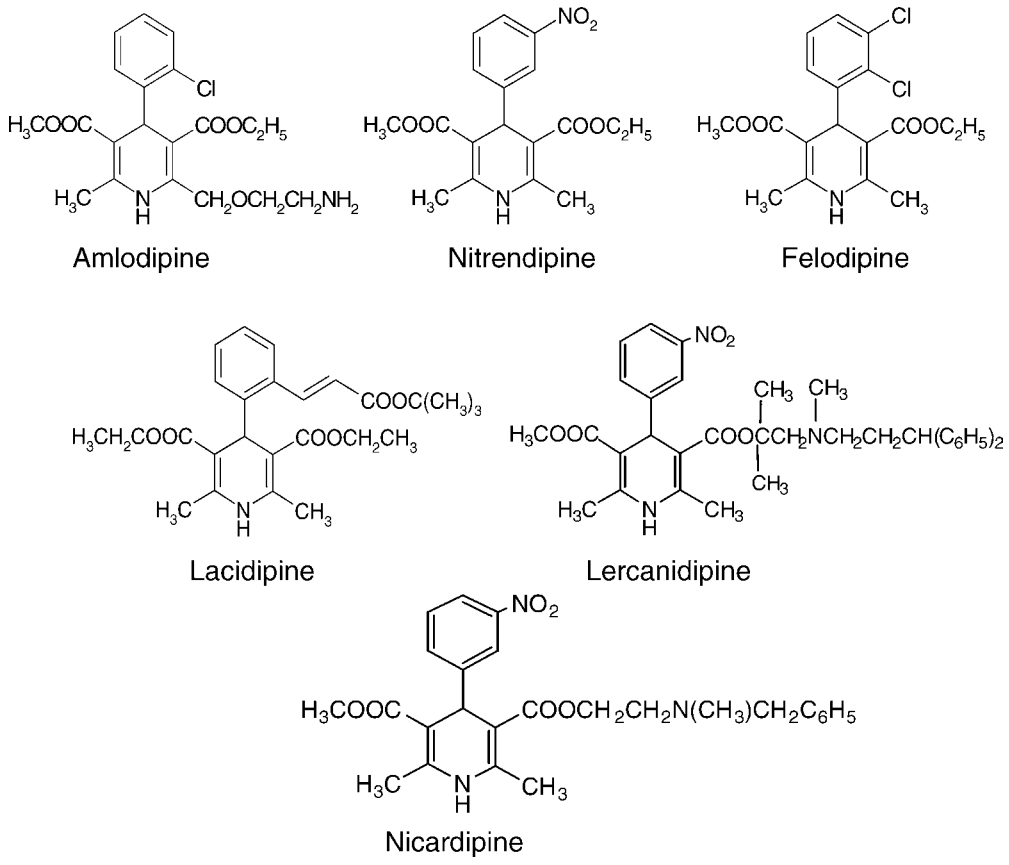

Fig. 1. Structure of the studied compounds and IS nicardipine.

In case of analysis of these compounds in biological fluids, it is necessary to perform a sample clean-up procedure prior to its introduction in the chromatographic system. For this process, it is found in literature that liquid–liquid extraction (LLE) [7,10,11,16,18–26] procedures are more commonly used than the solid-phase extraction methods [8,9,12–15,17].

Experimental design procedures are very useful in pharmaceutical developments, as well as in many other fields. It is found to be more effective than the classical one-variable-at-time approach, since it gives a fixed amount of information with much less effort as well as information about interactions. This kind of knowledge is an important clue in the achievement of optimum experimental conditions in the development of analytical methods [27–30].

The aim of this work was to optimize a LLE procedure for the above-mentioned compounds trying to get the best recovery for all of them simultaneously. In order to achieve this objective, experimental design was used to optimize the experimental conditions in which the procedure should be carried out.

There are several factors that can influence the LLE procedures. Among them, three variables were considered to be studied for the optimization of the LLE method: concentration of NaOH used as reagent to control pH of the plasma samples, volume of the organic solvent (diethyl ether) used as extractant, and finally, the shaking time of the mixture. Therefore, the optimization by means of experimental design was developed in three steps. After a preliminary screening investigation (factorial design), the knowledge about the system allowed a more detailed study to be performed in order to get the optimal conditions (central composite design), as well as the response surface describing the procedure. The different designs used in this study and the evaluation of the regression methods were done by means of The Unscrambler program (v. 7.5, Camo, Norway) [31]. Finally, to establish the optimal conditions for all the compounds, the Multisimplex program was used, since it allowed a simultaneous study of the variables at the same time [32].

An HPLC method with diode-array detection [33] was used for the extraction procedure monitoring.

2. Experimental

2.1. Apparatus and column

The HPLC system consisted of Waters 510 (Mildford, MA, USA) pump, a Rheodyne (Coati, CA, USA) Model 7125 injector fitted with a 20 μl loop and a Waters 996 photodiode-array detector. Wavelength was set at 237 and 360 nm. Chromatographic separation was accomplished using a Supelcosil ABZ+Plus, 250 mm × 4.6 mm i.d., 5 μm (Supelco, Barcelona, Spain) HPLC column with a

μBondapack C18 precolumn module (Waters). The column was kept at a constant temperature using a Waters TMC temperature control system.

pH of 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 3 M saturated in AgCl.

Plasma samples were centrifuged at constant temperature (4 °C) after the LLE procedure in an Eppendorf Model 5804R centrifuge (Hamburg, Germany). The extracted plasma samples were evaporated to dryness under a nitrogen stream using a Zymark Turvovap LV Evaporator (Barcelona, Spain).

2.2. Softwares

Millennium 3.2 Software was used for the acquisition and treatment of chromatographic data. The optimization by experimental design was performed with The Unscrambler program (v. 7.5, Camo, Norway) and with the Multisimplex program [32].

2.3. Reagents and solutions

Amlodipine and nitrendipine, were kindly supplied by Bayer (Barcelona, Spain), felodipine by Astra-Zeneca (Barcelona, Spain), lacidipine by Glaxo-Wellcome (Madrid, Spain), lercanidipine by Recordatti (Madrid, Spain) and nicardipine hydrochoride, which was used as internal standard (I.S.), by Roger (Barcelona, 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.

The buffer solution for the mobile phase was 10 mM CH_3COOH/CH_3COONa (pH 5). In order to achieve the desired pH value, volumes of 1 M HCl or 1 M NaOH solutions were added.

Standard stock solutions of every drug (1000 μg/ml) were prepared in methanol, stored at 4 °C and protected from light to avoid degradation. Working solutions of nicardipine hydrochloride (internal standard) (10 μg/ml) and of a mixture of amlodipine, nitrendipine, felodipine, lacidipine and lercanidipine (10 μg/ml) were prepared by appropriate dilution from stock solutions in mobile phase.

Drug-free control human plasma was purchased from Blood Bank of Galdakao Hospital (Bizkaia, Spain).

2.4. Chromatographic conditions.

The mobile phase consisted of acetonitrile–water (70:30, v/v) containing 10 mM acetate buffer (pH 5). The mobile phase was filtered through a 0.45 μm membrane under vacuum, and then degassed by flushing helium through the solution. This composition of the mobile phase allows the simultaneous analysis of all the compounds and it was optimized by experimental design [33].The mobile phase was pumped isocratically at a flow rate of 1 ml/min during analysis.

The 1,4-dihydropyridines have two absorption maxima at 237 and 360 nm. The fact that metabolites from the studied drugs are absorbent at 237 nm, as well as many other endogenous compounds from plasma, lead us to perform the experiments at 360 nm, since the whole procedure was optimised using blood samples. At this value of wavelength, the resulted metabolites are not absorbent due to the loss of the dihydropyridinic structure along the metabolic pathway [34–38].

It was decided to use an internal standard to correct possible errors due to reconstitution or injection of the samples. Among the 1,4-DHPs tested for this purpose (nifedipine, nisoldipine, nicardipine and barnidipine), only nicardipine and nisoldipine presented retention times that enables the quantification of all the peaks because of the appropriate resolution. Nicardipine was chosen as internal standard, since it is not so photolabile as nisoldipine.

2.5. Analytical procedure

Sample preparation was carried out as follows: once thawed and vortex were mixed, a certain volume of drug-free human plasma was spiked with 200 ng/ml of a mixture of all the studied compounds, shaked and divided in samples of 1 ml. The samples were then alkalinized by addition of 100 μl of NaOH, vortex-mixed for 30 s and a certain volume of diethyl ether was added. The mixture was gently shaked and centrifuged at 3500 × *g* for 10 min. The supernatant organic layer was transferred to a 10 ml glass tube and 20 μl of I.S. (nicardipine hydrochloride, 10 μg/ml) were added. Contents were evaporated under a stream of pure nitrogen at 37 °C. The residue was reconstituted in 100 μl of mobile phase and vortex mixed for 30 s. Aliquots of 20 μl were injected into the chromatographic system. The same procedure was carried out for blank plasma samples to check the cleanness of the extracts.

Concentration of NaOH, volume of organic solvent and shaking time were fixed, depending on the experiment that should be carried out.

2.6. Recovery and reproducibility of the optimized extraction procedure

The reproducibility and efficiency of the extraction procedure were determined by extracting replicate-spiked plasma samples (200 ng/ml of each compound, $n = 6$).

The extraction efficiency, expressed in terms of percentage of recovery, was estimated by comparing the peak areas of the compounds in spiked plasma samples with those from the blank plasma samples to which the compounds were added before evaporation. Data collected as peak areas and peak area ratio of each compound against I.S. were considered for all the calculations.

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

2.7. Specificity of the extraction procedure

Specificity of the assay was established with six independent sources of blank control plasma and comparing these with control plasma spiked with the studied compounds (200 ng/ml). The chromatograms were visually inspected for interfering chromatographic peaks from plasma endogenous substances.

3. Results

The LLE is a simple process which involves the adjustment of the pH value of the plasma sample by the addition of a basic or an acid aqueous solution, the extraction of the compounds after the addition of a solvent non-miscible with the aqueous phase and the shaking of the mixture.

To set each variable, some considerations were done considering the chemistry and characteristics of the compounds, as well as the previously reported LLE methods.

It is known that the modification of the pH value can enhance the selectivity of the process improving its efficiency. Two of the studied compounds, amlodipine and lercanidipine, present acid–base characteristics with pK_a values of 7 and 9, respectively [39,40]. Moreover, the dihydropyridine structure can appear as an ionized or a neutral structure depending on the pH value of the sample. Thus, an influence of the pH is expected. It is found in literature that the LLE of this kind of compounds has been performed at alkaline medium by addition of borate, phosphate or carbonate buffers and NaOH in the most of the cases [7,10,11,16,18–26].

The employment of solvents of different polarities can influence the efficiency of the extraction, as well as the selectivity of the process. It is also found that larger volumes of solvent, as well as larger times for the shaking of the sample enhance the efficiency of the extraction.

Thus, the variables to study were: the concentration of NaOH (M) used for the basification of the sample in the first step of the procedure, the extraction solvent, the volume of organic solvent (ml) and the shaking time (min).

In order to simplify the design, the variable "extraction solvent" was defined after a previous study. Therefore, the extraction procedure was performed, as it is described in Section 2.5 using common organic solvents or mixtures of solvents found in literature for the extraction of this group of drugs [7,10,11,16,18–26]. The pH value of the plasma sample was adjusted by additions of 100 μl of 5 M NaOH. A volume of 5 ml of organic solvent was used in every case and the mixture was shaken for 45 min.

The tested solvents (diethyl ether, ethyl acetate, toluene, hexane, hexane–diethyl ether (1:1, v/v), hexane–ethyl acetate (98:2, v/v) and hexane–dichloromethane (7:3, v/v) were evaluated in terms of best recovery and the cleanest extracts. The efficiency of the extraction was calculated as it is described in Section 2.6.

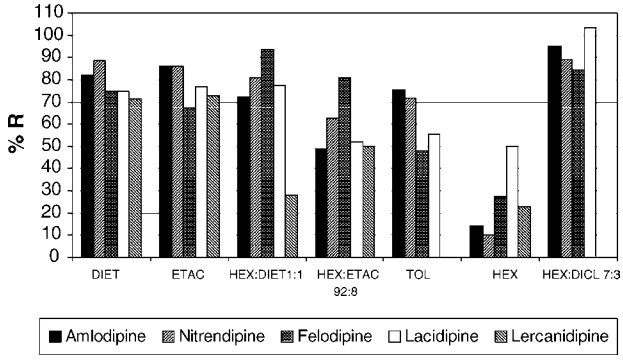

Fig. 2. Recovery of the extraction (%R) of the five 1,4-DHPs from human plasma with the tested solvents and mixture of solvents. Diethyl ether (DIET), ethyl acetate (ETAC), hexane (HEX), toluene (TOL), dichloromethane (DICL).

The best recoveries were achieved using diethyl ether and ethyl acetate for all the studied compounds as it can be seen in Fig. 2.

The mixture hexane–dichloromethane (7:3, v/v) also provided good values of recovery for all the compounds except for lercanidipine for which no amount of extracted compound was detected. This mixture of solvents, as well as toluene, provided chromatograms of extracted plasma samples which presented an interfering peak at the same retention time as of felodipine. Hexane is the solvent with the poorest recoveries but gave the cleanest chromatograms (Fig. 3).

Although the mixture of diethyl ether or ethyl acetate with hexane gave cleaner extracts than the use of diethyl ether or ethyl acetate in their pure form, diethyl ether was chosen as a solvent for the optimisation of the LLE extraction, due to the good recoveries obtained for all the studied 1,4-DHPs and the cleaner extracts obtained compared with ethyl acetate.

3.1. Optimization of the LLE method: experimental designs and calculations

The optimization of the LLE of the 1,4-dihydropyridines was carried out by experimental design. The different designs used in this study and the evaluation of the regression methods were done by means of The Unscrambler program (v. 7.5, Camo, Norway). Since each dihydropyridine may show a different regression model, the use of the Multisimplex program was introduced to explore simultaneously all the response surfaces and to obtain the best conditions to extract all the analytes.

In both kinds of designs, all the experiments were performed in random order to avoid trends, and three replicates were done for all the performed extractions to check the reproducibility of every experiment in order to avoid unreliable data.

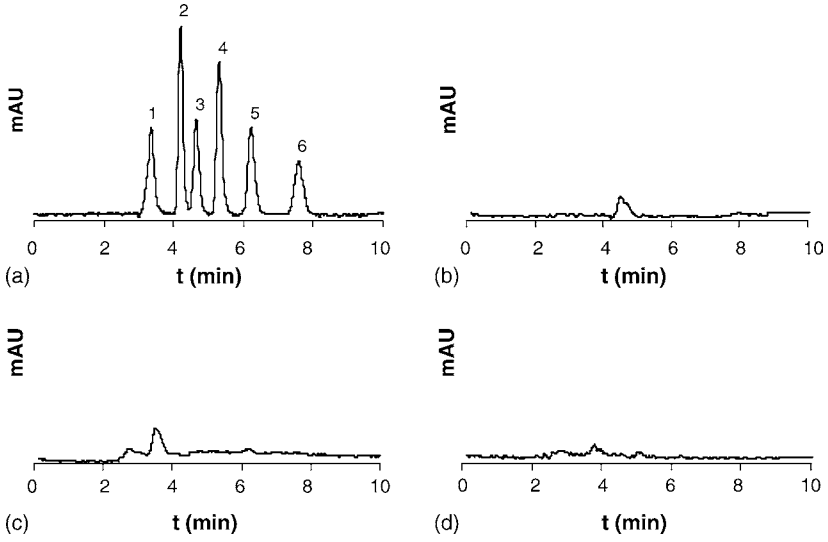

Fig. 3. Chromatograms obtained after the injection of (a) multicomponent standard solution (2 μg/ml of 1-amlodipine, 2-nitrendipine, 3-nicardipine hydrochloride (I.S.), 4-felodipine, 5-lacidipine and 6-lercanidipine), (b) extract of a blank plasma sample with toluene, (c) extract of a blank plasma sample with diethyl ether, and (d) extract of blank plasma sample with hexane-diethylether (1:1, v/v). Preconcentration 1:10 for samples b, c and d.

The efficiency of the extraction procedure expressed in terms of percentage of recovery (%R) was considered as the response of the system to be studied (Eq. (1)):

$$\%R = \frac{\text{Area}_{i_{\text{sp}}}}{\text{Area}_{i_{\text{bp}}}} \times 100 \quad (1)$$

where $\text{Area}_{i_{\text{sp}}}$ is the area of each compound quantified at 360 nm in spiked plasma samples; $\text{Area}_{i_{\text{bp}}}$ is the area of each compound quantified at 360 nm after direct injection of an standard solution at the same concentration and considering a recovery of 100%.

First of all, a full factorial design ($2^n + 1$) including all the potentially affecting variables, was accomplished. In this case, the effect of the concentration of NaOH (X_1, M) in the aqueous phase, the volume of diethyl ether (X_2, ml) and the shaking time (X_3, min) were studied. The factor space of this full factorial design was expanded within the following ranges: the concentration of NaOH was varied from 1 to 10 M, the volume of solvent from 2 to 6 ml and the shaking time from 10 to 30 min. The design matrix included two replicates of the central level to estimate the experimental variance. The obtained results are given in Table 1 where factors and levels of the factorial design appear as a legend.

The results of the full factorial design showed rather different patterns. On one side, the most effective variable was the concentration of NaOH (p-level <0.10) while the shaking time did not seem to have any effect. On the other side, the analysis of the regression models showed a significant lack of fit with some of the compounds. This meant that the models including only the interactions between variables are not enough to explain the experimental variance. In order to obtain better models, squared terms could be included in the regression models instead of some of the interactions. In

Table 1
Design matrix and recoveries from the factorial design (2^3)

Trial	Variables			Recovery (%)				
	X_1	X_2	X_3	Amlodipine	Nitrendipine	Felodipine	Lacidipine	Lercanidipine
1	−1	−1	−1	56 ± 7	84 ± 10	65 ± 3	59 ± 4	37 ± 1
2	+1	−1	−1	71 ± 4	89 ± 3	82 ± 2	68 ± 7	67 ± 3
3	−1	+1	−1	75 ± 1	94 ± 5	89 ± 5	76 ± 8	48 ± 10
4	+1	+1	−1	84 ± 2	92 ± 5	96 ± 6	79 ± 3	84 ± 5
5	−1	−1	+1	64 ± 4	91 ± 2	82 ± 1	77 ± 2	68 ± 1
6	+1	−1	+1	94 ± 4	88 ± 4	95 ± 2	55 ± 2	73 ± 1
7	−1	+1	+1	71 ± 6	92 ± 4	82 ± 4	78 ± 1	59 ± 2
8	+1	+1	+1	95 ± 1	94 ± 4	99 ± 7	59 ± 8	88 ± 8
9	0	0	0	95 ± 8	95 ± 2	93 ± 2	68 ± 7	70 ± 9
10	0	0	0	93 ± 10	99 ± 2	92 ± 1	58 ± 8	77 ± 4

Where X_1 (NaOH) ranges from 1 to 10 M (−1 and +1 in codified values, respectively); X_2 (volume of diethyl ether) ranges from 2 to 6 ml (−1 and +1 in codified values, respectively); X_3 (shaking time) ranges from 10 to 30 min (−1 and +1 in codified values, respectively).

Table 2
Significant parameters ($p < 0.10$) of the full factorial design experiments

Compound	r^2	Parameter
Amlopidine	0.960	X_1, X_2, X_3, X_1^2
Nitrendipine	0.804	X_1^2
Felodipine	0.814	X_1
Lacidipine	0.841	X_1, X_3
Lercanidipine	0.943	X_1, X_3

that case, the models did not show a significant lack of fit. In Table 2, the most significant parameters of the regression models for a given p-level ($p < 0.10$) are summarized.

From those results, it could be established that the responses for all the calcium channel antagonists were affected by the studied variables. As mentioned before, the concentration of NaOH seems to be the most significant variable for all the compounds, but its effect is not equivalent for all the compounds. While higher recoveries of amlopidine, felodipine and lercanidipine were obtained at increasing concentrations of NaOH, the recovery of nitrendipine and lacidipine were shown as a maximum and a minimum, respectively. Since, NaOH was used to adequate the pH of the aqueous phase while the organic solvent and time of the extraction played a more important role in the extraction procedure, it was decided to fix the concentration of the NaOH at 5 M (100 μl). Therefore, the response surface of the central composite design was studied taking into account the variation of the volume of diethyl ether, which was expanded from 5 to 9 ml, and the shaking time which remained from 10 to 30 min. Table 3 shows the design matrix of the central composite design as well as the experimental recoveries.

As it was done in the previous design, The Unscrambler program was used to study the regression models, including all the squared and interaction terms in order to get the response surface of the five 1,4-DHPs.

It can be concluded that the most meaningful parameter is the shaking time and the importance of the squared terms is reassured in this new design. Once the regression models were established, the next step is to explore the response surface to conclude with the optimum conditions. In order to show this, the different response surfaces can be seen in Fig. 4.

The analysis of the response surfaces can be done in several ways. The most immediate way of concluding the optimum extraction conditions is the graphical inspection of the five surfaces, since the 3D pictures give the complete overview of the systems. But when the number of factors are higher than 2, the response surfaces are projections of the response over two of the factors, and the graphical approach is not a correct way to get the optimum. In those cases, the use of the Multisimplex program was successful [41–43]. In order to carry out this analysis, all the regression models are written in a spreadsheet and the optimization procedure is defined in Multisimplex. In this way, the experiments suggested by Multisimplex are calculated in the spreadsheet and the responses are given again to Multisimplex. In this iterative way, Multisimplex evolves towards the optimum defined according to the optimization procedure. Multisimplex makes use of the membership value which range 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 experiments proposed by the Multisimplex program and the membership value of each of the experiments (ordered in terms of increasing membership value) are summarized in Table 4.

In this study, the Multisimplex program gave 23 min of shaking time and 6 ml of solvent as the optimal values for the studied variables in which the extraction should be carried out.

3.2. *Efficiency of the extraction and reproducibility*

When optimum conditions were established, a final LLE procedure was carried out ($n = 6$) with the proposed values obtained from the Multisimplex optimization for every variable to compare the experimental results with those given by Multisimplex program. The reproducibility of the extraction was also tested ($n = 6$) and expressed in terms of %R.S.D. The results are shown in Table 5.

Table 3
Composite central design matrix and efficacy of the extraction in terms of percentage of recovery for the studied compounds

Trial no.	Variables		Recovery (%)				
	X_1	X_2	Amlodipine	Nitrendipine	Felodipine	Lacidipine	Lercanidipine
1	−1	−1	67 ± 2	96 ± 9	96 ± 2	97 ± 9	68 ± 1
2	+1	−1	59 ± 8	95 ± 5	98 ± 8	88 ± 9	86 ± 15
3	−1	+1	94 ± 9	99 ± 1	94 ± 6	96 ± 5	94 ± 1
4	+1	+1	74 ± 8	99 ± 3	94 ± 4	93 ± 3	75 ± 11
5	−2	0	82 ± 4	99 ± 1	98 ± 6	97 ± 3	78 ± 5
6	+2	0	84 ± 11	92 ± 10	99 ± 2	97 ± 4	82 ± 10
7	0	−2	93 ± 4	72 ± 1	80 ± 1	69 ± 3	50 ± 10
8	0	+2	80 ± 6	99 ± 5	94 ± 1	92 ± 4	63 ± 9
9	0	0	66 ± 7	99 ± 2	90 ± 4	89 ± 5	84 ± 1
10	0	0	68 ± 11	97 ± 3	92 ± 8	88 ± 2	84 ± 5

Where: X_1 (volume of solvent) ranges from 5 to 9 ml (−2 and +2 in codified values, respectively); X_2 (shaking time) ranges from 10 to 30 min (−2 and +2 in codified values, respectively).

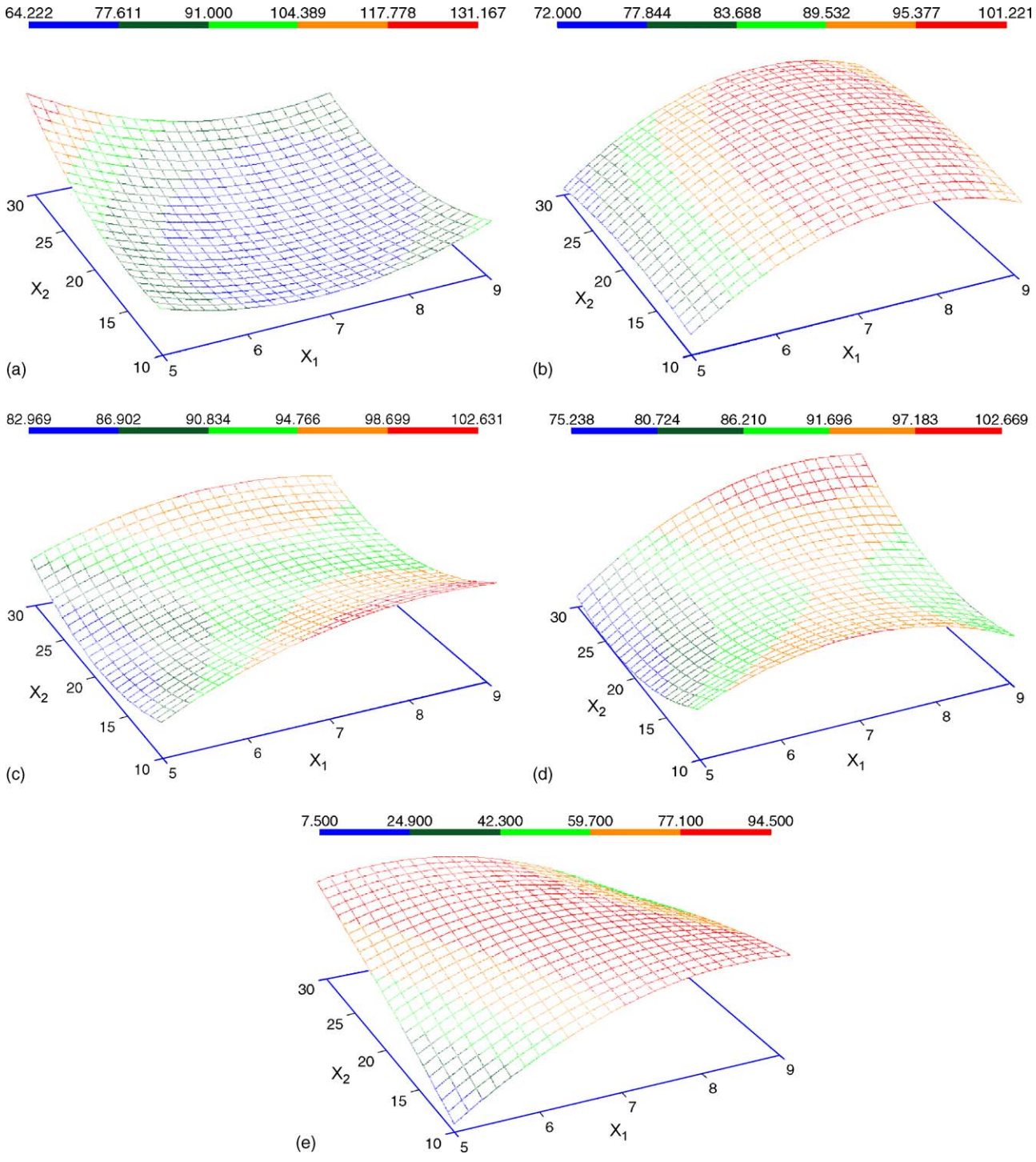

Fig. 4. Response surfaces for (a) amlodipine, (b) nitrendipine, (c) felodipine, (d) lacidipine and (e) lercanidipine.

3.3. Specificity

The studied compounds were extracted from spiked plasma from six different sources using the LLE procedure optimized in this work. The absence of interference from plasma matrix in the chromatograms obtained for drug-free and spiked plasma samples can be seen in Fig. 5.

4. Discussion

Due to an intense metabolic transformation of these compounds in the liver, the plasma concentrations are very low (ng/ml). Due to this, in the development of bioanalytical methods for their determination, not only a sensitive analytical method has to be performed, but also an extraction procedure which yield high percentages of

Table 4
Proposed experimental conditions, responses and membership values for each experiment performed by Multisimplex program

Trial no.	X_1	X_2	Responses (% recovery)					
			Amlodipine	Nitrendipine	Felodipine	Lacidipine	Lercanidipine	Membership
12	−2.06	1.38	126.5	68.5	86.7	71.1	73.8	0.784
8	0.06	0.22	65.8	99.9	93.3	91.6	86.6	0.865
9	−0.04	0.22	66.3	99.4	93.1	91.3	86.7	0.866
2	0.88	−0.14	69.15	100.1	94.1	91.1	82.5	0.873
4	1.37	−0.44	75.3	97	94.2	88.5	78.4	0.875
3	1.53	−0.54	79	95.5	94.2	87.3	76.8	0.876
6	−1.29	0.88	92.2	85.3	89.4	82	80.9	0.876
16	−1.45	1.09	100.1	82	89.5	80.3	81.2	0.878
13	−0.55	0.51	73.1	95.5	91.9	88.9	85.8	0.880
14	−0.45	0.51	71.8	96.4	92.3	89.6	86.3	0.880
10	−1.10	0.80	86.4	88.4	90.2	84.2	82.8	0.882
1	1.06	−0.49	71.5	99	94.7	90.3	83.8	0.883
18	−0.80	0.66	78.6	92.6	91.2	87	84.8	0.884
17	−0.70	0.66	76.9	93.7	91.7	87.9	85.7	0.886
7	−0.90	0.80	82.2	91.1	91.3	86.3	85	0.889
15	−0.90	0.80	82.2	91.1	91.3	86.3	85	0.889
11	−1.00	0.80	81.05	92.4	92.3	87.9	88	0.896
5	−1.00	0.80	77.9	95	93.8	90.5	88	0.904

recovery, is required. The developed method is applicable to the extraction of the studied compounds from blood samples from patients undergoing antihypertensive therapy, since it yields percentages of recovery up to at least 80% and low %R.S.D. (<10%) which makes it suitable for this purpose in accordance with international guidelines [44].

At the same time, its shows several advantages if compared with some of the LLE procedures found in literature, such as the use of lower volumes of solvent and just one extraction step, which implies a saving of reagents and time. For some compounds (lacidipine and lercanidipine), better recoveries were found with the optimised method.

The fact, the optimisation of the procedure, carried out using experimental design, offers other advantages if compared to those methods optimised by the widely used traditional step-by-step approach, which involves (i) a large number of independent runs; (ii) the interactions between variables cannot be detected and taken into account; (iii) only a very narrow range within all possible combinations of values is examined; and (iv) as a consequence, the detected optimum

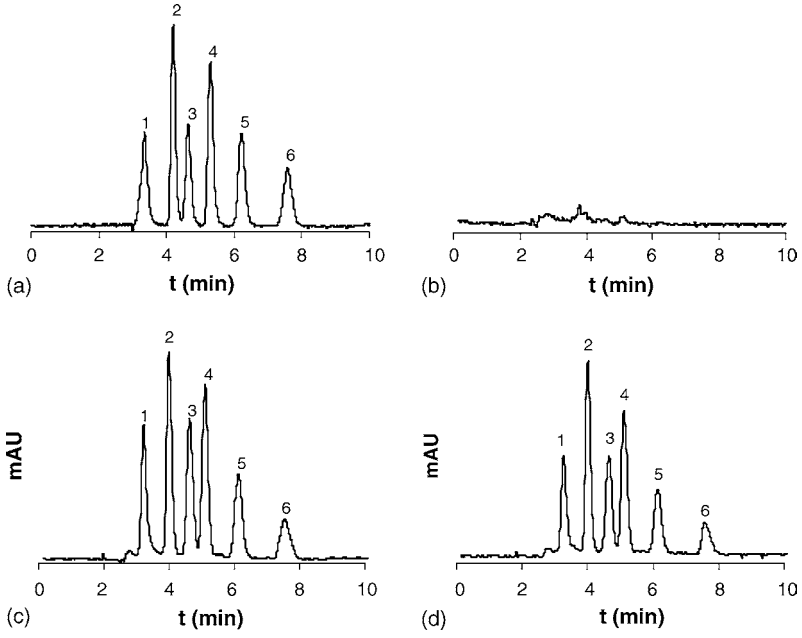

Fig. 5. Chromatograms obtained after the injection of (a) multicomponent standard solution (2 μg/ml of each compound), (b) extract of blank plasma sample, (c) extract of blank plasma sample spiked with 200 ng/ml of each compound after the extraction, and (d) extract of a spiked plasma sample in concentration 200 ng/ml of each compound. Peaks: 1-amlodipine, 2-nitrendipine, 3-nicardipine hydrochloride (I.S.), 4-felodipine, 5-lacidipine and 6-lercanidipine. Conditions for the LLE procedure are described in Section 2.5. Chromatographic conditions are described in Section 2.4.

Table 5
Recovery of 1,4-DHP drugs from human plasma samples spiked with 200 ng/ml of each compound

Compound	Experimental recovery (%) ($n = 6$)	R.S.D. (%) ($n = 6$)
Amlodipine	83 ± 6	9.2
Nitrendipine	95 ± 5	7.0
Felodipine	96 ± 2	4.2
Lacidipine	98 ± 3	5.4
Lercanidipine	91 ± 11	8.6

in many cases is not identical with the real optimum. Thus, the optimization using experimental design strategies allowed an efficient development of the extraction method, show that a correct use of an appropriate experimental design is of considerable benefit in the set up of experimental conditions.

This procedure will be used in a future project as a basis for the development of analytical methods for these dihydropyridines in plasma samples.

Acknowledgements

A.B.G. thanks the Ministry of Education and Science for a FPU grant, and the authors thank the University of Basque Country and Ministry of Science and Technology for the financial support (projects UPV 00171.310-15359/2003 and BQU 2002-03098) and the pharmaceutical companies for the kind supply of the 1,4-dihydropyridines studied.

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