Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 1192–1202

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Developing and optimizing a validated isocratic reversed-phase high-performance liquid chromatography separation of nimodipine and impurities in tablets using experimental design methodology

Panagiotis Barmpalexis[a], Feras Imad Kanaze[b], Emanouil Georgarakis[a,*]

[a] Department of Pharmacy and Drug Control, School of Pharmacy, Aristotle University of Thessaloniki, 541 24 Thessaloniki, Greece
[b] Pharmathen S.A., Pharmaceutical Industry, Athens, Greece

ARTICLE INFO

Article history:
Received 24 November 2008
Received in revised form 13 February 2009
Accepted 3 March 2009
Available online 20 March 2009

Keywords:
Nimodipine
Impurities
Reversed-phase HPLC
Statistical experimental design
Multiple response optimization

ABSTRACT

In the present study an isocratic reversed-phase high-performance liquid chromatography was investigated for the separation of nimodipine and impurities (A, B and C) using statistical experimental design. Initially, a full factorial design was used in order to screen five independent factors: type of the organic modifier – methanol or acetonitrile – and concentration, column temperature, mobile phase flow rate and pH. Except pH, the rest examined factors were identified as significant, using ANOVA analysis. The optimum conditions of separation (optimum values of significant factors) determined with the aid of central composite design were: (1) mobile phase: acetonitrile/H_2O (67.5/32.5, v/v), (2) column temperature 40 °C and (3) mobile phase flow rate 0.9 ml/min. The proposed method showed good prediction ability (observed–predicted correlation). The analysis was found to be linear, specific, precise, sensitive and accurate. The method was also studied for robustness and intermediate precision using experimental design methodology. Three commercially available nimodipine tablets were analyzed showing good % recovery and %RSD. No traceable amounts of impurities were found in all products.

1. Introduction

Calcium antagonists are a heterogeneous group of cardiovascular drugs used to block the entry of calcium ions into nerve cells, producing a reduction in peripherical vascular resistance [1]. This class of compounds (dihydropyridines) are 4-aryl-1,4-dihydropyridine 3,5-dicarboxylates. The ester functions in the 3,5-positions may vary widely without a significant reduction in potency [1].

Nimodipine (Nim) is a 1,4-dihydropyridine (DHP) with calcium channel antagonistic activity [2]. It was identified as having cerebrovasodilatory and neuronal effects at doses that had little or no effect on peripheral circulation [3–5]. Experimental studies in animals and humans suggested its effectiveness in the treatment of subarachnoid haemorrhage [6], focal or global ischemia [4,7–9], as well as epilepsy [2].

The chemical structure of nimodipine and its impurities is shown in Fig. 1. The pyridine derivatives (impurities) of nimodipine are [1]: (1) the photo-degradation product of nimodipine impurity A (Imp A): 2-methoxyethyl 1-methylethyl-2,6-dimethyl-4-(3-nitrophenyl) pyridine-3,5-dicarboxylate, (2) impurity B (Imp B): bis (1-methylethyl)-2,6-dimethyl-4-(3-nitrophenyl), 1,4-dihydro-pyridine-3,5-dicarboxy-late and (3) impurity C (Imp C): bis (2-methoxyethyl)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydro pyridine-3,5-dicarboxylate.

There are several literature reports for the determination of nimodipine using high-performance liquid chromatography (HPLC) [10–14]. However, there is only one attempt made for the simultaneous determination of all substances (nimodipine and all impurities) using a preparative HPLC [1].

Developing and optimizing an isocratic HPLC method is a complex procedure that requires simultaneous determination of several factors (e.g. type and composition of the organic phase, column temperature, flow rate, pH, type of the stationary phase, etc.). For decades HPLC separations were based on a trial and error methodology. The traditional approach entails studying the influence of the corresponding factors by Changing One Single (or Separate) factor at a Time (COST), whilst keeping the others constant [15]. The technique, at times, is also known as OFAT (One Factor at a Time) [15–17]. Many years of experience have showed that these COST methods are inefficient and time consuming as they require a great amount of effort (planned experiments) and time without actually (in many cases) being able to identify the optimum conditions [15,18].

A great amount of effort has been made in order to overcome these inefficiency problems. Snyder suggested a systematic methodology for selecting the "best" mobile phase, based on

* Corresponding author. Tel.: +30 2310997641; fax: +30 2310997652.
E-mail address: georgara@pharm.auth.gr (E. Georgarakis).

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doi:10.1016/j.jpba.2009.03.003

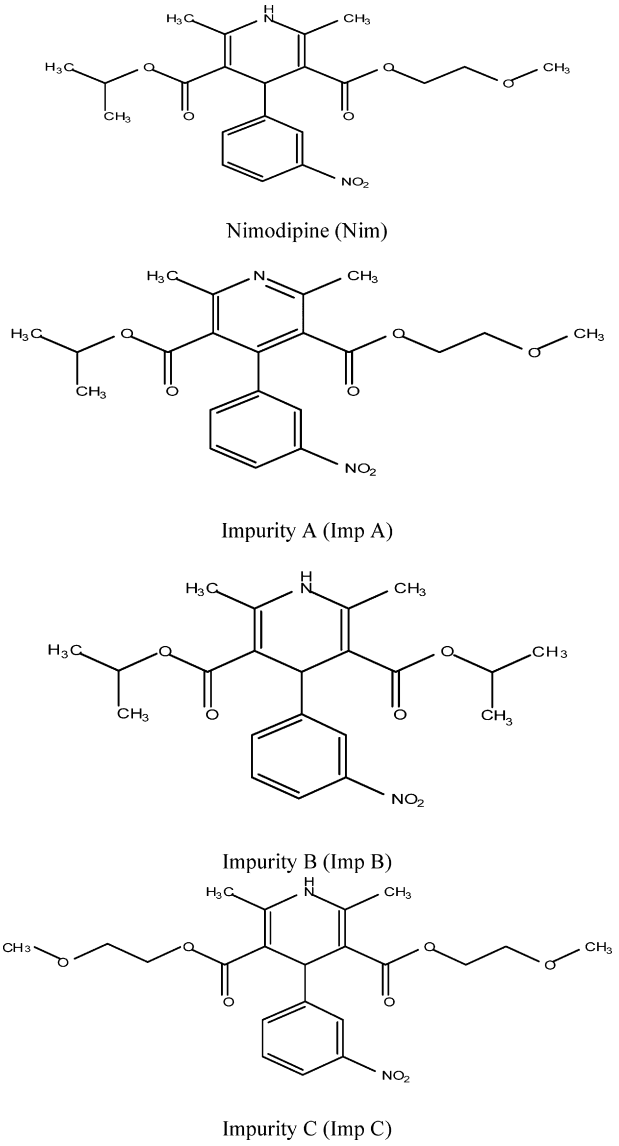

Fig. 1. Chemical structures of nimodipine and impurities A, B and C.

organic solvent's selectivity (Snyder's solvent-selectivity triangle) [19]. Although this method reduced (to some extend) the deficiency of COST methodology (using a systematic approach to identify optimum mobile phase type and composition) the rest of the factors were still being determined by trial and error. These drawbacks forced scientists to consider more efficient systematic optimization techniques such as experimental design.

The principles behind these techniques (known as Design of Experiments (DoE)), encompasses the use of experimental design, generation of mathematical equations and graphic outcomes [15]. Employing various rational combinations of factors, statistical experimental design fits experimental data into mathematical equations (known as models) in order to predict and optimize the examined responses. Examples of HPLC development and optimization attempts with the aid of DoE have shown important advantages [20–23].

The aim of the present paper was to develop and optimize an isocratic high-performance liquid chromatography method for the determination of nimodipine and impurities, using experimental design. The significance of the studied factors was evaluated with the aid of a full factorial design (full FD) whilst the optimum chromatographic conditions were estimated by a central composite design (CCD) using both a graphical (overlay contour plots) and a mathematical (Derringer's desirability function) global optimization approach. Finally, the proposed method was tested for linearity, specificity, inter and intra-day precision, accuracy, robustness and intermediate precision (using experimental design). Three commercially available nimodipine tablets were analyzed in order to check the validity of the proposed method.

2. Experimental

2.1. Apparatus

Experiments were performed on a Shimadzu Prominence HPLC system (HPLC 1) consisted of: degasser (Model DGU-20A5), pump (Model LC-20AD), total-volume injection-type auto-sampler (Model SIL-20AC), variable wavelength UV–vis detector (Model SPD-20A), and column oven (Model CTO-20AC). Chromatographic analyses were done on an Interchrom analytical column C8 (5 μm particle size, 250 mm × 4.6 mm I.D.). A C18 analytical column (150 mm × 4.6 mm I.D. and 3.6 μm particle size) was also tested but was unable to separate the examined substances. The entire HPLC system was controlled using LC solutions, ver. 1.21 SP1 PC software (Shimadzu Corporation, Kyoto, Japan). All eluents were filtered though 0.45 μm membrane filter (Whatman). The volume injected into the chromatographic system was 10 μl. UV detection was performed at 236 nm. A second Shimadzu Prominence HPLC system (HPLC 2) was used for intermediate precision, consisting of: communication bus module (Model CBM-20A), diode array detector (Model SPD-M20A), degasser (Model DGU-20A5), pump (Model LC-20AD), total-volume injection-type auto-sampler (Model SIL-20AC), and column oven (Model CTO-20AC).

2.2. Materials and reagents

Nimodipine and all impurities (A, B and C) were supplied by Union Quimico Farmaceutical S.A. (Barcelona, Spain). Acetonitrile and methanol (HPLC-grade) were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). The excipients used for specificity were: microcrystalline-cellulose (Avicel PH101, FMC International, Little Island, Cork, Ireland), starch (Sigma Chemical Co., Steinheim, Germany), povidone and crospovidone (BASF Co., Ledgewood, NJ), magnesium stearate (Katayama, Osaka, Japan), hydroxypropylmethyl-cellulose (Dow Chemical Co., Midland, MI, USA), polyethylene (CLARIANT, Sulzbach, Germany), iron oxide and titanium dioxide (Aldrich Chemical Co., Milwaukee, WI). Nimodipine tablet products (30 mg nominal content) were purchased form a local pharmacy store in Greece: Nimotop® (Bayer, Germany), Nimovac-V® (Pharmathen S.A., Greece) and Nortolan® (Anfarm, Greece). Double-distilled water was used during the analysis.

2.3. Standard solution

Stock standard solution of nimodipine and impurities (A, B and C) were prepared in methanol at a concentration of 2 mg/ml. The prepared stock solution was stored at 4 °C and protected from light. In the development and optimization phase, working mixture solutions of all compounds, containing 20 μg/ml of nimodipine and 300 ng/ml of each impurity, were freshly prepared by diluting the stock standard solution with methanol during the day of analysis.

2.4. Validation

The proposed method was validated according to ICH guidelines "Q2(R1), Validation of Analytical Procedures: Text and Methodology" [24]. For specificity study, placebo containing microcrystalline-cellulose, maize starch, povidone, crospovidone,

magnesium stearate, hydroxypropylmethyl-cellulose, polyethylene glycol, titanium dioxide and iron oxide was used. Before injecting the prepared solutions, the analytical column was equilibrated for at least 20 min with mobile phase. Calibration curves reporting peak areas versus drug concentrations were established in the range of 7.5–37.5 μg/ml for nimodipine and 18.75–300 ng/ml for all impurities. Linearity was studied by testing mixtures of the examined substances at seven concentration levels. The limits of detection (LODs) were estimated based on the standard deviation of the *y*-intercepts of regression analysis (σ) and the slope (S), by the following equation LOD = $3.3\sigma/S$ [24]. Similarly the limits of quantification (LOQs) were estimated by LOQ = $10\sigma/S$ [24]. Accuracy and intra/inter-day precision were also examined. The intra-day accuracy and precision were assessed from the results of six replicate analyses at three concentration levels (7.5, 22.5 and 30 μg/ml for nimodipine and 18.75, 75 and 300 ng/ml for impurities) on a single assay day. The inter-day accuracy and precision were determined from the same QC samples analyzed on 6 consecutive days. Precision was expressed as % relative standard deviation (RSD), whilst accuracy (%) was expressed as [(calculated amount/predicted amount) × 100]. Robustness and intermediate precision were examined using a two-level fractional (one-half 1/2 fraction) factorial and a full factorial experimental design, respectively. A stability study for nimodipine/methanol solution (20 μg/ml) was conducted in the following conditions: 0.5, 1, 2, 3, 5, 24, 48, 72, 96, 120 and 300 h in laboratory light and 25 °C. Three commercially available tablet products of nimodipine were investigated to identify drug's % recovery and %RSD. Six tablets (n = 6) from each product were granulated, weighted, dissolved in methanol (30 μg/ml concentration) and sonicated for 30 min. Samples from the prepared dilutions were filtered and assayed by the proposed HPLC method.

2.5. Statistical tools

Work on experimental design, data analysis, response surfaces and contour diagrams, was performed by Design Expert Version 6.0.4 (Stat-Ease Inc., Minneapolis, MN). The rest of the graphs were made using SigmaPlot Version 8.0 (Systat Software Inc., San Jose, CA, USA).

3. Results and discussion

3.1. Screening experiments with the aid of full factorial design

Before starting an optimization procedure, it is important to identify the crucial factors affecting the quality of the derived outcomes. In the present study the significance of five independent factors on the quality of the separation was investigated using a two-level full factorial design.

Screening designs can identify significant main effects rather than interaction effects. Therefore, these are usually first-order designs with low resolution [15]. Two-level full factorial designs (2^k) are the simplest form of orthogonal design employed for screening k number of factors [25]. The mathematical model associated with design consists of main and (possible) interaction effects (Eq. (1)):

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \quad (1)$$

where n is the number of factors, X is the factor examined, Y is the measured response, and β_0, β_i, β_{ij} represent the coefficients for each main or interaction effect.

In the present study five factors (Table 1) were examined in two levels (2^5 = 32 experiments): (1) type of organic modifier (X_1), (2) concentration of organic phase (X_2), (3) column temperature (X_3), (4) flow rate of mobile phase (X_4), and (5) pH (X_5). The high and low levels of factors were determined based on preliminary experiments.

Table 1
Factors examined in the screening phase (full FD).

Independent factors	Levels	
	Level (−1)	Level (+1)
X_1 = type of organic modifier	Acetonitrile	Methanol
X_2 = concentration of organic phase (%)	65	70
X_3 = column temperature (°C)	30	40
X_4 = Flow rate of the mobile phase (ml/min)	0.8	1
X_5 = pH	3.5	7.5

The experimental domain of the 2^5 full factorial design is given in Table 2. All experiments where conducted in randomized order and in triplicate.

Statistical analysis tools (analysis of variance (ANOVA)) was used in order to identify significant effects. The response factors chosen were: (1) the resolution of the worst separated peak Rs_2 (nimodipine and impurity A), (2) the retention time of the first eluted peak Rt_C (impurity C), (3) the retention time of the last peak Rt_B (impurity B), and (4) the chromatographic optimization function (COF). COF is calculated according to Eq. (2) [26]:

$$\mathrm{COF} = \sum_{i=1}^{k} A_i \ln\left(\frac{Rs_i}{Rs_{id}}\right) + B(t_M - t_L) \quad (2)$$

where A_i and B are weighted parameters (equal to unity in this study), Rs_i is the resolution of the ith pair, Rs_{id} is the desired resolution for the specific pair (equals to 1.5), t_M represents the desired maximum analysis time (here assumed 10 min), and t_L is the actual time of the last eluted peak. COF was used in the present study because it has the ability to reduce data from each chromatogram to a single number which can be used in the optimization procedure.

Table 2
Experimental domain of a 2^5 full factorial design.

Std	Run	X_1	X_2	X_3	X_4	X_5
21	1	+1	+1	+1	+1	+1
5	2	+1	+1	−1	+1	+1
19	3	−1	−1	+1	−1	+1
12	4	−1	+1	−1	+1	−1
16	5	+1	−1	+1	+1	+1
32	6	−1	−1	−1	+1	−1
14	7	+1	−1	+1	+1	−1
26	8	−1	−1	+1	+1	+1
25	9	−1	+1	+1	−1	+1
3	10	+1	−1	+1	−1	−1
31	11	−1	−1	−1	−1	−1
27	12	+1	+1	+1	−1	−1
13	13	+1	+1	−1	−1	+1
1	14	−1	+1	+1	−1	−1
8	15	+1	+1	−1	+1	−1
30	16	+1	−1	−1	−1	−1
9	17	+1	+1	−1	−1	−1
11	18	−1	+1	−1	−1	+1
18	19	+1	−1	+1	−1	+1
15	20	+1	−1	−1	−1	+1
17	21	−1	−1	+1	−1	−1
10	22	−1	+1	+1	+1	−1
28	23	+1	+1	+1	+1	−1
22	24	−1	−1	−1	+1	+1
4	25	−1	−1	+1	+1	−1
7	26	+1	+1	+1	−1	+1
20	27	−1	−1	−1	−1	+1
24	28	−1	+1	−1	+1	+1
6	29	+1	−1	−1	+1	+1
29	30	+1	−1	−1	+1	−1
23	31	−1	+1	−1	−1	−1
2	32	−1	+1	+1	+1	+1

Table 3
ANOVA results for full FD. A 5% level of significance was desired. Insignificant interaction effects were excluded.

Factors	Rs_2		Rt_C		Rt_B		COF	
	F	p	F	p	F	p	F	p
X_1	19004.00	<0.0001	3892.46	<0.0001	14037.66	<0.0001	4926.88	<0.0001
X_2	543.03	<0.0001	1377.98	<0.0001	4681.32	<0.0001	1681.15	<0.0001
X_3	1385.53	<0.0001	210.13	<0.0001	729.03	<0.0001	275.21	<0.0001
X_4	264.75	<0.0001	286.55	<0.0001	4414.47	<0.0001	380.50	<0.0001
X_5	6.84×10^{-4}	0.4206	0.63	0.4376	0.50	0.4875	9.79×10^{-2}	0.5221
X_1X_2	306.72	<0.0001	566.76	<0.0001	1484.92	<0.0001	885.68	<0.0001
X_1X_3	–	–	89.78	<0.0001	184.92	<0.0001	118.80	<0.0001
X_1X_4	8.13	0.0002	35.88	<0.0001	101.25	<0.0001	53.13	<0.0001
X_2X_3	–	–	6.64	0.0010	45.09	<0.0001	31.26	<0.0001
X_2X_4	–	–	5.74	0.0019	75.10	<0.0001	25.47	<0.0001
R^2_{adj} [a]	0.9998		0.9996		0.9996		0.9997	

[a] Value close to 1 shows perfect fit to Eq. (1).

Table 4
Conducted experiments and measured responses for central composite design.

Std	Run	Factors			Responses			
		A (v/v)	B (°C)	C (ml/min)	Rs_2	Rt_B (min)	COF	k_1
7	1	65.0	40.0	1.0	2.340	9.410	4.463	1.918
4	2	70.0	40.0	0.8	2.391	9.915	3.703	2.345
20	3	67.5	35.0	0.9	2.293	10.637	3.360	2.258
3	4	65.0	40.0	0.8	2.512	13.042	1.235	2.822
6	5	70.0	30.0	1.0	1.989	8.646	4.845	1.799
10	6	71.7	35.0	0.9	2.223	8.549	4.909	1.930
8	7	70.0	40.0	1.0	2.231	7.960	5.446	1.692
9	8	63.3	35.0	0.9	2.296	13.357	1.072	2.651
2	9	70.0	30.0	0.8	2.138	10.765	2.951	2.478
16	10	67.5	35.0	0.9	2.293	10.637	3.360	2.258
12	11	67.5	43.0	0.9	2.535	9.934	4.013	2.155
1	12	65.0	30.0	0.8	2.136	14.281	0.024	3.001
17	13	67.5	35.0	0.9	2.293	10.637	3.360	2.258
5	14	65.0	30.0	1.0	2.003	11.431	2.677	2.212
14	15	67.5	35.0	1.1	2.188	8.986	4.873	1.753
11	16	67.5	26.6	0.9	2.010	11.358	2.659	2.362
18	17	67.5	35.0	0.9	2.293	10.637	3.360	2.258
15	18	67.5	35.0	0.9	2.293	10.637	3.360	2.258
13	19	67.5	35.0	0.7	2.412	13.104	1.038	3.011
19	20	67.5	35.0	0.9	2.293	10.637	3.360	2.258

Table 5
ANOVA results for CCD. A 5% level of significance was desired. Insignificant factors are excluded.

Factors	Rs_2		Rt_B		COF		k_1	
	F	p	F	p	F	p	F	p
A	9.32	0.0092	533.23	<0.0001	950.66	<0.0001	750.72	<0.0001
B	306.41	<0.0001	76.67	<0.0001	185.62	<0.0001	103.65	<0.0001
C	68.79	<0.0001	453.12	<0.0001	1077.45	<0.0001	2439.66	<0.0001
A^2	7.55	0.0166	–	–	10.65	0.0068	–	–
B^2	4.86	0.046	–	–	–	–	–	–
C^2	–	–	–	–	13.11	0.0035	26.34	0.0002
AB	5.69	0.033	7.52	0.0159	19.52	0.0008	8.52	0.0120
AC	–	–	14.68	0.0018	36.32	<0.0001	20.47	0.0006
BC	–	–	–	–	–	–	–	–
R^2_{adj} [a]	0.9542		0.9827		0.9918		0.9943	

[a] Value close to 1 shows perfect fit to Eq. (3).

Table 6
Response models and statistical parameters obtained from ANOVA for CCD.

Response	Regression model	Model p-value	%C.V.	Adequate precision
Rs_2	$2.29 - 0.027A + 0.15B - 0.073C - 0.023A^2 - 0.019B^2 - 0.027AB$	<0.0001	1.43	28.656
Rt_B	$10.73 - 1.39A - 0.53B - 1.28C + 0.22AB + 0.30AC$	<0.0001*	2.07	52.501
COF	$3.37 + 1.10A + 0.49B + 1.17C - 0.11A^2 - 0.12C^2 - 0.21AB - 0.28AC$	<0.0001	4.11	66.130
k_1	$2.26 - 0.27A - 0.078B - 0.38C + 0.038C^2 + 0.029AB + 0.045AC$	<0.0001	1.23	79.562

* The significant model for the retention time of impurity B was linear. Quadratic terms were not significant.

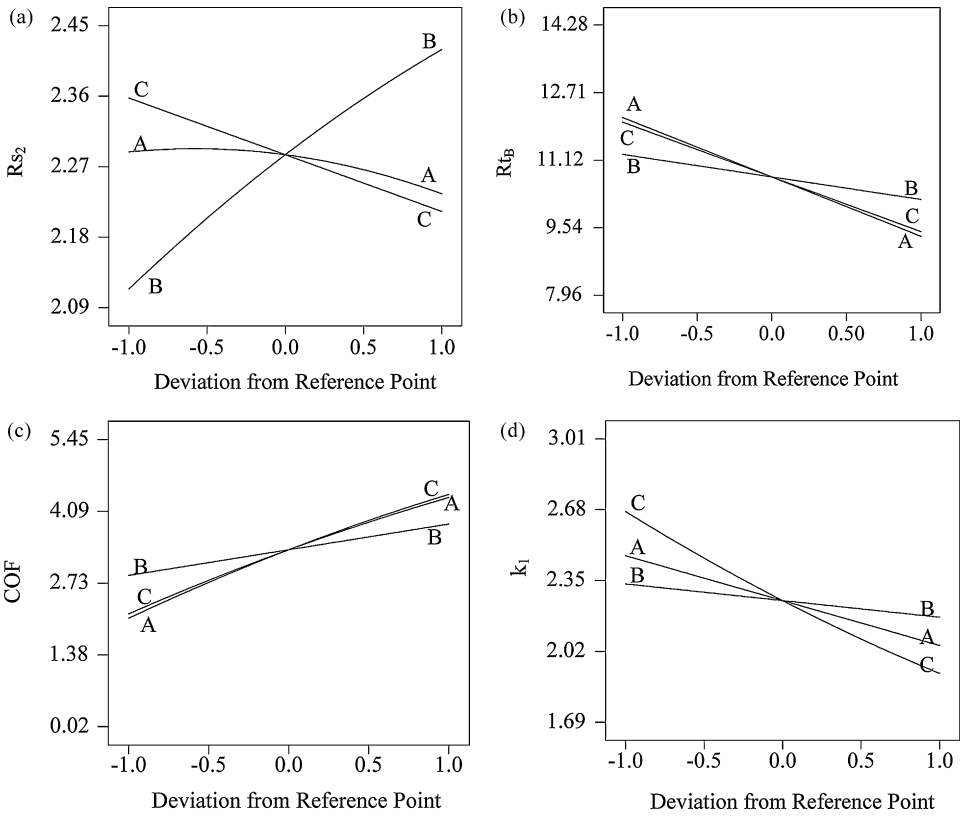

Fig. 2. Perturbation plots showing the effect of the examined factors on the responses (a) Rs_2, (b) Rt_B, (c) COF and (d) k_1. Where A is the concentration of acetonitrile, B the temperature of the column and C the mobile phase flow rate.

A good peak resolution and small elution time (desirable outcome) results to a greater COF value. It is crucial to remember that COF works satisfactory only when all peaks have the same relative order of retention in all conditions [26]. Response transformations were made when necessary.

The statistical analysis (ANOVA) is given in Table 3. An independent factor had significant effect on a given response when it had a p-value < 0.05.

The results indicated that all factors, except pH (X_5), had significant effect on the selected responses. Analytically, the type of organic modifier (X_1) and the column temperature (X_3) had the most significant influence on the resolution of the critical pair of peaks (Rs_2). Similarly, the type (X_1) and the concentration (X_2) of the organic modifier were mostly affecting the chromatographic optimization function (COF), whilst the retention times of the first and the last peak were mainly affected by the type of the organic modifier (X_1). Two-level interactions between the examined factors (X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4) also showed significant effects (Table 3). R^2_{adj} was greater than 0.999 in all cases, revealing good fit of the experimental data.

It is important to note that when acetonitrile was used as an organic modifier, the rest of the chromatographic parameters (capacity factor, tailing factor, etc.) had better results compared to methanol. Also the retention time of the last peak (impurity B, Rt_B) was minimized with acetonitrile rather than methanol. Therefore, acetonitrile was chosen as the proper organic modifier for the optimization phase.

An optimization study including three factors (concentration of acetonitrile, column temperature, and mobile phase flow rate) was carried out in order to identify the optimum chromatographic conditions. The value of pH (insignificant factor) was set constant at 7.5.

3.2. Optimization using central composite design

For nonlinear responses (requiring second-order mathematical models) central composite design (CCD) is the most frequently employed design [15]. This type of design (also known as the Box-Wilson design) contains an imbedded full factorial design (2^k for k number of factors investigated) with the addition of a group of star ($2k$) and central points [25]. In the present study, a rotatable CCD (RCCD) was used. In this type of design the star points are equal to $\pm(2^k)^{1/4}$. The information is equally generated from all directions, i.e. the variance of the estimated responses is the same at all points on a sphere centered at the origin [15]. The selected CCD was circumscribed, meaning that the star points were chosen at the same distance from the center [25].

CCD can be applied to optimize an HPLC separation by gaining better understanding of factor's main and interaction effects [20,21]. The key factors examined in the optimization phase (selected previously from the screening stage) were: the % concentration of acetonitrile in the mobile phase (A), the column temperature (B) and the mobile phase flow rate (C).

The resolution of the worst separated peak Rs_2 (nimodipine and impurity A), the retention time of the last peak Rt_B (impurity B), the chromatographic optimization function (COF) and the capacity factor for the first eluted peak (k_1) were selected as responses. All experiments where conducted in randomized order. Table 4 summarizes the conducted experiments and responses.

The quadratic mathematical model for three independent factors is given in Eq. (3):

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 \quad (3)$$

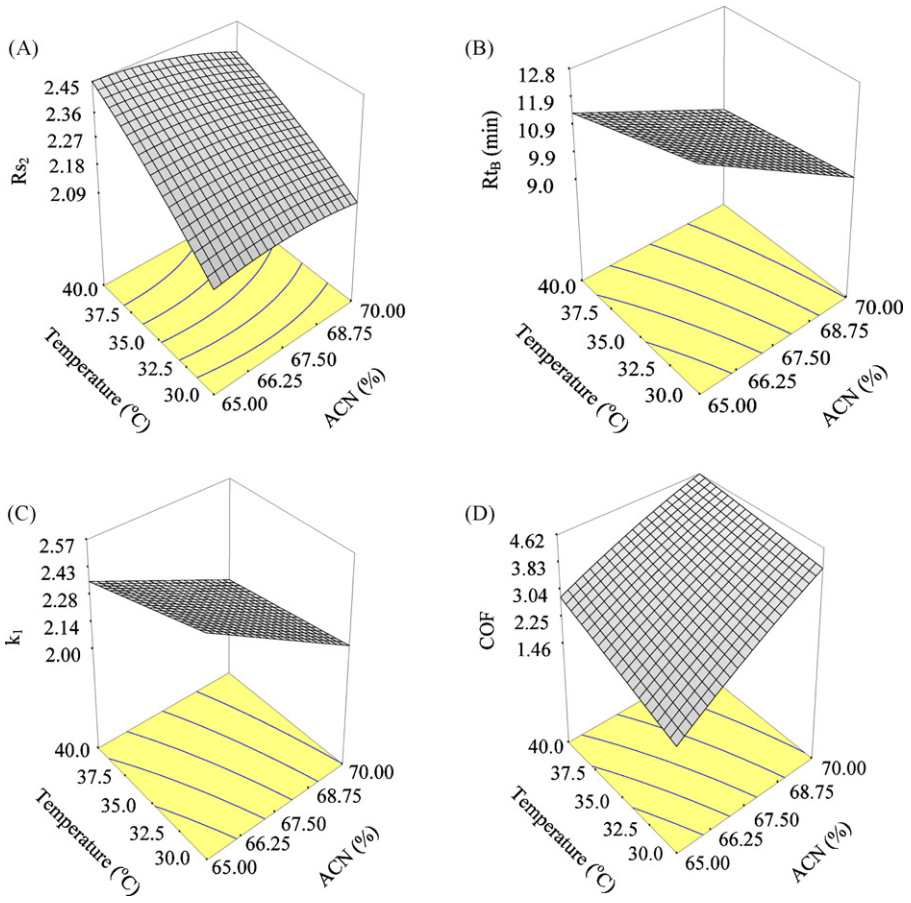

Fig. 3. Response surfaces related to acetonitrile concentration (%) and column temperature (°C): (A) resolution of the critical pair (Rs_2), (B) retention time of the last peak (Rt_B), (C) capacity factor of the first peak (k_1) and (D) chromatographic optimization function (COF). Flow rate was kept constant (0.9 ml/min).

where A, B and C are the factors examined, Y is the measured response, and β_i represent the regression coefficients.

Statistical parameters obtained from ANOVA are given in Table 5. The insignificant terms were eliminated from the model through backward elimination process. From Table 5 it was concluded that temperature (factor B) had the most important effect on the investigated resolution (Rs_2) whilst flow rate (factor C) mostly affected the capacity factor k_1. The concentration of the organic modifier (factor A) and flow rate (factor C) were the most significant factors affecting the rest responses (COF and Rt_B). Quadratic terms also created important effects (though less significant than the main effects). A^2 had significant effect on the investigated resolution Rs_2 and COF, whilst C^2 had significant influence on COF and k_1. Factor interactions (AB and AC) had also significant effect on the measured responses (Table 5).

The adequate precision (depicts the value of signal to noise ratio; ratio grater than 4 is preferred), the coefficient of variation (CV) (measures the reproducibility of the model; a value less than 10% is desirable) and the p-value of the models ($p < 0.05$ is needed), were all in the desirable limits (Table 6) [22]. The derived regression models are also shown in Table 6. A positive sign in the models showed a synergistic effect, whilst a negative sign indicated antagonistic effect.

In Fig. 2 perturbation plots are presented in order to gain a better understanding of the investigated procedure. This type of plots show the effect of an independent factor on a specific response, with all other factors held constant at a reference point [22]. A steepest slope (or curvature) indicates sensitiveness to a specific factor. Fig. 2(a) shows that temperature (factor B) had the most important effect on Rs_2. Increasing levels of B resulted to an increase in the investigated resolution. The rest of the examined factors (mobile phase concentration and flow rate) had significant effect on COF, Rt_B and k_1 (Fig. 2(b)–(d)). In Fig. 2(b) and (d), Rt_B and k_1 values decreased as the levels of factors A and C increased, whist in Fig. 2(c), the value of the chromatographic optimization function (COF) increased with increasing levels of A and C. Most of the examined perturbation plots showed small curvature indicating reduced quadratic significance.

Response surfaces and contour plots are shown in Figs. 3 and 4. Analytically, the interaction effects of column temperature and % acetonitrile concentration are illustrated in Fig. 3. Fig. 3(A) and (D) shows that Rs_2 and COF vary in a nearly linear descending pattern. Fig. 3(B) and (C) also exhibit a linear trend for Rt_B and k_1, but in an ascending order. Fig. 4 illustrates the response surfaces and contour plots for the interaction effect of mobile phase concentration and mobile phase flow rate. Rt_B (Fig. 4(A)) and k_1 (Fig. 4(B)) values were increasing as both examined factors decreased. Fig. 4(C) shows that COF reached its maximum value (COF = 5.11) when both % acetonitrile concentration and mobile phase flow rate were at their highest levels.

The fact that most of the examined response surfaces (Figs. 3 and 4) formed hillsides with small curvatures indicated that all factors (% acetonitrile concentration, column temperature and mobile phase flow rate) contributed mostly independently towards the separation of the compounds.

Before continuing, it was important to identify the criteria of optimization. In the present study these criteria were: (1) maximum resolution between the peaks, (2) increased peak quality (that is good capacity factor, good asymmetry factor, etc.) and (3) reduced elution time. Based on the analysis plots in Figs. 3 and 4, it was con-

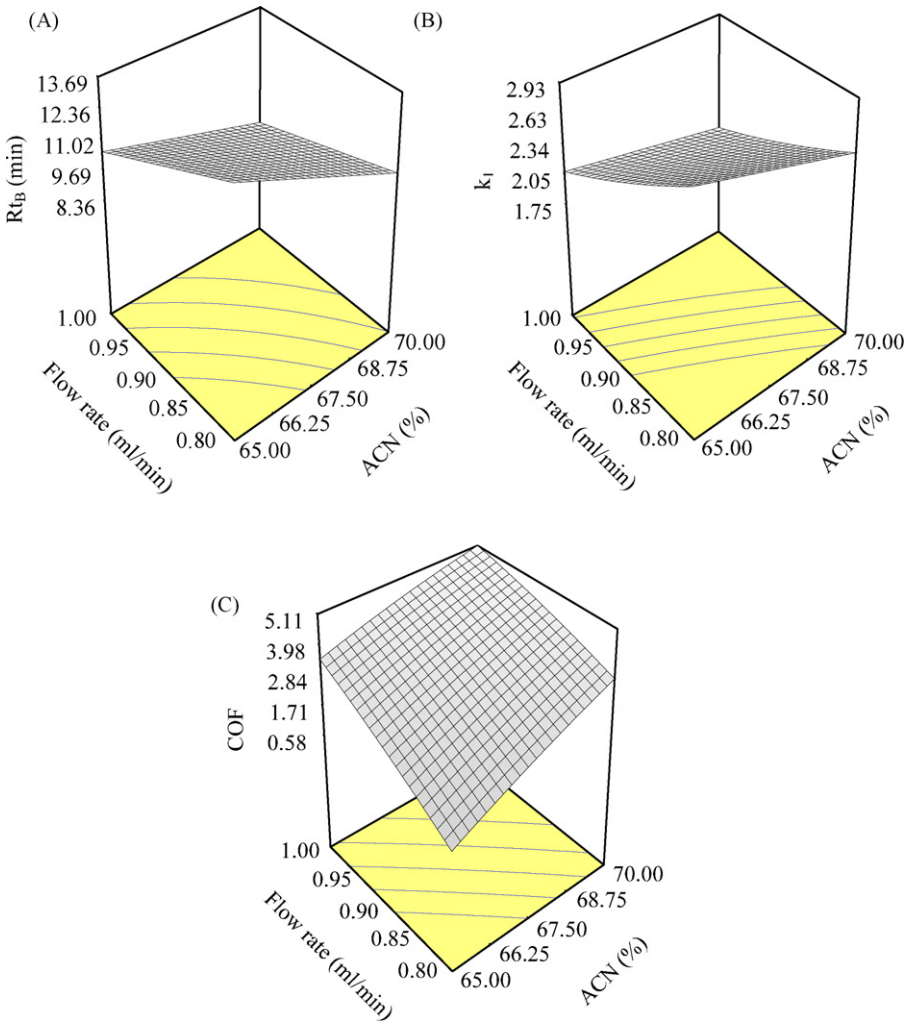

Fig. 4. Response surface related to acetonitrile concentration (%) and mobile phase flow rate: (A) retention time of the last peak (Rt_B), (B) capacity factor of the first peak (k_1) and (C) chromatographic optimization function (COF). Temperature was kept constant (35 °C).

cluded that, in order to reach the optimum conditions of separation, there has to be a compromise between the individual optimums of each response separately. Two alternative methods were used for global optimization: a graphical and a mathematical.

The first method was based on contour overlay plots (Fig. 5). These plots depict the alteration of all selected responses against two independent factors, whilst keeping the rest at a constant level. The optimum experimental conditions were defined by area Ω in Fig. 5. In this area the examined substances were separated in less than 11 min giving the maximum possible resolution for the critical pair of separation (Rs_2) whilst keeping the capacity factor for the first peak (k_1) above the critical value of 2. The optimum conditions identified with the aid of overlay contour plots were in the following area: acetonitrile concentration from 66.50% to 68.75%, column temperature from 38 °C to 40 °C.

The optimum conditions of separation were also estimated by Derringer's desirability function [22]:

$$D = [{d_1}^{p1} \times {d_2}^{p2} \times \cdots \times {d_n}^{pn}]^{1/n} \quad (4)$$

where p_i is the weight of the response, n the number of responses and d_i the individual desirability function of each response. In the present study all p_i values were set equal to 1. Derringer's desirability function (D) can take values from 0 to 1. A value close to unity indicates that the combination of the different criteria is matched in a global optimum [22]. The response surface obtained for the global

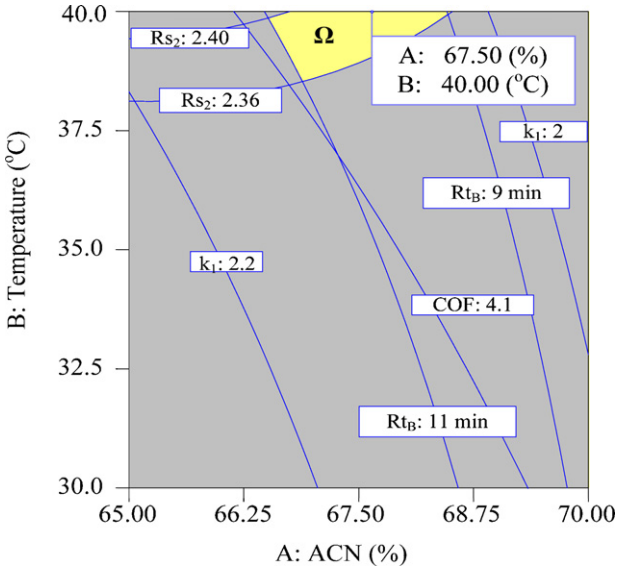

Fig. 5. Overlay contour plot of the investigated responses for % acetonitrile concentration (A) and column temperature (B). Mobile phase flow rate was kept constant (0.9 ml/min).

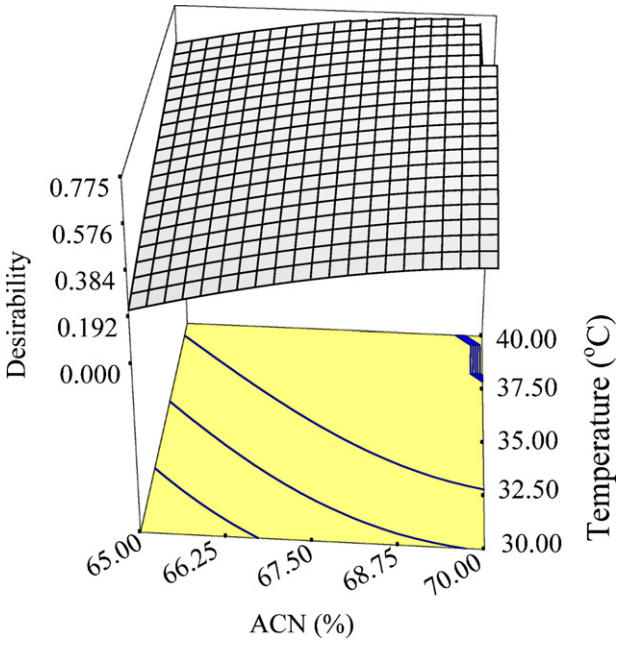

Fig. 6. Response surface obtained for desirability function. Flow rate of the mobile phase was 0.9 ml/min.

desirability function is presented in Fig. 6. Desirability increased when both column temperature and % acetonitrile concentration increased. From the figure it can be concluded that there was a set of coordinates producing high desirability values (from 0.754 to 0.771) allocated in a sub-sector of area Ω in Fig. 5 (determined by overlay plot methodology). Acetonitrile optimum concentration was from 67.00% to 69.70% and column temperature from 39 °C to 40 °C.

Therefore, the following conditions can be identified as optimal: acetonitrile/water 67.5/32.5 (v/v), column temperature 40 °C, and flow rate of the mobile phase 0.9 ml/min. The chromatogram obtained from the above conditions is shown in Fig. 7. Table 7 shows the chromatographic characteristics for nimodipine and impurities in the selected optimum conditions.

In order to investigate the predictability of the proposed model, the agreement between the predicted and the actual (observed) responses was examined. The percentage prediction error (P.E.) was calculated by Eq. (5):

$$\text{P.E.} = \frac{\text{Observed} - \text{Predicted}}{\text{Predicted}} \times 100 \quad (5)$$

The percentage prediction error (P.E.) was calculated equal to −0.882, −0.789, 0.758 and −1.505 for Rs_2, Rt_B, COF and k_1, respectively, indicating good correlation between the observed and the predicted responses.

3.3. Assay method validation

The last step of the present study was to check method's validation for specificity, linearity, accuracy, intra/inter-day precision, robustness, intermediate precision and stability in laboratory conditions.

The optimized HPLC method was specific in relation to the placebo used in this study. All placebo chromatograms showed no interference peaks.

The linearity of the proposed method was estimated by regression analysis at seven concentration levels in the range of 7.5–37.5 μg/ml for nimodipine and 18.75–300 ng/ml for impurities. The slope and intercept of calibration curves are shown in Table 8. The correlation coefficients (R^2) varied from 0.9990 to 0.9994. The LOD and LOQ were estimated at 0.09 and 0.28 μg/ml for Nim, 3.5 and 12 ng/ml for Imp C, 5.5 and 17 ng/ml for Imp A, and 4.8 and 15 ng/ml for Imp B, respectively.

Accuracy and intra/inter-day precision results are shown in Table 9. For each concentration level and day of experiment, six samples were prepared ($n = 6$). The results showed good accuracy and intra/inter-day precision. Accuracy (%) was within acceptable ranges (100 ± 2%).

Robustness was tested using experimental design methodology. Statistical experimental design methodology has proved to be a useful tool for robustness tests, as it simplifies the investigation of simultaneously changing factors parameters [27]. When a factor is not robust, one can decide whether to change the proposed method or to control the factor in question [27,28]. It is important to remember that, in robustness testing, factor's interactions (an interaction between two factors occurs when the effect of one factor depends on the level of the other) are usually considered negligible [29]. For robustness a two-level fractional (one-half 1/2 fraction) factorial design was used in order to identify possible significant effects from the following factors: % acetonitrile concentration (A), column temperature (B), mobile phase flow rate (C), and wavelength of detection (D). The method settings and the range investigated in robustness are shown in Table 10. Peak areas of nimodipine and impurities were selected as responses. A linear relationship (Eq. (6)) with no interaction effects was selected as a proper mathematical model, since interaction and quadratic effects were excluded:

$$y = b_0 + b_1A + b_2B + b_3C + b_4D \quad (6)$$

where y is the response measured (peak area), A, B, C and D are the factors investigated, and b_0, b_1, b_2, b_3 and b_4 are weight coefficients. The results are shown in Table 10. All measurements were conducted in triplicate. The effects of the examined factors were estimated by ANOVA. The results are represented in Table 11(I). Significant effects had p-value < 0.05. From the results it was concluded that in all cases (nimodipine and impurities) the only significant factor affecting robustness was mobile phase flow rate. Thus, the influence of mobile phase flow rate was examined separately at

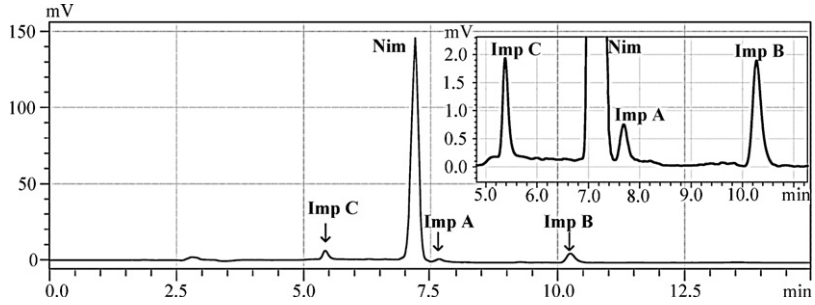

Fig. 7. Chromatogram of the optimum conditions for the separation of nimodipine (Nim) and its impurities: impurity A (Imp A), impurity B (Imp B) and impurity C (Imp C). Nimodipine concentration: 25 μg/ml. Impurities concentration: 300 ng/ml.

Table 7
Chromatographic characteristics for the analysis of nimodipine and impurities in the selected conditions (ACN/H_2O: 67.5/32.5 (v/v), column temperature 40 °C, flow rate 0.9 ml/min).

	Retention time (min)	Peak area	Peak resolution (*Rs*)	Theoretical plates	Capacity factor (*k*)
Imp C	5.374	14,190	–	10,453	2.212
Nim	7.098	1,328,014	7.716	13,150	3.242
Imp A	7.637	2,554	2.421	26,389	3.564
Imp B	10.270	24,257	8.999	15,861	5.138

Table 8
Linearity and sensitivity data of nimodipine and impurities.

	Slope ± SD	Intercept ± SD	R^2	LOD[a]	LOQ[b]	Range
Nim	$3.9 \times 10^4 \pm 52.52$	$-2674.2 \pm 1.1 \times 10^3$	0.9994	0.09 μg/ml	0.28 μg/ml	7.5–37.5 μg/ml
Imp C	42.1 ± 0.23	−24.08 ± 0.28	0.9993	3.5 ng/ml	12 ng/ml	18.75–300 ng/ml
Imp A	9.01 ± 0.05	6.66 ± 0.183	0.9990	5.5 ng/ml	17 ng/ml	18.75–300 ng/ml
Imp B	75.95 ± 1.69	−140.5 ± 0.019	0.9993	4.8 ng/ml	15 ng/ml	18.75–300 ng/ml

[a] LOD: limit of detection.
[b] LOQ: limit of quantification.

Table 9
Accuracy and intra/inter-day precision for nimodipine (Nim) and impurities (Imp) A, B and C.

	Nominal conc.	Inter-day			Intra-day		
		Found Conc. ± SD	RSD	Accuracy (%)	Found Conc. ± SD	RSD	Accuracy (%)
Nim (μg/ml)	7.5	7.5 ± 0.01	0.13	100.1	7.6 ± 0.14	1.87	101.7
	22.5	22.2 ± 0.06	0.27	98.6	22.4 ± 0.21	0.95	99.6
	30	30.1 ± 0.04	0.13	100.3	30.4 ± 0.56	1.86	101.5
Imp C (ng/ml)	18.75	19.1 ± 0.15	0.79	101.8	19.2 ± 0.38	1.99	102.3
	75	76.2 ± 1.01	1.33	101.6	76.5 ± 1.42	1.86	102.0
	300	300.8 ± 0.46	0.15	100.3	294.7 ± 1.03	0.34	98.3
Imp A (ng/ml)	18.75	19.0 ± 0.36	1.89	101.3	19.0 ± 0.37	1.95	101.3
	75	73.6 ± 1.34	1.83	98.1	73.3 ± 1.34	1.83	97.7
	300	305.2 ± 0.31	0.10	101.7	306.3 ± 2.84	0.92	102.1
Imp B (ng/ml)	18.75	18.4 ± 0.17	0.92	98.4	18.5 ± 0.36	1.95	99.0
	75	76.5 ± 0.95	1.24	101.6	76.5 ± 1.44	1.88	102.0
	300	299.8 ± 0.64	0.21	99.9	300.3 ± 0.66	0.22	100.1

a different (narrower) range (0.8–1.0 ml/min). Changes in the peak areas, retention times and resolutions were within acceptable limits (less than 4% [22]).

The objective of intermediate precision validation is to verify that in the same laboratory the method will provide the same results once the development phase is over. Intermediate precision is established when the proposed analysis is performed by multiple analysts, using multiple instruments, on multiple days in one laboratory [24]. Hence, it is important for the analyst to identify the effects of random changes/events on method precision [28–30]. In the present study, intermediate precision was studied using experimental design methodology. Three factors: (1) analyst (Z_1), (2) HPLC system (Z_2), and (3) day of analysis (Z_3) were studied, using a two-level full factorial design. The experimental domain is shown in Table 12. Peak areas of nimodipine and impurities were selected as responses. All measurements were conducted in a randomized order (and in triplicate). Table 12 shows the measured responses. Statistical analysis (ANOVA, Table 11) indicated method's intermediate precision (all examined factors were insignificant with p-value > 0.05).

Table 10
Method settings and responses (peak areas) measured in robustness test.

Factors examined	Optimum value	Range investigated
A: Mobile phase (ACN/H_2O)	67.5/32.5	62.5/37.5–72.5/27.5
B: Flow rate (ml/min)	0.9	0.7–1.1
C: Column temperature (°C)	40	35–45
D: Wavelength (nm)	236	234–238

Std	Run	Response (peak area) ± SD			
		Nimodipine	Impurity A	Impurity B	Impurity C
7	1	1,044,768 ± 25	4239 ± 29	17,486 ± 26	10,716 ± 81
1	2	1,653,300 ± 40	6687 ± 94	27,561 ± 3	17,423 ± 40
6	3	1,645,316 ± 41	6398 ± 79	29,118 ± 117	17,638 ± 57
4	4	1,037,510 ± 174	4341 ± 8	18,010 ± 120	11,058 ± 95
5	5	1,612,811 ± 45	6307 ± 12	29,501 ± 188	16,624 ± 69
3	6	1,021,973 ± 108	4355 ± 20	17,885 ± 11	9,712 ± 97
8	7	1,011,347 ± 82	4148 ± 48	17,896 ± 15	11,266 ± 110
2	8	1,589,235 ± 75	5735 ± 101	28,724 ± 25	17,139 ± 139

Table 11
ANOVA results for robustness (I); examined factors were: % ACN (A), mobile phase flow rate (B), column temperature (C), wavelength of detection (D); and intermediate precision (II); examined factors were: Analyst (Z_1), HPLC system (Z_2), and day of analysis (Z_3). Interaction effects were excluded in both cases. A 5% significance level was desired.

Nimodipine (factors)	Peak area		Imp A (factors)	Peak area		Imp B (factors)	Peak area		Imp C (factors)	Peak area	
	F	p		F	p		F	p		F	p
(I)											
A	2.607	0.2048	A	1.509	0.3069	A	0.532	0.5186	A	5.307	0.1046
B	6065.7	<0.0001	B	104.6	0.0020	B	585.3	0.0002	B	523.2	0.0002
C	0.159	0.7165	C	0.001	0.9757	C	1.019	0.3870	C	0.640	0.4821
D	22.583	0.0177	D	2.028	0.2496	D	1.031	0.3847	D	3.375	0.1635
(II)											
Z_1	0.593548	0.4840	Z_1	3.604288	0.1305	Z_1	2.951027	0.1610	Z_1	0.319735	0.6020
Z_2	1.517706	0.2854	Z_2	3.947368	0.1179	Z_2	1.478035	0.2909	Z_2	7.042673	0.0568
Z_3	0.650431	0.4652	Z_3	0.329435	0.5967	Z_3	0.040091	0.8511	Z_3	0.182064	0.6916

Table 12
Experiments conducted and responses (peak areas) measured for intermediate precision test.

Std	Run	Examined factors			Responses (peak area) ± SD			
		Analyst (Z_1)	HPLC (Z_2)	Day (Z_3)	Nimodipine	Impurity A	Impurity B	Impurity C
8	1	Analyst 2	HPLC 2	Day 2	1,186,239 ± 125	2878 ± 24	22,395 ± 123	13,211 ± 78
3	2	Analyst 1	HPLC 2	Day 1	1,233,933 ± 107	2899 ± 22	22,292 ± 107	13,226 ± 66
6	3	Analyst 2	HPLC 1	Day 2	1,166,967 ± 145	2877 ± 37	21,445 ± 89	12,941 ± 97
1	4	Analyst 1	HPLC 1	Day 1	1,166,748 ± 58	2871 ± 11	22,181 ± 76	12,946 ± 117
5	5	Analyst 1	HPLC 1	Day 2	1,191,448 ± 111	2878 ± 70	21,467 ± 57	12,946 ± 132
4	6	Analyst 2	HPLC 2	Day 1	1,185,852 ± 67	2872 ± 38	21,535 ± 135	12,982 ± 109
2	7	Analyst 2	HPLC 1	Day 1	1,195,546 ± 134	2869 ± 25	21,492 ± 91	12,874 ± 91
7	8	Analyst 1	HPLC 2	Day 2	1,188,864 ± 125	2891 ± 23	22,247 ± 116	13,053 ± 80

Table 13
Assay results obtained for three commercially available tablets of nimodipine (30 mg).

Product	Nominal conc. (μg/ml)	Obtained conc. (μg/ml)	% recovery	%RSD
Nimovac-C®	30	29.03 ± 0.47	96.76	1.616
Nimotop®	30	30.53 ± 0.52	101.77	1.708
Notrolan®	30	29.53 ± 0.43	98.42	1.457

Stability studies, conducted for nimodipine/methanol solution (20 μg/ml) in laboratory light and 25 °C revealed no instability problems (data not shown).

Finally, three commercial nimodipine tablet products were assayed by the proposed HPLC method. Nimodipine concentration was within the acceptable limits for all tested products (Table 13). Recovery ranged from 96.76% to 101.77%, whilst the %RSD was from 1.457% to 1.708%. The examined products did not show any traceable amounts of impurities.

4. Conclusion

An efficient isocratic reversed-phase high-performance liquid chromatography method was developed, optimized and validated to separate the calcium antagonist nimodipine and its impurities (A, B and C) using statistical experimental design. Time of analysis, chromatographic optimization function, resolution and quality of the peaks were simultaneously optimized using response surface methodology. The method was found to be specific, linear, sensitive, precise and accurate. The results showed good intermediate precision. Mobile phase flow rate appeared to have significant effect on robustness, and hence it was important to be carefully controlled.

It is concluded that the use of experimental design and response surface methodology is a flexible procedure, able to reduce the number of the needed experiments for the development, optimization and validation of an isocratic RP-HPLC separation of nimodipine and impurities.

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