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Optimization of a LC method for the enantioseparation of a non-competitive glutamate receptor antagonist, by experimental design methodology ☆

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Abstract

The aim of this work was to obtain the direct optical resolution of a new glutamate receptor antagonist ((*p*-chloro)1-aryl-6,7,-dimethoxy-1,2,3,4-tetrahydroisoquinoline, PS3), by liquid chromatography on Chiralcel® OD column. A response surface methodology (RSM) was employed to optimize the enantiomeric separation of the racemate with the lowest number of experiments; in particular, a face-centred design (FCD) was applied to evaluate the influence of critical parameters on the experimental response. Furthermore, in order to find the best compromise between several responses, a multicriteria decision-making approach, the Derringer's desirability function, was successful to simultaneously optimize the responses resolution and migration times of the two enantiomers.

The proposed LC method provided the baseline enantioseparation of the investigated drug. 9.3% (v/v) ethanol added to *n*-hexane as mobile phase, 1.0 mL min^{-1} flow rate, and 18 °C column temperature were the optimum experimental conditions allowing to achieve the highest enantioresolution of PS3 in less than 17 min.

Keywords: Chiral liquid chromatography; Enantioresolution; Chiralcel® OD; Tetrahydroisoquinoline derivative; Face-centred design; Desirability function

1. Introduction

Enantioselective separation has become one of the most important analytical tasks over the last decades, stimulated by the increasing demand for the production of enantiomerically pure compounds in such fields as pharmacology, chemistry, biotechnology, chemical engineering, etc. Nowadays, enantioselective analytical methods are frequently requested to meet regulatory guidelines for the development and manufacture of chiral drugs [1–3].

Many book chapters and review articles dealt with the separations of drug enantiomers using LC techniques [4], which are widely employed, both for analytical and preparative purposes. Direct methods using chiral mobile phase additives with an achiral stationary phase have limited applicability in the biomedical and pharmaceutical areas, because of purity of chiral selectors and detection problems. On the other hand, the use of chiral stationary phases (CSPs) is suitable for the analysis of enantiomers in a standard sample and pharmaceutical preparations, where the low amount of the antipode level should be determined [5].

Polysaccharide derivatives belong to the most widely used CSPs for LC enantioseparations, and can be successfully employed both on analytical and preparative scale enantioseparations. On the analytical scale, polysaccharide-type CSPs have been successfully used also in miniaturized techniques, such as capillary liquid chromatography and capillary electrochromatography. However, the complexity of column selection and parameters optimization, coupled with long analysis times, pose tremendous challenges to scientists responsible for chiral method development [6].

In a previous work, we had exploited the performance of two cellulose-based CSPs, Chiralcel® OD and OJ columns,

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in the enantioseparation of a new class of compounds of pharmaceutical interest, *N*-acetyl-1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline derivatives, recently synthesized in our laboratories [7]. A selective and non-competitive blockade of AMPA-type glutamate receptor was shown by these heterocyclic compounds, exhibiting pharmacological effects against acute and chronic neurodegenerative disease, with favourable lack of sedative–hypnotic activity with respect to classical 1,4-benzodiazepines [8]. The anticonvulsant activity of competitive 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo (F)-quinoxaline (NBQX) and non-competitive 2,3-benzodiazepines and tetrahydroisoquinolines (THIQs) AMPA/kainate receptor antagonists, was tested in different experimental seizure models and compared with diazepam, a conventional antiepileptic drug acting on GABAergic neurotransmission. In particular, the compounds were evaluated against audiogenic and maximal electroshock seizures (MES) test and pentetrazol (PTZ) seizures model, and all of them showed protective action [9]. These findings were in agreement with the results of previous molecular modelling studies, suggesting that tetrahydroisoquinolines might satisfy the structural requirements for AMPAR binding [10]. A sensitive and specific HPLC method for the determination of the studied compounds in rat plasma was afterwards developed, and successfully employed for the evaluation of their pharmacokinetic profile [11]. These molecules show a chiral centre at C-1, thus existing as racemic mixtures; since the single enantiomers are likely to have a different degree of specificity of action, direct optical resolution was required for further pharmacological evaluation.

A large number of experimental parameters proved to influence the separation selectivity and performance, and the best results were generally obtained with the use of OD column. However, further effort was needed to obtain baseline-separation for the non-acetylated, *p*-chlorinated derivative PS3, which had given very good results in pharmacological testing.

Generally less time-consuming than univariate procedure, design of experiments (DOE) is becoming an essential tool for the development and optimization of analytical methods, systematically applying statistics to experimentation. By performing a planned sequence of experiments, called a design, the effects of factors and their interactions on response variations can be established. The experimental design strategy can be chosen according to the particular objectives of the case studied, and it may be successfully employed in order to find the best experimental conditions for achieving the maximum of analytes resolution [12–14].

The aim of this study was to assess adequate experimental conditions in which chiral resolution of the tetrahydroisoquinoline derivative (PS3, structure in Fig. 1) could be achieved, in the shortest analysis time. Response surface methodology (RSM) seemed to be the most suitable experimental design strategy to optimize the enantiomeric separation of PS3 with the lowest number of experiments, by taking into account those parameters (amount of the organic modifier, flow rate, and column temperature) which more influenced the chromatographic separation. A cellulose-based column (Chiralcel® OD) was employed as the chiral selector, operated in the normal phase mode. A central composite design (CCD) was applied to determine the optimal conditions for the chiral separation. Furthermore, in order to find the best compromise between several responses, a multicriteria decision-making approach had to be used. The desirability function approach, as proposed by Derringer and Suich [15], was here employed to simultaneously optimize the responses resolution and migration times of the two enantiomers.

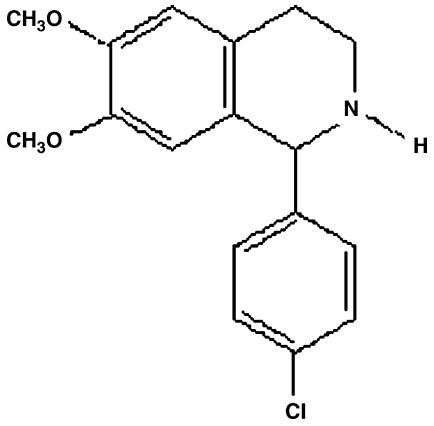

Fig. 1. Chemical structure of the investigated compound ((*p*-chloro)1-aryl-6,7,-dimethoxy-1,2,3,4-tetrahydroisoquinoline, PS3).

2. Experimental

2.1. Chemicals

HPLC grade *n*-hexane, isopropyl alcohol, and ethyl alcohol were used, from Merck (Germany). Solutions of the analyte (0.5 mg mL^{-1}) in ethyl alcohol were filtered trough the 0.45 μm membrane, stored at 4 °C, and replaced weekly. Solutions of the analytes were filtered prior to injection trough Sartorius Minisart®-SRP 15 PTFE 0.45 μm filters (Germany), using a 1 mL glass syringe (Poulten & Graf GmbH, Germany).

2.2. Apparatus and procedure

A Shimadzu® LC-10 AD *VP* solvent delivery module was employed for the analyses, connected to a SPD-M10A *VP* UV–vis photodiode array detector. A Rheodyne® 8125 injector with a 5 μL loop was used. The analytical column employed was: 5 μm, 25 cm × 4.6 mm i.d. stainless steel, packed with cellulose tris-(3,5-dimethyl-phenylcarbamate) absorbed on microporous silica gel (Chiralcel® OD, Daicel Chemical Industries, J.T. Baker, The Netherlands). Column was thermostated with a Merck Hitachi L-7003 column oven. Integration of the data was done by EZSTART v7.2 SP1 Chromatography Software by Shimadzu®. The detector was operated at 284 nm. Experimental design and statistical analysis were performed by Nemrod® software.

3. Results and discussion

On the basis of a previous study, our interest was here devoted to obtain the direct optical resolution of (*p*-chloro)1-aryl-6,7,-

dimethoxy-1,2,3,4-tetrahydroisoquinoline (PS3), using tris-(3,5-dimethyl-phenylcarbamate)-cellulose column (Chiralcel® OD) as a chiral selector. A large number of experimental parameters proved to influence the separation selectivity and performance; among these, the amount of organic modifier added to *n*-hexane in the eluent played a crucial role. Organic modifiers can in fact compete with the enantiomers for bonding at the chiral site of CSP; moreover, it can interact with a chiral site located nearby the chiral groove, thus producing a steric perturbation. A preliminary study was therefore carried out, to ascertain the influence of ethyl- and isopropyl alcohol on retention time, stereoselectivity and resolution factors obtained for the investigated compound; the former was chosen for the study. A response surface methodology was successively applied with the aim to achieve the highest resolution of the analyte, in the shortest analysis time.

3.1. Response surface methodology (RSM)

Response surface methodology permits to define empirical models (usually quadratic polynomials) that describe accurately how responses behave at all values of the studied variables in the experimental region [16]. The aim of RSM is to determine conditions that provide process improvement. In order to calculate quadratic regression model coefficients, each design variable has to be studied at three distinct levels at least and, consequently, the central composite design (CCD) is often used to provide estimation of a second-order equation. Among the standard designs applied in RSM, CCD represents a good choice because of its high efficiency with respect to the number of runs required. A CCD for k factors consists of 2^k factorial points, $2k$ axial or 'star' points and $n_0 \geq 2$ center points. The axial points are located at a distance, α, from the design center with a choice of $\alpha = \sqrt[4]{N_F}$, where N_F represents the number of factorial runs [17]. In order to study the variables at no more than three levels (−1; 0; +1), a face-centred design (FCD) was chosen for this study, with $\alpha = \pm 1$ [18].

Table 1
Experimental domain for the selected variables

Factors	Centre	Variation step
U_1: alcohol (%)	5.5	4.5
U_2: temperature (°C)	20.0	5.0
U_3: flow rate (mL min^{-1})	0.9	0.1

In the case of PS3 chromatographic enantioresolution, three key factors were selected in the optimization process: amount of the organic modifier, ethanol (U_1), flow rate (U_2), and temperature (U_3). The centre value and the variation step taken for each variable defined the cubical experimental domain, as reported in Table 1.

Three experimental responses were studied: Y_1 = enantioselectivity (α), Y_2 = resolution (Rs), and Y_3 = total analysis time. Since retention times of the first- and second-eluting enantiomers behaved identically, the latter was chosen, representing total time of the analyses. A three-factor FCD required 17 experiments, expressed in coded variables X_i, while the corresponding experimental plan gives the runs expressed in real variables U_i, as shown in Table 2. Three replicates were made at the centre point, to estimate the experimental error variance, and all experiments were performed in randomized order to minimize the effects of uncontrolled factors that may introduce a *bias* on the measurements.

A classical second-degree model was postulated for each experimental response Y_i, as follows:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \varepsilon_i,$$

Table 2
FCD: experimental plan and responses

No.	U_1	U_2	U_3	Enantioselectivity (α) Y_1	Resolution (Rs) Y_2	Retention time (Rt) Y_3
1	1.0	15.0	0.8	1.04	0.44	92.91
2	10.0	15.0	0.8	1.19	1.73	19.85
3	1.0	25.0	0.8	1.04	0.41	82.88
4	10.0	25.0	0.8	1.13	1.15	16.97
5	1.0	15.0	1.0	1.05	0.44	76.34
6	10.0	15.0	1.0	1.19	1.54	15.80
7	1.0	25.0	1.0	1.00	0.05	70.00
8	10.0	25.0	1.0	1.13	1.12	13.66
9	1.0	20.0	0.9	1.03	0.31	79.15
10	10.0	20.0	0.9	1.17	1.46	16.40
11	5.5	15.0	0.9	1.22	1.87	26.97
12	5.5	25.0	0.9	1.12	1.03	21.96
13	5.5	20.0	0.8	1.19	1.80	27.70
14	5.5	20.0	1.0	1.18	1.66	22.17
15[a]	5.5	20.0	0.9	1.18	1.75	24.35
				1.20	1.80	24.60
				1.16	1.70	23.90

The relation $U_i = U_i^0 + X_i \Delta U_i$ allows to switch from coded variables to real variables. U_i^0, value of the real variable, i, at the centre of the experimental domain; ΔU_i, variation step of the real variable, i, for a unit variation of the coded variable X_i.

[a] Experiment 15 was made in triplicate to estimate the experimental error variance.

where X_1, X_2, and X_3 are the coded variables, β_i represents the model coefficient, and ε_i is the experimental error.

All experimental results were computed by Nemrod® software [19]. The coefficients of the second-order polynomial model were estimated by the least squares regression. The equation models for Y_1, Y_2, and Y_3 were as follows:

$$Y_1 = 1.180 + 0.065X_1 - 0.027X_2 - 0.004X_3 - 0.080X_1^2 - 0.010X_2^2 + 0.005X_3^2 - 0.009X_1X_2 + 0.004X_1X_3 - 0.006X_2X_3;$$

$$Y_2 = 1.684 + 0.535X_1 - 0.226X_2 - 0.072X_3 - 0.749X_1^2 - 0.184X_2^2 + 0.096X_3^2 - 0.073X_1X_2 + 0.017X_1X_3 - 0.025X_2X_3;$$

$$Y_3 = 24.296 - 31.860X_1 - 2.640X_2 - 4.234X_3 + 23.469X_1^2 + 0.159X_2^2 + 0.629X_3^2 + 1.419X_1X_2 + 2.761X_1X_3 + 0.554X_2X_3.$$

Classical statistical tools, as analysis of variance (ANOVA) and residual analysis, were employed to validate each model [20,21]. The statistical analysis showed that the models represented the phenomenon quite well and fitted accurately to the experimental data.

Following validation of the model, graphs of surface responses could be drawn, by plotting the response variation against two of the factors, while the third was held constant at a specified level, namely the centre value.

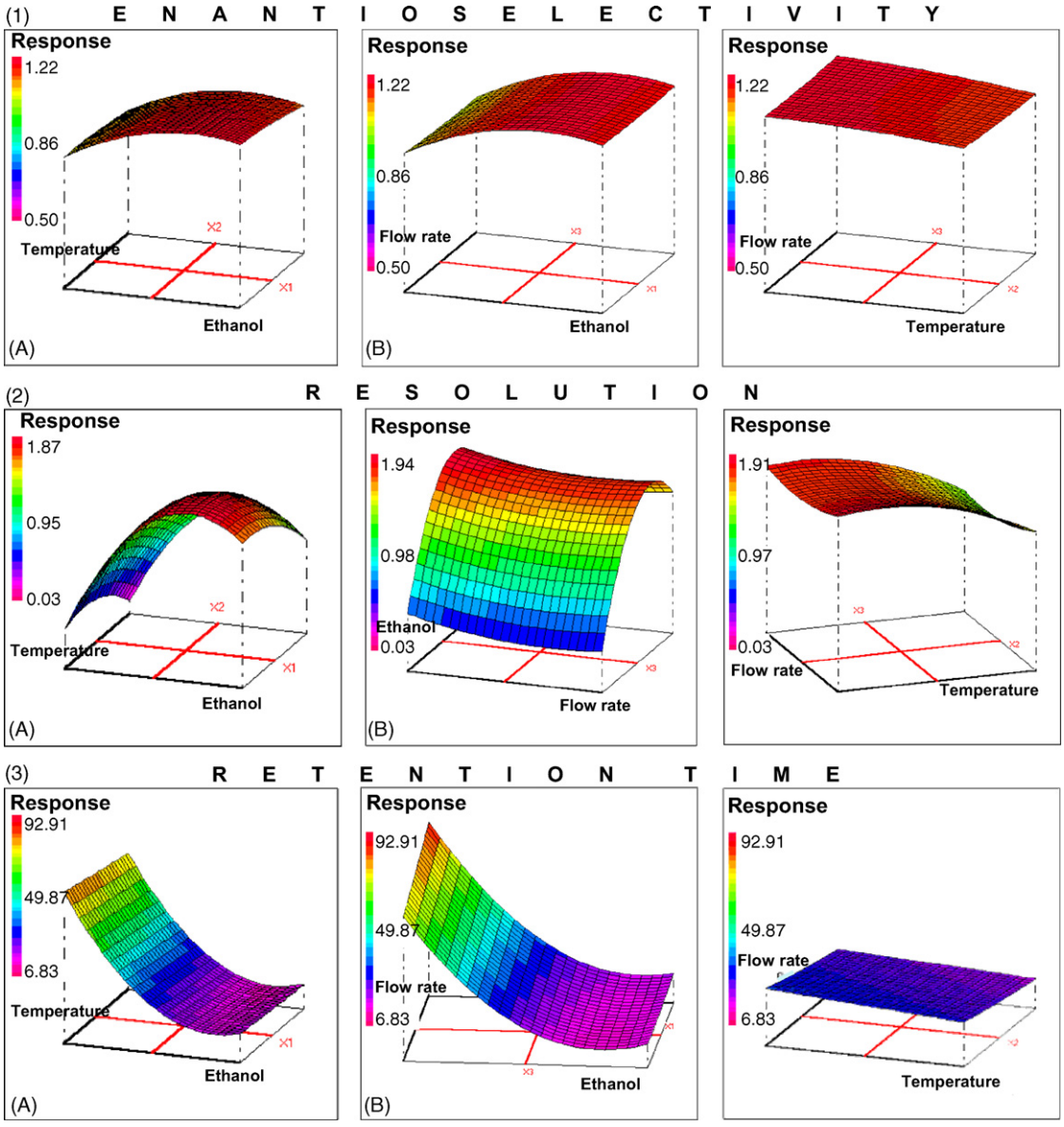

Fig. 2. Upper row: three-dimensional plot of α (Y_1). 1-A: response plot of %alcohol (X_1) vs. temperature (X_2); 1-B: response plot of %alcohol (X_1) vs. flow rate (X_3); 1-C: response plot of temperature (X_2) vs. flow rate (X_3). Middle row: three-dimensional plot of Rs (Y_2). 2-A: response plot of %alcohol (X_1) vs. temperature (X_2); 2-B: response plot of %alcohol (X_1) vs. flow rate (X_3); 2-C: response plot of temperature (X_2) vs. flow rate (X_3). Lower row: three-dimensional plot of Rt (Y_3). 3-A: response plot of %alcohol (X_1) vs. temperature (X_2); 3-B: response plot of %alcohol (X_1) vs. flow rate (X_3); 3-C: response plot of temperature (X_2) vs. flow rate (X_3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

The response surfaces for α (Y_1), Rs (Y_2), and Rt (Y_3) are reported in Fig. 2. The areas of interest for minimization of the retention time and maximization of enantioselectivity and resolution were examined. As it can be seen from the plots, an increase in the alcohol percentage (U_1) results in an increase of α (Y_1), while temperature (U_2) and flow rate (U_3) have no important effect in the studied domain on the considered response. As regards resolution, a similar behaviour is observed: Rs (Y_2) is highest near the centre (around 20 °C, and 7–8% ethyl alcohol). For Rs to be >1, % ethanol (U_1) must be >6 and temperature (U_2), the lowest. On the contrary, flow rate (U_3) scarcely affects the response. As regards the analysis time, Rt (Y_3), Fig. 2 shows as flow rate (U_3) has to be considered at the upper levels for its minimization (around 1), while a temperature (U_2) variation, between 15 and 25 °C, has no significant effect on the studied response. Once again, increasing the amount of the organic modifier (U_1) has a positive effect on the response.

3.2. Derringer's desirability function

The desirability function approach to multiresponse optimization was a useful technique for analysing these experiments, in which several responses had to be optimized simultaneously. The measured properties of each response Y_i, $i = 1, 2, \ldots, m$, were transformed to a dimensionless desirability scale (d_i), defined as *partial desirability function*. This made possible to combine results obtained for properties measured on different scales. The scale of the desirability function ranged between $d = 0$, for a completely undesirable response, and $d = 1$, if the response was at the target value. Thus, the responses α, Rs, and Rt were transformed into an appropriate desirability scale d_1, d_2, and d_3, having regard that enantioselectivity and resolution had to be maximized (α: minimum, 1.15, optimum, 1.19; Rs: minimum, 1.5, optimum, 1.6), while the analysis time had to be minimized (Rt: maximum, 20 min, optimum, 13). Once the function d_i was defined for each of the m responses of interest, an overall objective function (D), representing the *global desirability function*, was calculated by determining the geometric mean of the individual desirabilities in the experimental domain, as follows: $D = \left(\prod_{i=1}^{m} d_i\right)^{1/m}$.

By taking into account all requirements for the m responses, we chosen the conditions on the design variables that maximize D. A value of D different from zero implies that all responses are in a desirable range simultaneously and consequently, for a value of D close to 1, the combination of the different criteria is globally optimal, so far as the response values are near target values. After calculation by Nemrod® software, an optimal separation with an amount of ethanol equal to 9.3% (*v/v*), temperature of 18.0 °C, and flow rate 1.0 mL min^{-1} was predicted using the desirability function. The optimal conditions were obtained with a global degree of satisfaction of D for the three responses equal to 83.05%, and were validated experimentally. The three-dimensional plot of D is presented in Fig. 3, where the rather flat area around the optimal conditions means the values of the three responses near this point are stable. This represents the robustness of the predicted optimal conditions. According to International Conference on Harmonization (ICH), robustness of a method is in fact evaluated by making little variations of the experimental parameters, around the optimum conditions. From the three-dimensional plot of D (desirability function) presented in Fig. 3, a rather flat area around the optimal conditions can be seen, meaning that the values of the three responses (enantioselectivity, resolution, and retention time) near this point are stable. This represents the robustness of the predicted optimal conditions, i.e. the experimental range of flow rate, temperature, and ethanol percentage values for which a high degree of satisfaction is obtained. Fig. 4 shows the chromatogram obtained for PS3 under the optimized conditions. It is clear that the experimental design strategy, chosen according to the particular objectives of the case, was successfully employed to find the best experimental conditions for the enantioresolution of the new glutamate receptor antagonist, with respect of a reduced number of experiments. By means of RSM and multiresponse optimization, the three considered responses (enantioselectivity, resolution, and migration time) were modelled in the experimental domain with a good fitness. In addition, the use of an appropriate chemometric methodology during optimization study gave an indication of the method robustness.

Being less time-consuming than the classic univariate procedure, design of experiments (DOE) is an essential tool for

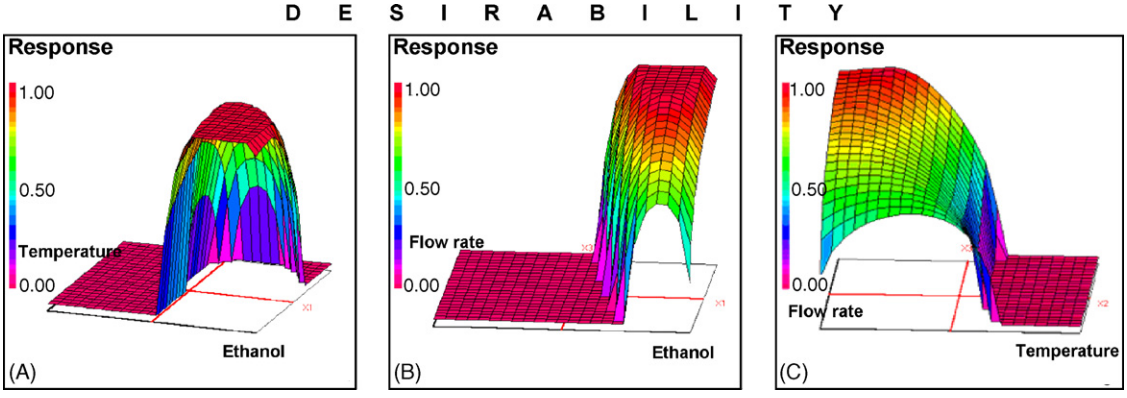

Fig. 3. Graphical representation of the overall desirability function D: degree of satisfaction vs. experimental conditions in the plane. A: temperature vs. flow rate; B: flow rate vs. %ethanol; C: flow rate vs. temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

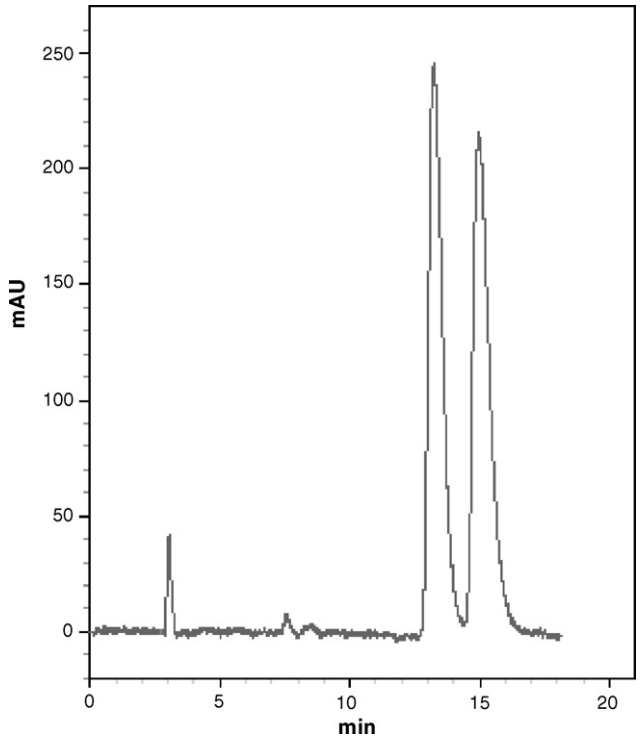

Fig. 4. Chromatographic enantioseparation of PS3 ((*p*-chloro)1-aryl-6,7,-dimethoxy-1,2,3,4-tetrahydroisoquinoline) enantiomers under the optimized conditions: 9.3% (v/v) ethanol in *n*-hexane, 18.0 °C column temperature, and 1.0 mL min^{-1} flow rate. UV detection at 284 nm. 5 μm, 25 cm × 4.6 mm i.d. Chiralcel® OD column.

the development and optimization of analytical methods, and can be successfully realized systematically applying statistics to experimentation. Also, an appropriate use of DOE ensures that the effects of factors and their interactions on response variations can be established, thus providing experimental data at high quality information level.

Acknowledgments

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