Short communication

Robust Optimization of Psychotropic Drug Mixture Separation in Hydrophilic Interaction Liquid Chromatography

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Received: 05-09-2012

Abstract

This paper presents multiobjective optimization of complex mixtures separation in hydrophilic interaction liquid chromatography (HILIC). The selected model mixture consisted of five psychotropic drugs: clozapine, thioridazine, sulpiride, pheniramine and lamotrigine. Three factors related to the mobile phase composition (acetonitrile content, pH of the water phase and concentration of ammonium acetate) were optimized in order to achieve the following goals: maximal separation quality, minimal total analysis duration and robustness of an optimum. The consideration of robustness in early phases of the method development provides reliable methods with low risk for failure in validation phase. The simultaneous optimization of all goals was achieved by multiple threshold approach combined with grid point search. The identified optimal separation conditions (acetonitrile content 83%, pH of the water phase 3.5 and ammonium acetate content in water phase 14 mM) were experimentally verified.

Keywords: Hydrophilic interaction liquid chromatography, multiple threshold optimization, robustness

1. Introduction

Hydrophilic interaction chromatography (HILIC) is an alternative approach to efficiently separate small polar compounds.[1] HILIC separations are carried out on polar stationary phases, such as bare silica while the mobile phase contains a mixture of a certain amount of water (typically at least 2.5 vol %) and a less polar solvent (typically > 70% acetonitrile) where water is the strongest solvent.[2–4]

The retention mechanism in HILIC involves partitioning between the organic part of the mobile phase and the water–enriched liquid layer immobilized on the polar stationary phase. However, ionic interactions, hydrogen bonding, dipole–dipole interactions and hydrophobic interactions also significantly contribute to the retention of the analyte.[1,2] Method development in this type of chromatography is demanding task and can be performed by design of experiments methodology (DoE) which was poorly used for HILIC method development and only few papers can be found in literature so far.[5–8]

Once the chromatographic behavior of investigated system is described, the optimization goals (adequate separation, minimal analysis duration etc.) should be defined. Recently, regulatory affairs in pharmaceutical industry highlighted the robustness of an optimum as inevitable quality criteria, as well.[9,10] Since the implementation of the robustness aspect at early stage of method development diminish the risk of method failure. The multiobjective optimization evaluating several optimization goals simultaneously can be achieved through the application of different chemometric strategies, including chromatographic response functions (CRF),[8,11–13] Derringer's desirability function[14–16] or multiple threshold approach (MTA).[17,18] Chromatographic response functions and Derringer's desirability function consist of individual quality measures which are combined into single numerical value. Therefore, it can happen that extremely satisfactory value of one quality criterion masks the unsatisfactory result of another criterion. On the other hand, MTA is based on setting the admissible threshold level for certain goals while the other goals are let to tend to the best pos-

sible values. Therefore, the satisfactory value for the most important criteria is guaranteed and cannot be masked. MTA is not suitable when the number of goals of interest is high, however, when there are only few goals it can provide good and reliable results. The benefit of such optimization is especially noticed when the separation of critical peak pair is one of the set goals.

The aim of this paper is to present the strategy for method development in HILIC applying design of experiments (DoE) methodology and multiple threshold approach where three simultaneous optimization goals will be achieved: the adequate separation of the analyzed substances, the minimal analysis duration and the maximal robustness of the obtained optimum. The final identification of optimal separation conditions will be achieved by grid point search.

As a model mixture for the investigation, five psychotropic drugs were chosen: clozapine (pKa = 7.6), thioridazine (pKa = 9.5), sulpiride (pKa = 10.2), pheniramine (pKa = 9.3) and lamotrigine (pKa = 5.7). Their chemical structures are presented in Figure 1.

2. Experimental

Working standards of clozapine, thioridazine, sulpiride, pheniramine and lamotrigine were used for the preparation of the standard solutions. All reagents used were of an analytical grade. Acetonitrile–HPLC gradient grade (Sigma, St. Louis, MO, USA), ammonium acetate obtained from Riedel–de Haen, Seelze, Germany and water–HPLC grade were used to prepare a mobile phase. Glacial acetic acid (Zorka, Šabac, Serbia) was used to adjust pH of the mobile phase. The solutions of investigated substances are prepared dissolving each of them in the mixture acetonitrile–water (85:15 v/v) in order to achieve the concentration of 100 μg mL^{-1}.

The chromatographic system Waters Breeze consisted of 1525 Binary HPLC Pump, 2487 UV/VIS dual absorbance detector and Breeze Software Windows XP for data collection. Separations were performed on Betasil Silica–100 4.6 mm × 100 mm, 5 μm column (Thermo Fisher Scientific Inc., Waltham, MA, USA). UV detection was performed at 254 nm. Flow rate was 1 mL min^{-1} and the column temperature 30 °C.

2. 1. Software

Experimental design and data analysis were performed using Design-Expert® 7.0.0. (Stat-Ease Inc., Minneapolis). The M-files were written in MATLAB for partial derivatives calculation and *grid point search* optimization. Theoretical chromatogram was constructed in Microsoft Excel.

3. Results and Discussion

In order to develop HILIC method which provide optimal and robust separation of the investigated mixture

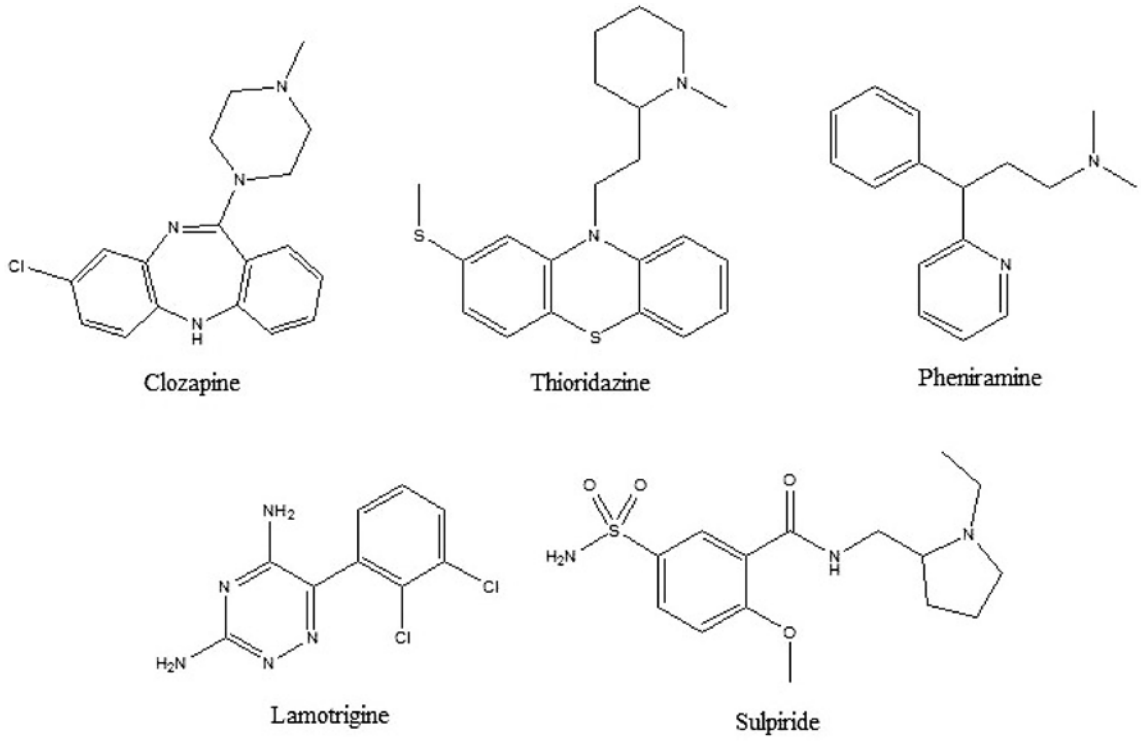

Figure 1. Chemical structures of the investigated substances

within a minimal possible time DoE methodology was applied. During the preliminary investigation bare silica column is selected as a stationary phase. Mobile phase composition included high percentage of acetonitrile and small amount of water modified by adding ammonium acetate buffer and its pH was adjusted by glacial acetic acid. Three factors related to the mobile phase composition are identified as potentially significant and their influence is investigated thoroughly applying Box-Behnken experimental design (Figure 2). The selected factors, their levels and experimental plan are presented in Table 1.

As responses to be followed, retention factors of analyzed substances were selected (k_1 to k_5), and the obtained results are presented in Table 1. The separation of two last eluting substances is identified as critical and measured calculating the selectivity factor $\alpha_{4,5} = k_5/k_4$. Therefore, the maximization of selectivity factor $\alpha_{4,5}$ is identified as the first optimization goal. Consequently, the robustness of this response is identified as the second optimization goal. Finally, as third goal minimal analysis duration (measured as retention factor of the last eluting peak (k_5)) is defined.

The applied DoE methodology allowed construction of second order polynomial models[19] for responses $\alpha_{4,5}$ and k_5. The obtained equations were:

$$\alpha_{4,5} = 1.2 - 0.039*A - 0.019*B + 0.059*C - 0.031*A*B + 0.015*A*C + 0.028*B*C - 0.026*A^2 - 0.069*B^2 - 0.008*C^2$$

$$k_5 = 4.74 - 4.18*A + 0.81*B - 1.72*C - 0.45*A*B - 1.33*A*C - 1.19*B*C + 1.54*A^2 - 0.096*B^2 - 1.21*C^2$$

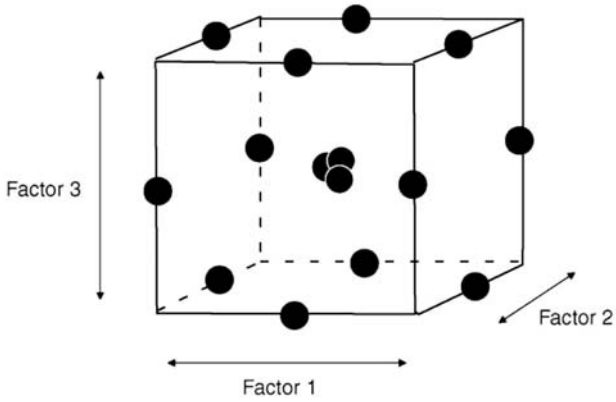

Figure 2. Graphical presentation of experimental points defined by Box-Behnken design for three factors

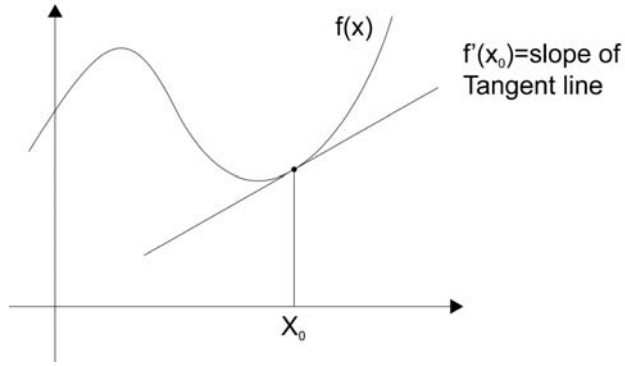

Figure 3. Partial derivative of the response function

Table 1. Plan of experiments and the obtained results

No	A	B	C	k_1	k_2	k_3	k_4	k_5	$\alpha_{4,5}$
1	80 (–1)[a]	2.5 (–1)[a]	20 (0)[a]	0.66	0.99	1.43	1.95	2.24	1.15
2	90 (+1)	2.5 (–1)	20 (0)	0.93	4.49	6.34	9.15	10.19	1.11
3	80 (–1)	4.5 (+1)	20 (0)	0.05	1.08	1.08	2.66	3.08	1.16
4	90 (+1)	4.5 (+1)	20 (0)	0.05	1.74	3.58	9.21	9.21	1.00
5	80 (–1)	3.5 (0)	5 (–1)	0.27	1.47	1.88	2.75	3.14	1.14
6	90 (+1)	3.5 (0)	5 (–1)	0.00	4.80	7.07	14.75	15.45	1.05
7	80 (–1)	3.5 (0)	35 (+1)	0.16	0.73	1.10	1.73	2.17	1.25
8	90 (+1)	3.5 (0)	35 (+1)	0.09	2.89	3.16	7.53	9.18	1.22
9	85 (0)	2.5 (–1)	5 (–1)	1.18	2.38	3.15	4.16	4.64	1.11
10	85 (0)	4.5 (+1)	5 (–1)	0.05	2.29	4.55	10.01	10.35	1.03
11	85 (0)	2.5 (–1)	35 (+1)	0.67	1.52	2.29	3.24	3.74	1.15
12	85 (0)	4.5 (+1)	35 (+1)	–0.05	1.12	1.46	3.96	4.69	1.19
13	85 (0)	3.5 (0)	20 (0)	0.11	1.83	2.29	3.93	4.72	1.20
14	85 (0)	3.5 (0)	20 (0)	0.14	1.60	2.05	3.43	4.11	1.20
15	85 (0)	3.5 (0)	20 (0)	0.26	1.43	1.67	4.61	5.53	1.20
16	85 (0)	3.5 (0)	20 (0)	0.16	1.78	2.25	3.83	4.60	1.20

A – concentration of acetonitrile in mobile phase (%); B – pH of the water phase; C –concentration of ammonium acetate in water phase (mmol L^{-1}); $k_1 - k_5$ retention factors of lamotrigine, thioridazine, clozapine, pheniramine and sulpiride, respectively; $\alpha_{4,5}$ –selectivity factor between pheniramine and sulpiride

[a] coded factor levels

Both models were characterized with satisfactory R^2 and adjusted R^2 values (higher than 0.9) so they can be used for navigating the design space.

The modelling of $\alpha_{4,5}$ robustness was performed by calculation of partial derivatives of obtained response function with respect to the investigated factors x.[17] $d\alpha/dx$ provide the information on the impact of small variations in factors on the response and tell us how critical or robust is the obtained optimum (Figure 3).

The created models for $d\alpha_{4,5}/dA$, $d\alpha_{4,5}/dB$, $d\alpha_{4,5}/dC$ were:

$$d\alpha_{4,5}/dA = 0.039 - 0.031*B + 0.015*C - 0.052*A$$

$$d\alpha_{4,5}/dB = 0.019 - 0.031*A + 0.028*C - 0.138*B$$

$$d\alpha_{4,5}/dC = 0.059 + 0.015*A + 0.028*B + 0.016*C$$

These equations enable the investigation of experimental space in order to find A, B and C which provide the most robust $\alpha_{4,5}$.

Further on, multiple threshold approach is applied in order to achieve the following optimization goals:

Separation quality: $\alpha_{4,5} > 1.2$

Robustness of critical selectivity factor:

$$d\alpha_{4,5}/dA < 0.05;\ d\alpha_{4,5}/dB < 0.05;\ d\alpha_{4,5}/dC < 0.05$$

Total analysis duration: minimization of k_5

It can be seen that no compromise are acceptable in terms of separation quality and robustness of that separation. On the other hand, the compromise in terms of total analysis duration can be made, although the minimization of this response is desirable, as well.

The optimal separation conditions were found by grid point search method. Grid point search method consists of dividing the design space by a grid and then searching the response functions values only in grid nodes. This approach involves discretization of the investigated factors intervals. The grid density for investigated problem was defined by increments of all three factors of 0.2 (in terms of coded factors values). The total number of levels for each factor was eleven. Consequently the total number of investigated experimental points was:

11 levels for acetonitrile content*11 levels for pH*11 levels for ammonium-acetate concentration = 1331 points.

Further on, for each of five optimization goals defined by MTA, following the theoretical models, 1331 values were predicted. According to the set thresholds, the values having $d\alpha_{4,5} > 1.2$, $d\alpha_{4,5}/dA < 0.5$, $d\alpha_{4,5}/dB < 0.5$ and $d\alpha_{4,5}/dC < 0.5$ are considered. Among the extracted points the one with minimal total analysis duration was searched and identified as: acetonitrile content 83%, pH of the water phase 3.5 and ammonium acetate content 14 mM.

The identified optimal conditions (Betasil Silica–100 4.6 mm × 100 mm, 5 μm column; mobile phase: acetonitrile – water phase (14 mM ammonium acetate, pH 3.5 adjusted by glacial acetic acid); column temperature 30 °C, flow rate 1 mL min^{-1}, UV detection at 254 nm) were experimentally checked and compared with theoretical chromatogram (Figure 4). The summarized results are presented in Table 2.

High matching between theoretical and experimentally obtained chromatogram verified the adequacy of applied response functions for modeling the investigated system. Figure 4 demonstrate that, under the defined optimal conditions, the separation of the investigated com-

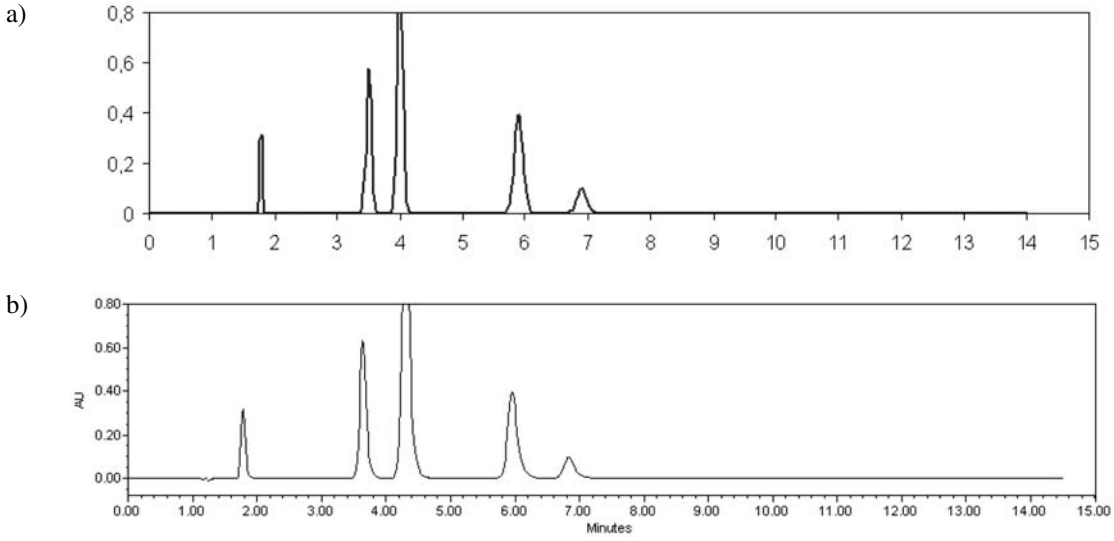

Figure 4. a) Predicted and b) experimentally obtained chromatogram (acetonitrile content in mobile phase 83%; pH of the water phase 3.5; ammonium acetate concentration in water phase 14 mM).

Table 2. Comparison between theoretical and experimentally obtained results

	experimental values	theoretical values	error (%) error (%)
$\alpha_{4,5}$	1.2	1.2	0
k_5	3.9	4.0	2.5
Run time (min)	6.8	6.9	1.5

pounds is achieved within 7 minutes. Moreover, the identified optimum is found to be robust enough indicating the reliability of the proposed method in routine application. It can be seen that DoE methodology, MTA and grid point search provided robust optimization of the investigated mixture separation.

4. Conclusion

This paper presented the development and optimization of method for separation of five psychotropic drugs in hydrophilic interaction liquid chromatography. Design of experiments methodology was applied for construction of mathematical relationship between investigated factors and selected responses. Three simultaneous goals (maximal separation of critical peak pair, robustness of this separation and minimal analysis duration) are traced by multiple threshold approach and grid point search optimization. The experimental results under identified optimal conditions highly correlated with theoretically predicted results. It is proved that the synergy of Design of experiments methodology, multiple threshold approach and grid point search provide valuable assistance in HILIC method development.

5. Acknowledgements

The authors thank to Ministry of Education and Science of Republic of Serbia for supporting these investigations in Project 172052.

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Povzetek

Članek predstavlja večpredmetno optimizacijo ločbe kompleksnih zmesi s hidrofilno interakcijsko tekočinsko kromatografijo (HILIC). Izbrana modelna zmes je bila sestavljena iz petih psihotropnih učinkovin: klozapin, tioridazin, sulpirid, feniramin in lamotrigin. Optimizirali smo tri faktorje, povezane s sestavo mobilne faze (delež acetonitrila, pH vodne faze in koncentracija amonijevega acetata), z namenom, da dosežemo maksimalno kvaliteto ločbe, minimalen skupen čas analize in robustnost optimuma. Z upoštevanjem robustnosti v začetnih fazah razvoja metode razvijemo zanesljivo metodo z majhnim tveganjem, da validacija ne bi uspela. Simultano optimizacijo vseh ciljnih parametrov smo dosegli z večnivojskim pristopom v kombinaciji z iskanjem mrežnih točk. Izbrane optimalne pogoje ločbe (delež acetonitrila 83 %, pH vodne faze 3,5 in koncentracija amonijevega acetata v vodni fazi 14 mM) smo tudi eksperimentalno potrdili.