Technical paper

Central Composite Design with/without Artificial Neural Networks in Microemulsion Liquid Chromatography Separation Robustness Testing

Biljana Jančić-Stojanović,[1]* Andjelija Malenović,[1] Darko Ivanović[1] and Mirjana Medenica[2]

[1] *Faculty of Pharmacy, Institute of Drug Analysis, Vojvode Stepe 450, Belgrade, Serbia*

[2] *Faculty of Pharmacy, Institute of Physical Chemistry, Vojvode Stepe 450, Belgrade, Serbia*

* *Corresponding author: E-mail: jancic.stojanovic@pharmacy.bg.ac.yu*

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Abstract

In past few years, for overcoming some analytical problems in liquid chromatography, the microemulsion as eluent was employed. Due to the strict regulatory requirements, robustness testing became important especially when proposing completely new method such as microemulsion liquid chromatography (MELC). In this paper robustness testing of MELC method, proposed for carbamazepine and its impurities (iminostilben and iminodibenzyl) separation, was done using two different approaches both based on experiments defined using central composite design (CCD). Input and output data from CCD were either handled as second order polynomials and tested with Analysis of variance (ANOVA), or as variables in Artificial Neural Networks (ANN). From both approaches appropriate conclusions about system robustness were distinguished, e.g. that the influence of surfactant content on chromatographic retention was the largest for all analytes, meaning that small changes in its concentration will strongly influenced on chromatographic retention. On the other hand influence of the pH of the mobile phase proved to be negligible, meaning that the substances are mainly distributed in the interfacial layer. ANN gave better results and proved to be better tool for explanation and understanding of investigated factors effects on the chromatographic system and for definition of the robustness limits.

Keywords: Robustness, experimental design, artificial neural networks, microemulsion liquid chromatography

1. Introduction

Microemulsions are chemically, structurally and functionally completely different eluents than mobile phases used in conventional RP-HPLC methods. For that reason in microemulsion liquid chromatography (MELC) robustness testing, as important part of method validation, is more complex and definitely more demanding. Complex eluent composition (organic phase, surfactant, co-surfactant and water phase) as well as complex interactions among substance-eluent-stationary phase, mean that factor selection for robustness testing must be done with more attention than in conventional RP system. Having on mind such kind of MELC system complexity, the aim of this study was the analysis of central composite design (CCD) application with/without artificial neural networks (ANN) in robustness testing of carbamazepine and its two related substances (iminostilben – IS and iminodybenzil – ID) chromatographic retention. The robustness testing can be conducted using different kinds of experimental design e. g. CCD,[1] Plackett-Burman design,[2] full and fractional factorial design,[3] etc. Experimental design is very useful in many aspects of method development and evaluation. For that reason many authors used experimental design in robustness/ruggedness testing.[4,5] In some papers combination of appropriate experimental design and neural networks was used in various phases of method development.[6,7] For that reason, this paper presents comparison of experimental data with data predicted by derived second order polynomials, as well as with data predicted by ANN in aim to examine robustness of novel analytical method.

In point of carbamazepine analysis some papers were found. Some of them present application of RP-HPLC method for carbamazepine and its impurities analysis in bulk substances and/or tablets.[8,9] Also, two stability indicating RP-HPLC methods are described in literature.[10,11] Other methods for analysis of carbamazepine[12,13] could also be found in literature Many papers deal with analysis of carbamazepine and others antiepileptic drugs in biological samples.[14–20]

2. Experimental

The chromatographic system *Waters* Breeze consisted of Waters 1525 Binary HPLC Pump, *Waters* 2487 UV/VIS detector and Breeze Software, Windows XP, for data collection. Sodium dodecyl sulphate (SDS), Brij 35 (polyoxyethylene 23-lauryl ether) and sodium dioctyl sulphosuccinate (SDOSS; dowsate sodium, BP 93) were obtained from *Sigma* (St. Louis, MO, USA). Diisoprophyl ether, *n*-butanol and *n*-propanol – HPLC grade were manufactured from *Riedel-deHäen* (Seelze, Germany). Heptane and cyclohexane – HPLC grade were obtained from *Fluka* (Buchs, Switzerland). Water – HPLC grade, triethylamine (TEA) *Acros Organic* (Geel, Belgium) and ortophosphoric acid *Carlo Erba* (Milan, Italy) were used to prepare a water phase. Separations were performed on the X-TerraTM 4.6 mm × 50 mm, 3.5 μm particle size column with UV detection at 230 nm. Mobile phases were prepared by mixing all the microemulsion components and treating them on an ultrasonic bath for 30 min. The resulting transparent microemulsion was filtered through a 0.45 μm membranes filter *Alltech* (Lokeren, Belgium). Flow rate was 0.3 mL $min.^{-1}$. Mobile phases consisted of different ratio of diisopropylether, SDS, *n*-propanol and water phase containing 1% of TEA. pH of the mobile phase was adjusted with orthophosphoric acid.

3. Results and Discussion

Central composite design is a progression from the factorial designs and it has been widely used in response-surface modeling and optimization. This kind of design proved to be very useful in chemistry for modelling. It produces a detailed quantitative model which is used for mathematical prediction of how a response relates to the values of various factors. The first step is to code factors where the central point for each factor is assigned as 0 and the design is symmetrically configured around it. Next step is building up of experimental matrix. The CCD consists of three parts. First part is fractional factorial or full factorial design, second is star design and the last part is replications. Building up CCD with full factorial design is recommendable because in that way interaction estimates are provided. Star design, in fact "one factor at a time", is easy feasible. Finally, experimental error estimation can be very useful and one of the experimental possibilities is extra replications in the central point.[21] Experimentally obtained results can be expressed as mathematical relationship, namely second order polynomial equation, or graphically as three D graph. On the other hand, same data (coded inputs and obtained outputs) could be used for creation of appropriate neural network.

In this paper, CCD is applied for robustness testing of carbamazepine and its impurities retention when microemulsion is used as eluent. Since the robustness test examines potential sources of variability in one or a number of method responses, it can be viewed as a part of method validation that is performed at the end of method development or at the beginning of the validation procedure.[4,22]

Whereas relatively new eluent was applied for the separation of investigated substances some previous experiences must be illustrated. In our previous papers some general characteristics of microemulsion as eluent including application in drug analysis were given.[23,24]

During the preliminary investigations as inner phase hexan, cyclohexan and diisopropyl ether were analysed. In the same time, as co-surfactant *n*-butanol and *n*-propanol were tested. SDS was chosen as surfactant because all other combinations (SDS-SDOSS, SDOSS-Brij 35) resulted in low retention and deterioration of peak shape of all substances in the mixture. In water phase 1% TEA was added in order to prevent peak tailing. The satisfactory separation was obtained using diisopropyl ether as inner phase, SDS as surfactant, *n*-propanol as co-surfactant, water phase with 1% TEA and acid pH of all microemulsion adjusted with orthophosphoric acid. As factors which can have influence on chromatographic behavior of analyzed substances SDS, *n*-propanol and pH of the mobile phase were selected. Variation domain for SDS, *n*-propanol and pH of the mobile phase were 2.4 ± 0.9% w/v, 7.0 ± 1.0% w/v and 3.5 ± 1%, respectively. Factors and their levels are presented in Table 1.

Table 1. Factors and their levels

Factors		Factor levels				
		–1	–0.5	0	+0.5	+1
x_1	SDS content (% w/v)	1.5	1.9	2.4	2.8	3.3
x_2	*n*-propanol content (% w/v)	6.0	6.5	7.0	7.5	8.0
x_3	pH of the mobile phase	2.5	3.0	3.5	4.0	4.5

As output variable retention factor for carbamazepine, IS and ID were chosen. Experiments for CCD built for three chosen factors as well as results for retention factors are presented in Table 2.

Example of suitable separation obtained with eluent composed of 2.8% w/v of SDS, 6.5% w/v of *n*-propanol and pH of the final mobile phase was adjusted at 3.0 is given in Figure 1.

Table 2. Plan of experiments for CCD and obtained results

	Exp. No	Factors			Responses		
		x_1	x_2	x_3	k_1	k_2	k_3
Full factorial design	1	–0.5	–0.5	–0.5	2.436	12.506	17.026
	2	–0.5	+0.5	–0.5	2.252	11.728	16.02
	3	+0.5	+0.5	–0.5	1.618	6.854	9.152
	4	+0.5	–0.5	–0.5	1.830	8.006	10.744
	5	–0.5	–0.5	+0.5	2.388	12.294	16.706
	6	–0.5	+0.5	+0.5	2.218	11.53	15.738
	7	+0.5	+0.5	+0.5	1.552	6.500	8.646
	8	+0.5	–0.5	+0.5	1.798	7.822	10.466
Star design	9	–1	0	0	2.702	14.918	20.352
	10	+1	0	0	1.616	7.496	10.124
	11	0	–1	0	2.124	10.628	14.53
	12	0	+1	0	1.858	9.244	12.568
	13	0	0	–1	2.042	10.006	13.616
	14	0	0	+1	2.020	10.044	13.640
Replications	15	0	0	0	2.206	10.832	14.594
	16	0	0	0	2.218	10.854	14.648
	17	0	0	0	2.220	10.88	14.654
	18	0	0	0	2.222	10.886	14.672

k_1 – retention factor of carbamazepine; k_2 – retention factor of iminostilben; k_3 – retention factor of iminodibenzyl

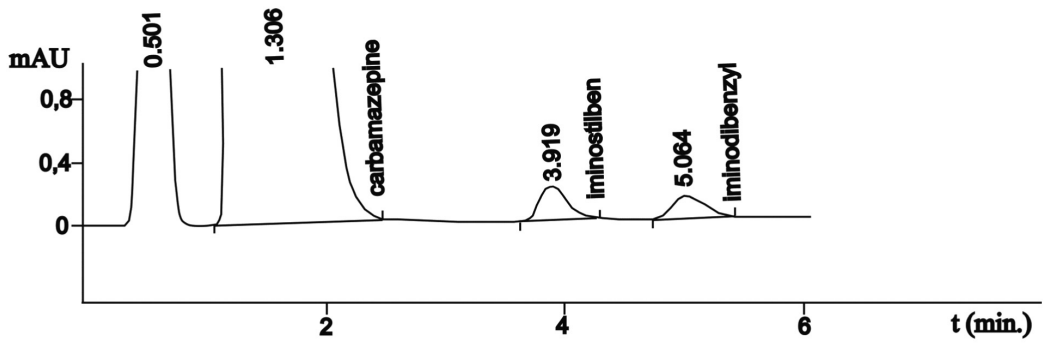

Figure 1. Chromatogram of laboratory mixture (X TerraTM 50 × 4.6 mm, particle size 3.5 μm column; temperature 35 °C; λ = 230 nm; mobile phase containing 0.5% w/v of diisopropylether, 2.8% w/v of SDS, 6.5% w/v of *n*-propanol, 1% of TEA and 89.2% w/v of water; pH of the mobile phase was adjusted to 3.0 with ortophosphoric acid)

On the basis of plan of experiments and obtained outputs appropriate calculations in Design-Expert 7.0.0 and Statistica Neural Networks were done.

Using usual statistical approach, the coefficients for second order polynomial equations which have next form:

$$y = b_o + b_1x_1 + b_2x_2 + b3x3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{123}\,x_1x_2x_3$$

were calculated and presented in Table 3.

According to values for coefficients it can be seen that the influence of factor x_1 on chromatographic retention is the largest for all three compounds. That is confirmed by p-value lower than 0.001 for all three outputs. The other two factors, especially factor x_3, have significantly lower influence. Further, using analysis of variance (ANOVA) model adequacy was confirmed. Namely, mod-

Table 3. Coefficients and effect estimate

	Effect estimate		
	k_1	k_2	k_3
b_0	2.32	10.68	14.25
b_1	–0.62	–4.22	–5.87
b_2	–0.13	–0.85	–1.17
b_3	–0.066	–0.12	–0.18
$b_1\,b_2$	–0.19	–0.47	–0.73
$b_1\,b_3$	0.13	–0.056	–0.079
$b_2\,b_3$	–0.14	–0.07	–0.083
b_1^2	–0.21	0.068	0.36
b_2^2	–0.38	–1.2	–1.33
b_3^2	–0.35	–1.13	–1.29
$b_1\,b_2\,b_3$	0.23	–0.17	–0.242

b_0 – intercept, b_i (b_1, b_2 and b_3), b_{ij} (b_{12}, b_{13} and b_{23}) and b_{ijk} (b_{123}) represent the coefficient for the second order polynomial

k_1 – retention factor of carbamazepine; k_2 – retention factor of iminostilben; k_3 – retention factor of iminodibenzyl

el F-value for C, IS and ID were 16.9, 14.2 and 14.6, respectively which implies that the model is significant. The calculated "lack of fit" F-value for C, IS and ID, 1.2, 4.97 and 8.5 respectively, imply that the "lack of fit" is not significant relative to the pure error which means that the terms in the model capture all of the assignable-cause variation of the response. Calculated coefficient of determination (R^2) values for all three compounds were grater than 0.94 proving that over 94% of the total variations are explained by the model.

Data obtained using conventional approach gave enough information. We can conclude from this results that the method is robust when factors x_2 and x_3 changes in investigated region but the factor x_1 must be strictly controlled if we want to save method performance. But, one interesting question was imposed. What we could be obtained by importing date in some kind of software which supports Neural Networks? Taking into considaration the fact that ANNs present digitized model of a human brain it would be interesting and useful to apply it for robustness testing. The basic processing unit in an ANN is called a node, which simulate neuron. These nodes can form multiple layers arranged so that each node in one layer is connected with each node in the next layer, and so on. The entire group of layerd nodes makes up a complete ANN.[25]

In this paper application of multilayer perceptrons (MLP) in purpose of robustness testing is presented. MLP as architecture with supervised learning, can be trained with different kinds of algorithm, but the most often used in analytical application is backpropagation algorithm (BP). Such kind of network has one input layer, one or more hidden layers and one output layer.[25] Each layer has a few nodes corresponding to neurons. The strengths of connections between two units are called "weights".[26] Training of ANN is performed by adjusting weights in order to minimize the root mean square (RMS) of the training data and on that way prevent the same error happening again. When the network fulfills the appropriate demands, it is presumed that it has good predictive capabilities and ability to accurately describe the system.

In order to get optimal network from the data obtained by CCD three different sets were made, one for net-

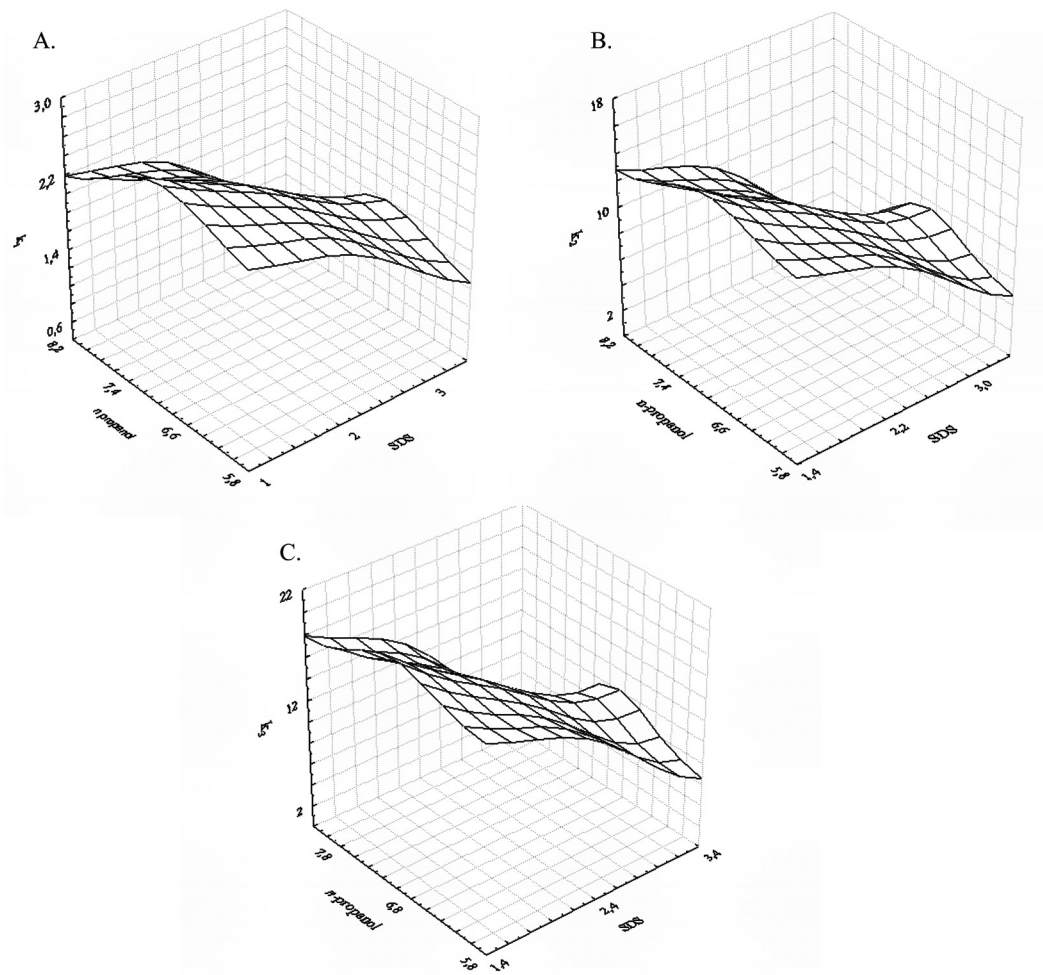

Figure 2. Three-D graph: A. k_1 (carbamazepine) = f (SDS, *n*-propanol); B. k_2 (IS) = f (SDS, *n*-propanol); C. k_3 (ID) = f (SDS, *n*-propanol).

work training, second for network verification and third for network testing. The network was trained until the smallest value for RSM was got. The optimal network had three layers with architecture 3-8-3. Input layer, which corresponded to the factors (SDS, *n*-propanol and pH of the mobile phase), had a three nodes, output layer with three nodes were retention factors of C, IS and ID and hidden layer with eight nodes.

The smallest (RMS) error was obtained after network training with back propagation (BP) algorithm in 50^{th} epoch and conjugate gradient descent algorithm in the 1st epoch. Difference between RMS error for training and verification sets was the smallest and correlations in verification, training and testing sets were satisfactory for all three substance.

The weigts were distributed among –1.5 and +1.5. Sensitivity analysis of obtained network proved the same order of factor's influence on chromatographic retention of carbamazepine and its impurities.

In both cases, appropriate three-D graphs, as suitable way of results presention, could be produced. They present very appropriate modality of factor's influence vizualization and some important information about analysed system are provided to analyst. Chemically relevant conclusions can be also derived helping in better understanding of processes in the investigated system. For the three-D graph presentation one factor must be excluded and two other analyzed. As previously concluded pH of the water phase had the smallest influence so the three-D graphs were constructed as k = f (% SDS, % *n*-propanol). Three-D graphs are presented in Figure 2 (Figure 2A for carbamazepine, Figure 2B for IS and Figure 2C for ID).

Changes in SDS and *n*-propanol content imposed alike chromatographic behavior of analytes. Obviously great similarity in chemical structure was graphically reprinted and confirmed by three-D graphs. However, no matter how small differences in structure exist they were effectual on the distribution of analytes between stationary and mobile phase which resulted in satisfactory separation.

4. Conclusion

In this paper, authors present the application of CCD alone or in combination with ANN in study of carbamazepine and its impurities separation robustness when microemulsion is used as eluent. As quantitative factors SDS, *n*-propanol and pH of the mobile phase were selected, CCD was built and experiments were done. Data analysis by conventional statistics, gave coefficients of second order polynomials followed by factor's estimate and ANOVA analysis. On the other hand, same data were imported in Statistics Neural Networks and network with acceptable characteristics was built. Comparing results from classical statistical evaluation and ANN, almost the same conclusions were done. Finally, the question was, which approach is easier for analyst? Is it enough to explain system using polynomial equations and some statistical estimation or have some additional confirmations by ANN? Generally, if there is possibility to use ANN, that approach combining experimental design and ANN is more useful and therefore recommendable one. Namely, date in ANN could be easily widen, than unite with some new or previous experiences and so on, giving more possibilities and enabling better understanding of processes being on investigated system.

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Povzetek

V zadnji letih so mikroemulzije vedno bolj v uporabi pri kromatografskih ločbah. Pri uvajanju novih metod kot je mikroemulzijska tekočinska kromatografija (MELC) pa so zaradi predpisov postala testiranja robustnosti zelo pomembna. V tem prispevku predlagamo metodo testiranja robustnosti za ločitev karbamazepina in nečistoč (iminostilbena in iminodibenzila), pri čemer smo uporabili dva različna pristopa, oba na osnovi centralne sestavljene oblike (CCD – central composite design). Preverili smo vpliv prisotnosti površinsko aktivnih sredstev na kromatografsko retencijo, ki se je izkazala kot zelo pomembna. Po drugi strani je bil vpliv pH majhen, kar pomeni, da so snovi porazdeljene predvsem po medfaznem sloju. Umetna živčna omrežja so dala boljše rezultate in vodila do boljšega razumevanja vplivov na kromatografsko ločbo.