Chemometrical Tools in the Study of the Retention Behavior of Azole Antifungals

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Certain chemometrical tools allow an efficient way to provide valuable data to evaluate the retention behavior of analytes in liquid chromatography. In this study of the retention behavior of azole antifungals, the experimental design was applied in combination with artificial neural networks (ANNs). Three potentially significant factors (methanol content, pH of the mobile phase and column temperature) were incorporated in the plan of experiments, defined by central composite design. As the system outputs, the retention factors of all six investigated substances (fluconazole, ketoconazole, bifonazole, clotrimazole, econazole and miconazole) were determined. The pattern for the analyzed behavior of the system was created by employing ANNs. The final, optimized topology of the highly predictive network was 3–8–6. Twelve experiments were used in a training set, whereas a back-propagation algorithm was optimal for network training. The ability of the defined network to predict the retention of the investigated azoles was confirmed by correlations higher than 0.9912 for all analytes. The presented approach allowed the adequate prediction of the retention behavior of azoles, in addition to the extraction of important information for a better understanding of the analyzed system.

Introduction

To meet the demands of modern chromatographic analysis, the employment of chemometrical tools such as experimental design and artificial neural networks allows the achievement of an unbiased assessment of the analytical methods. The experimental design (ED), among other things, offers a systematical data collection that might be used in further evaluation by artificial neural networks (ANNs). ANNs are biologically inspired computer programs designed to simulate the information processing of a human brain. Currently, ANNs represent a powerful tool in science, especially when it is necessary to create a pattern for system behavior. In chromatographic science, it is very important to have possibilities to predict the chromatographic retention of the analyzed substances. Up to now, the capabilities of ED and ANNs have been described in different areas of chromatographic analysis. For example, they proved to be very useful in process optimization (1–6) or retention modeling in liquid chromatography (LC) (7–12). Also, the authors' previous experience in ED-ANN application showed great potential in LC (13–16). By applying ANN, different problems have been solved: in the study by Jančić-Stojanović *et al.* (13), ANN allowed the retention behavior of an active pharmaceutical ingredient and its impurities to be determined. The successful optimization of a high-performance liquid chromatography (HPLC) method compatible with mass spectrometry (MS) was achieved with ANN (14). Moreover, there are examples of other successful optimizations, not only for LC methods (14, 15), but also for micellar LC methods (16).

The aim of this study was to investigate the retention performance of selected antifungal azoles to track and predict their chromatographic behavior, by employing ANNs. The investigated substances are presented in Figure 1.

In previously published papers, no references were found that suggested such an approach. A critical review of all chromatographic and electrophoretic techniques used in the analysis of triazole antifungal agents can be found (17). Suitable HPLC methods have been developed for the analysis of ketoconazole, clotrimazole, tioconazole, bifonazole, isoconazole, econazole, miconazole and fenticonazole in pharmaceutical formulations (18); for clotrimazole and its two degradation products in spray formulation (19); and econazole nitrate and its primary impurities in cream formulation (20). Densitometric and reversed-phase (RP)-HPLC methods have been described as stability indicating for the determination of ketoconazole, clotrimazole, miconazole nitrate and econazole nitrate, along with the degradation products of each analyte (21). Also, an HPLC method was provided as a stability-indicating method for the determination of miconazole nitrate in bulk and cream formulations (22). On the other hand, there is one paper dealing with the influence of the eluent composition on the retention factors of various derivatives of nitrogen-containing five-membered heterocycles (diazole and triazole) in RP-LC (23).

However, this is the first time that an in-depth analysis of the chromatographic retention of azoles is explained by utilizing ED-ANNs. The set of experiments for network evaluation was defined by central composite design (CCD). To obtain a network with strong predictive ability, the critical parameters of ANNs were optimized; namely, the number of nodes in the hidden layer and the number of experimental data points in the training set were simultaneously varied and their importance was estimated with suitable statistical parameters. Also, the optimal algorithm for training was chosen. The network obtained in this way provides its own pattern of the chromatographic retentions of antifungal azoles and possesses an excellent ability for the prediction of system behavior.

Theory

Experimental design

The ED is a tool that offers a systematic assessment of the different types of factors that might affect a certain system. The application of experimental design in chromatography facilitates the identification of significant variables, thus allowing the optimization of method parameters and the elucidation of factors that govern a separation. Also, the ED is usually used to
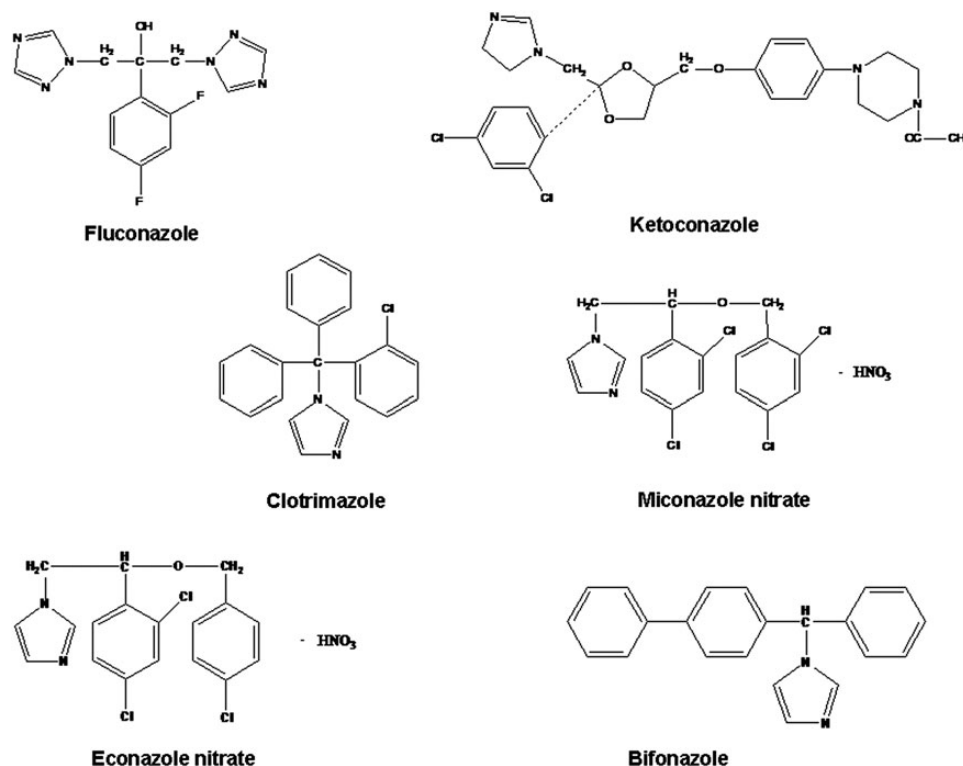
Figure 1. Chemical structures of the investigated antifungal azoles.

Table I

Experimental Plan Defined by CCD and the Obtained System Outputs*

| Experiment	Factor $x_1^\dagger$	Factor x_2	Factor x_3	k_F	k_K	k_B	k_C	k_E	k_M
1	56.00 (−1.0)‡	2.40 (−1.0)	30.0 (−1.0)	0.546	3.707	6.199	5.583	15.616	32.596
2	59.00 (+1.0)	2.40 (−1.0)	30.0 (−1.0)	0.408	2.198	3.760	3.482	9.054	18.454
3	56.00 (−1.0)	3.40 (+1.0)	30.0 (−1.0)	0.542	7.286	13.018	17.985	24.630	52.696
4	59.00 (+1.0)	3.40 (+1.0)	30.0 (−1.0)	0.432	5.408	9.862	13.826	17.818	37.18
5	56.00 (−1.0)	2.40 (−1.0)	40.0 (+1.0)	0.472	2.917	4.809	4.809	11.029	22.108
6	59.00 (+1.0)	2.40 (−1.0)	40.0 (+1.0)	0.371	1.940	3.311	3.311	7.260	14.279
7	56.00 (−1.0)	3.40 (+1.0)	40.0 (+1.0)	0.483	6.852	12.152	17.119	21.474	44.376
8	59.00 (+1.0)	3.40 (+1.0)	40.0 (+1.0)	0.409	5.468	9.856	13.798	16.825	33.988
9	55.00 (−1.7)	2.90 (0.0)	35.0 (0.0)	0.551	5.344	9.101	11.583	18.772	39.383
10	60.00 (+1.7)	2.90 (0.0)	35.0 (0.0)	0.354	2.566	4.513	5.788	8.896	17.844
11	57.50 (0.0)	2.05 (−1.7)	35.0 (0.0)	0.424	2.010	3.616	2.956	8.997	18.160
12	57.50 (0.0)	3.75 (+1.7)	35.0 (0.0)	0.468	10.121	18.042	23.740	31.430	65.858
13	57.50 (0.0)	2.90 (0.0)	26.5 (−1.7)	0.511	4.165	7.083	7.872	15.939	33.519
14	57.50 (0.0)	2.90 (0.0)	43.5 (+1.7)	0.387	2.931	5.069	6.719	9.695	19.306
15	57.50 (0.0)	2.90 (0.0)	35.0 (0.0)	0.438	3.323	5.711	7.043	11.834	24.268
16	57.50 (0.0)	2.90 (0.0)	35.0 (0.0)	0.437	3.377	5.806	7.127	12.003	24.566
17	57.50 (0.0)	2.90 (0.0)	35.0 (0.0)	0.452	3.474	5.969	7.328	12.348	25.271
18	57.50 (0.0)	2.90 (0.0)	35.0 (0.0)	0.442	3.401	5.841	7.170	12.078	24.717

*Note: retention factor for fluconazole (k_F); retention factor for ketoconazole (k_K); retention factor for bifonazole (k_B); retention factor for clotrimazole (k_C); retention factor for econazole (k_E); retention factor for miconazole (k_M).

†Factor x_1: methanol (%); Factor x_2: pH; Factor x_3: temperature (°C).

‡Coded values for factor levels are given in parentheses.

set the necessary experiments for ANNs. Different kinds of designs could be employed for these purposes. In this paper, a CCD was chosen. Generally, CCD combines two-level full factorial or fractional factorial design with additional axial or star points, and at least one point in the center of the investigated experimental region (24). It allows the determination of both linear and quadratic models. In general, CCD consists of the

following parts: (i) points defined by 2^{k-p} design, where k denotes the number of factors, whereas p is usually, but not necessarily, equal to zero; (ii) axial (star) points, i.e., the experiments positioned along the axes at a distance α from the central point; (iii) central points (25). All sections are variable, which means that even CCD, usually presented with the full factorial portion composed of the points defined by 2^k design, could be
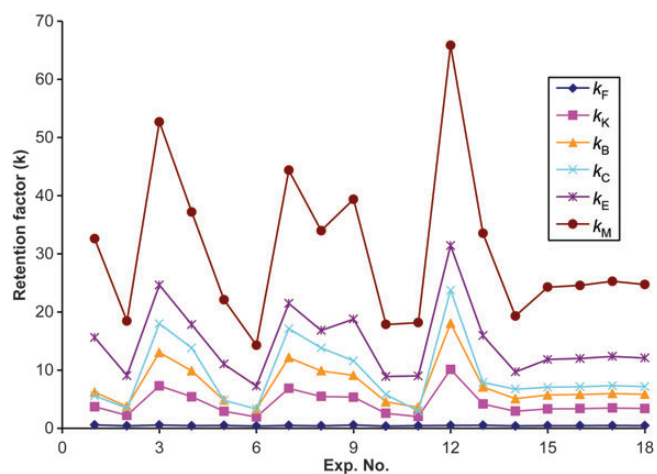
Figure 2. Change of retention factors with the change in experimental conditions designed by ED (k_F: retention factor for fluconazole; k_K: retention factor for ketoconazole; k_B: retention factor for bifonazole; k_C: retention factor for clotrimazole; k_E: retention factor for econazole; k_M: retention factor for miconazole).

determined with the fractional factorial (2^{k-p}). Some examples of its successful employment in chromatographic analysis have been presented in previous papers (14, 15, 26–28).

Artificial neural networks

ANNs present a digitized model of human brain. The basic processing unit in an ANN is called node, which simulates a 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 layered nodes makes up a complete ANN (29). Generally, ANNs consist of the input devices, the interconnected processing elements or nodes and the output devices. Typically, there is one input layer, one output layer and one or more hidden layers between them (30).

In this paper, the multilayer perceptron (MLP) was applied. This is likely the most popular architecture in use. The MLP is a feed-forward ANN model that maps sets of input data onto a set of the appropriate outputs and presents some kind of supervised learning. It is a modification
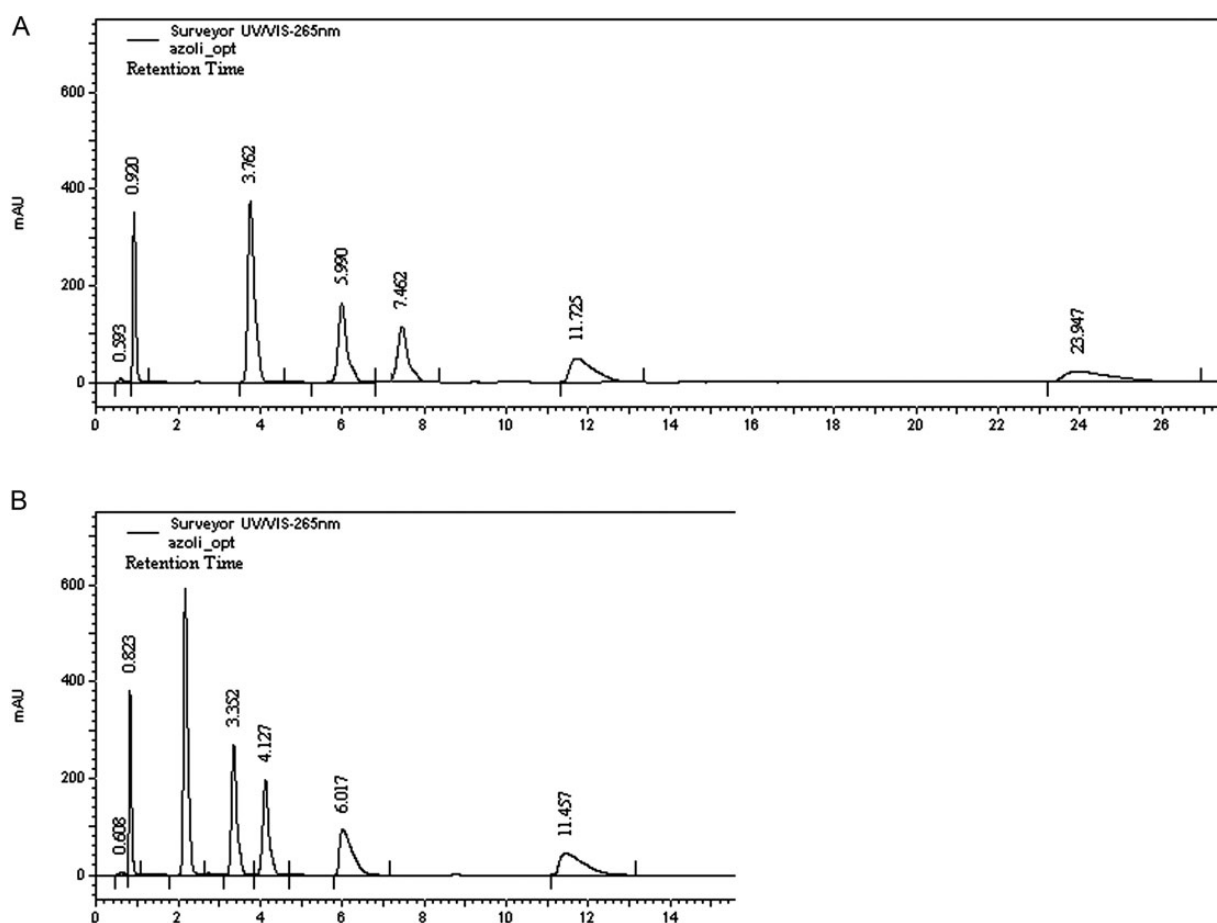
Figure 3. Influence of the content of methanol on the retention behavior of the investigated azole antifungals. Mobile phase: methanol−water (1% TEA, pH 2.9) (55:45 v/v), 35°C; fluconazole (0.992 min), ketoconazole (3.762 min), bifonazole (5.990 min), clotrimazole (7.462 min), econazole (11.725 min) and miconazole (23.947 min) (A); mobile phase: methanol−water (1% TEA, pH 2.9) (60:40 v/v), 35°C; fluconazole (0.823 min), ketoconazole (2.168 min), bifonazole (3.352 min), clotrimazole (4.127 min), econazole (6.017 min) and miconazole (11.457 min) (B).
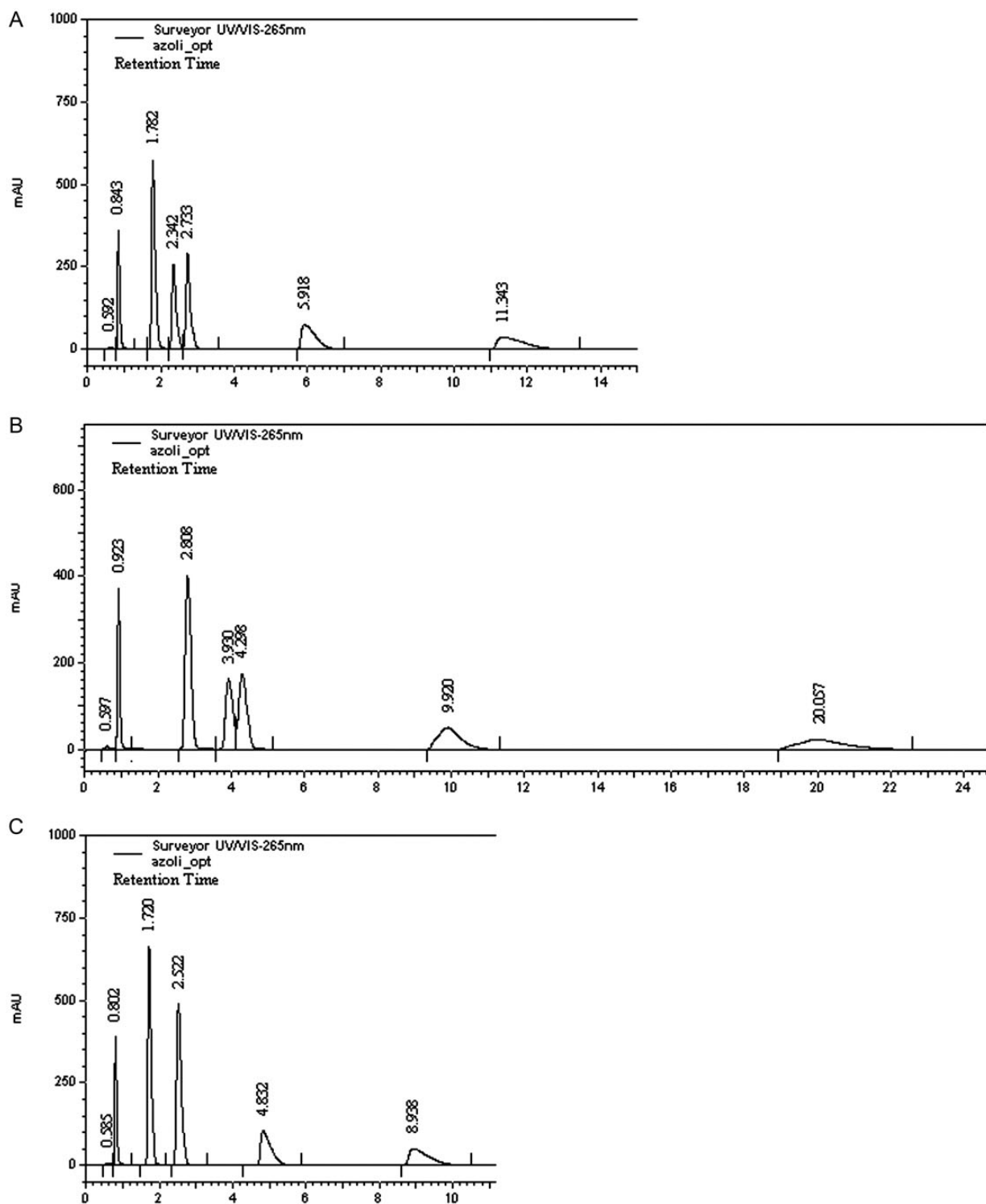
Figure 4. Influence of the pH value of the water phase on the retention behavior of the critical pair bifonazole and clotrimazole. Mobile phase: methanol–water (1% TEA, pH 2.05) (57.5:42.5 v/v), 35°C; fluconazole (0.843 min), ketoconazole (1.782 min), clotrimazole (2.342 min), bifonazole (2.733 min), econazole (5.918 min) and miconazole (11.343 min) (A); mobile phase: methanol–water (1% TEA, pH 2.4) (56:44 v/v), 30°C; fluconazole (0.923 min), ketoconazole (2.808 min), clotrimazole (3.930 min), bifonazole (4.298 min), econazole (9.920 min) and miconazole (20.057 min) (B); mobile phase: methanol–water (1% TEA, pH 2.4) (59:41 v/v), 40°C; fluconazole (0.802 min), ketoconazole (1.720 min), clotrimazole and bifonazole (2.522 min), econazole (4.832 min) and miconazole (8.938 min) (C).

of the standard linear perceptron that uses three or more layers of neurons (nodes) with nonlinear activation functions, and is more powerful than the perceptron that can distinguish data that are not linearly separable (31). This kind of network has one input layer, one or more hidden layers and one output layer. Each layer has a few nodes

Table II
Equation Coefficients in Terms of Coded Factors*

	k_F	p-value	k_K	p-value	k_B	p-value	k_C	p-value	k_E	p-value	k_M	p-value
b_0	+0.44		+3.40		+5.84		+7.16		+12.07		+24.73	
b_1	−0.056	< 0.0001	−0.76	0.0002	−1.25	< 0.0001	−1.52	< 0.0001	−2.80	< 0.0001	−6.13	< 0.0001
b_2	+0.009	0.0192	+2.03	< 0.0001	+3.73	< 0.0001	+5.87	< 0.0001	+5.51	< 0.0001	+11.75	< 0.0001
b_3	−0.031	< 0.0001	−0.26	0.0540	−0.45	0.0283	−0.28	0.0231	−1.53	0.0022	−3.65	0.0010
b_{12}	+0.009	0.0565	−0.097	0.5322	−0.19	0.4120	−0.49	0.0056	−0.14	0.7644	−0.49	0.6170
b_{13}	+0.011	0.0241	+0.13	0.4132	+0.23	0.3336	+0.18	0.2005	+0.62	0.2109	+1.43	0.1687
b_{23}	+0.006	0.1895	+0.084	0.5863	+0.12	0.5958	+0.006	0.9618	+0.28	0.5572	+0.39	0.6878
b_{11}	+0.006	0.1104	+0.17	0.1716	+0.31	0.1041	+0.55	0.0006	+0.55	0.1611	+1.21	0.1402
b_{22}	+0.003	0.3056	+0.91	< 0.0001	+1.71	< 0.0001	+2.16	< 0.0001	+2.76	< 0.0001	+5.85	< 0.0001
b_{33}	+0.005	0.1940	+0.034	0.7780	+0.061	0.7298	+0.068	0.5205	+0.20	0.5920	+0.45	0.5597

*Note: retention factor for fluconazole (k_F); retention factor for ketoconazole (k_K); retention factor for bifonazole (k_B); retention factor for clotrimazole (k_C); retention factor for econazole (k_E); retention factor for miconazole (k_M).

corresponding to neurons. The nodes in the neighboring layers are fully interconnected with links, corresponding to synapses. The strengths of internode connections are called weights (32). Learning occurs in the perceptron by changing connection weights, depending on the algorithm used for training. Training of the ANN is performed by adjusting weights to minimize the root-mean-square (RMS) error of the training data, and thus prevent the same error from recurring. Training algorithms are an integral part of ANN model development. An appropriate topology may still fail to provide a good model, unless trained by a suitable training algorithm. A good training algorithm will shorten the training time, but will achieve better accuracy. Network training can be conducted by employing different kinds of algorithms. In the present study, the back-propagation (BP) algorithm was used. This is a supervised learning method that requires a teacher that knows, or can calculate, the desired output for any given input. It requires the activation function used by the artificial neurons (nodes) to be differentiable. As the name implies, the errors and, therefore, the learning, propagate backward from the output nodes to the inner nodes. Thus, BP is used to calculate the gradient of the network error with respect to the network's modifiable weights. This gradient is almost always used in a simple stochastic gradient-descent algorithm to find weights that minimize the error. BP usually allows the quick convergence on satisfactory local minima of error in the suited kinds of networks.

Experimental

All reagents were of analytical grade. The investigated mixture consisted of the antifungals bifonazole (10 µg/mL), fluconazole, ketoconazole, clotrimazole (250 µg/mL), econazole and miconazole (500 µg/mL). The experiments were performed on the chromatographic system Finnigan Surveyor Thermo Scientific. The analytical column was SunFire C18, 100×3.0 mm, with 3.5 µm particle size. The flow rate was 0.75 mL/min and the detection wavelength was 265 nm. The mobile phases consisted of methanol and water (1% triethylamine, pH adjusted with ortho-phosphoric acid) and their compositions were defined by the CCD using the DesignExpert 7.0 software. In addition to the content of methanol and pH of the water phase, the temperature was chosen as a factor to be varied. The real and coded values of the factors are given in Table I.

Results and Discussion

In HPLC analysis, the information about the retention behavior of analytes is very important. Moreover, the provision of data that might help in the evaluation of the most influential chromatographic parameters, or the creation of a pattern for system behavior, would be the most preferable. The employment of certain chemometrical tools is the most efficient way to achieve these goals. In this study of the retention behavior of azole antifungals, the application of ED was selected in combination with ANNs.

The study started with the selection of the type of stationary phase and mobile phase constituents. Based on the polarity of the investigated substances, the SunFire C18 column, 100×3.0 mm, with 3.5 µm particle size was chosen. The neutral to basic character of the investigated compounds, if they are in their molecular form, would impose long retention times due to the characteristics of the column. On the other hand, the advantage of the selected column is its stability under low pH, which offers the possibility to manipulate the ionization of the analytes using mobile phases of different pH, and thus manipulate their retention. Preliminary studies were conducted to determine significant factors and their domains for the ED. The experiments showed that methanol as organic solvent at approximately 57%, pH of the water phase lower than 4.0 and the presence of 1% of triethylamine (TEA) resulted in an acceptable run time, adequate separation of the investigated substances and satisfactory peak appearances. In addition to these factors, the column temperature was recognized as a factor that could affect the retention. To conduct a smaller number of experiments, TEA was excluded from the design of experiments because it only affects the shapes of peaks.

For the analysis of three chosen factors (methanol content, pH of the mobile phase and column temperature), the changeable CCD was chosen. The lower (−1) and upper (+1) levels were set based on the preliminary experiments, whereas the final experimental space was programmed by DesignExpert 7.0 software. Therefore, due to the high sensitivity of retention factors on the content of methanol, it was varied in a narrow interval (56–59%). The pH value was varied from 2.4 to 3.4 and the change of temperature was in the range from 30 to 40°C. The total number of experiments for three selected factors was 18, in which four experiments represent the replications in the central point to minimize the risk of missing a nonlinear

Table III

Experiments and Results for the Optimization of the Number of Nodes in the Hidden Layer and Number of Experiments in the Training Set

Number	Number of nodes in hidden layer	Number of experiments in training set	RMS for verification set	Regression ratio
1	4	5	0.4813	1.1260
2	4	8	0.5058	1.1970
3	4	12	0.8342	1.0337
4	6	5	0.4839	0.9011
5	6	8	0.8522	1.2415
6	6	12	0.5946	0.9049
7	8	5	0.4235	0.9474
8	8	8	0.5022	1.0388
9	8	12	0.8371	0.6399
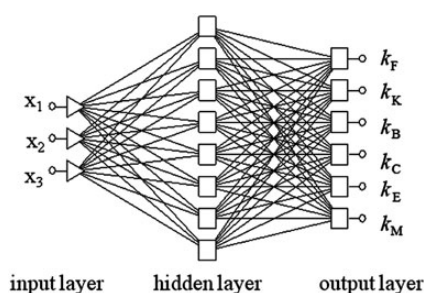
Figure 5. Optimized ANN topology.

relationship within the intervals. As the system outputs, the retention factors, k, of all six investigated substances were chosen. The experimentally obtained data are given in Table I. Also, Figure 2 depicts changes of retention factors for all investigated substances, and the change of experimental conditions designed by the experimental plan is presented in Table I. Also, the chromatograms are given that present the influence of the content of methanol (Figures 3A and 3B) and the pH value of the water phase (Figures 4A, 4B and 4C) on the retention behavior of the investigated azole antifungals.

The next step was the calculation of the effects of the factors, by employing DesignExpert 7.0 software, and the establishment of the appropriate mathematical relationship between the inputs (for three factors) and outputs. Table II shows the equation coefficients in terms of coded factors. Although the results for analysis of variance (ANOVA) tests proved models to be significant (p-value < 0.05) and corresponding R^2 satisfactory, the lack of fit for certain models was also significant (p-value < 0.05). For that reason, the coefficients for those models cannot be trusted, and the significant variables cannot be identified by the application of the ED. The situation is not hopeless, because the obtained set of data can be further evaluated by ANN and the pattern for the analyzed system behavior can be created.

When ANNs are used to produce a network with high predictive ability, the optimization of the network should be conducted first. Generally, the optimization of the network means a choice of the optimal number of neurons in the hidden layer, the number of experiments used for network training and the optimal algorithm for the network training. In the first phase of the investigation, the topology of the network should be set. However, the networks consist of three layers (input, hidden and output layer). In this study, the influence of three

Table IV

Regression Statistics of ANN*

	Tr k_F	Ve k_F	Te k_F	Tr k_K	Te k_K	Ve k_K	Tr k_B	Te k_B	Ve k_B	Tr k_C	Te k_C	Ve k_C	Tr k_E	Te k_E	Ve k_E	Tr k_M	Ve k_M	Te k_M
Data mean	0.453	0.428	0.477	4.541	4.712	2.619	7.952	8.264	4.498	10.026	10.813	4.828	15.583	16.327	9.907	32.205	19.858	34.177
Data SD	0.066	0.042	0.057	2.306	2.230	0.528	4.181	4.118	0.775	6.155	6.212	1.881	6.644	7.193	1.032	14.134	2.031	16.041
Error mean	0.007	0.014	0.018	−0.30	0.425	−0.245	−0.591	0.622	−1.007	−0.967	0.084	−1.416	−0.779	0.176	−1.49	−1.739	−2.994	2.935
Error SD	0.016	0.013	0.008	0.880	0.340	1.124	1.38	0.211	2.262	1.798	1.817	3.146	2.429	1.446	3.198	5.366	7.513	1.621
Absolute error mean	0.016	0.014	0.018	0.630	0.425	0.798	1.05	0.622	1.882	1.494	1.424	2.427	1.829	1.446	2.71	3.957	6.051	2.935
SD ratio	0.253	0.308	0.149	0.382	0.152	2.129	0.330	0.051	2.919	0.292	0.293	1.672	0.365	0.0244	3.098	0.379	3.699	0.101
Correlation	0.9689	0.9987	0.9912	0.9244	0.9997	0.9554	0.9441	0.9998	0.9555	0.9565	0.9998	0.9973	0.9311	0.9997	0.5250	0.9253	0.4871	0.9997

*Note: training (Tr); verification (Ve); testing (Te); retention factor for fluconazole (k_F); retention factor for ketoconazole (k_K); retention factor for bifonazole (k_B); retention factor for clotrimazole (k_C); retention factor for econazole (k_E); retention factor for miconazole (k_M).

chromatography related factors on the retention factors of six azoles was followed. This means that three nodes in the input layer and six nodes in the output layer are defined. In that manner, the basic topology of network was set: 3– × –6. In the next step, the number of nodes in the hidden layer should be optimized.

Based on the preliminary tests, three levels (4, 6 and 8) were assigned for the number of nodes in the hidden layer. In addition, three levels were defined (5, 8 and 12) for the number of experiments in the training set. Factors were simultaneously changed through nine experiments. The experimental setup is presented in Table III, along with the results obtained for the chosen responses.

The lower value of regression ratio was obtained when 12 experiments were used in a training set and with eight nodes in a hidden layer, so the final topology of network was 3–8–6 (Figure 5).

The obtained network was trained with a back-propagation algorithm and regression statistics of the final neural network are presented in Table IV.

For all substances, for the testing set, the correlations were higher than 0.9912, which confirmed the ability of the network to predict the retention of the investigated azoles. Therefore, the presented approach, which consists of two steps, the first when the network was optimized and the second when the optimized network was applied, was successful for the prediction of the retention of azoles in LC. Namely, the obtained network is able to predict the system output (retention factor), not only for investigated factor values, but for any input in the investigated factor range.

Conclusion

In this paper, the retention behavior of the selected antifungal azoles in the liquid chromatographic system was investigated by employing ED in combination with ANNs. A network with satisfactory predictive ability was obtained by network optimization, thus ensuring that the created network could predict any input in the investigated factor range. The lower value of regression ratio was obtained when 12 experiments were used in a training set and with eight nodes in a hidden layer. The final topology of network was 3–8–6. In this way, utilizing ED-ANNs, a pattern was created for analyzing the behaviour of the system.

Acknowledgments

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