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Five different columns in the analysis of basic drugs in hydrophilic interaction liquid chromatography

Research Article

Marko Jovanović[1*], Biljana Jančić Stojanović[*1], Tijana Rakić[1], Anđelija Malenović[1], Darko Ivanović[1], Mirjana Medenica[2]

[1]*University of Belgrade – Faculty of Pharmacy, Department of Drug Analysis, 11221 Belgrade, Serbia*

[2]*University of Belgrade – Faculty of Pharmacy, Department of Physical Chemistry and Instrumental Methods, 11221 Belgrade, Serbia*

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Abstract: Five different columns (two silica, two cyanopropyl and one diol) were investigated in hydrophilic interaction liquid chromatography (HILIC). For the assessment of columns behavior in HILIC mode, six basic drugs (lamotrigine, thioridazine, clozapine, chlorpheniramine, pheniramine and sulpiride) were chosen. The assessment of the influence of the concentration of organic modifier on analytes' retention on each column was provided by fitting the retention data into theoretical models. Utilizing the statistical analysis, the selection of the model that provides better prediction of the retention behavior was enabled. Dual RP–HILIC mechanism was suggested on cyanopropyl and diol columns, therefore the transition points between the two mechanisms on these columns were calculated. Furthermore, in order to investigate the impact of three factors simultaneously on the retention behavior of the analyzed substances on Betasil Silica column, chemometrically-aided empirical models were built. The experiments were conducted according to the matrix of Box–Behnken design and on the basis of the retention data, six quadratic models were obtained and their adequacy was confirmed using ANOVA test. The obtained coefficients of quadratic models enabled the elucidation of both single factor and factor interactions influence. This was also graphically presented in 3D response surface plots.

Keywords: *Hydrophilic interaction chromatography • Retention prediction • Retention behavior • Basic drugs • Box–Behnken design*

1. Introduction

Hydrophilic interaction chromatography (HILIC), which was originally used for the analysis of carbohydrates, amino acids and peptides [1,2], has become increasingly popular lately. This method has specially found the widest application in the analysis of small polar compounds, such as polar drugs that exhibited serious difficulties in the analysis by reversed phase high–performance liquid chromatography (RP–HPLC) [3]. HILIC separation is carried out on polar columns, such as bare silica, similar as in normal phase high–performance liquid chromatography (NP–HPLC), however mobile phase consists of aqueous–organic mixture (more than 50% of organic solvent, usually acetonitrile) in which water is considered the strongest eluting solvent. This powerful technique provides better separation efficiency of small polar compounds than in RP–HPLC method and as a result of a presence of water in mobile phase, their poor solubility in solely organic solvents used in NP–HPLC method is also surpassed. Other reasons for increasing popularity of HILIC are low back pressures during the separation procedures due to low viscosity of the organic–rich mobile phases typically used and its exceptional compatibility with mass spectrometry (MS) [4].

* *E-mail: jancic.stojanovic@pharmacy.bg.ac.rs*

Alpert proposed that the mechanism of HILIC retention involves partitioning of the analyzed compounds between the bulk mobile phase and the partially immobilized water–enriched layer on the surface of the polar stationary phase [5]. However, it has also been suggested that apart from partitioning, surface adsorption and ion–exchange with charges on the stationary phase are included in the retention mechanism as well. The driving force for partitioning is hydrogen bonding [3,6], whereas in the adsorption and in the ion–exchange in HILIC, ionic interactions and dipole–dipole interactions are also included. However, it is also mentioned that hydrophobic interactions in some cases can have a significant influence on the separation [6,7]. The mechanism and the type of forces involved in the particular case depend on the nature of the stationary phase, the polarity and ionization of the analyzed compounds and the composition of the mobile phase [6]. There is a variety of polar columns that could be utilized in chromatographic separations in HILIC mode. Most commonly in use are bare silica columns [8–10] and silica phases modified with diol [11,12], aminopropyl [8,13], amide [14], cyanopropyl [15], zwitterionic [16] and many other functional groups. Their properties, possible applications in HILIC, and potential separation mechanisms can be found in the literature [3,4,17,18].

In this study the mixture of 6 drugs with basic functional groups in their structures were analyzed. The chemical structures of the analyzed substances are presented in Fig. 1. So far, the retention behavior of many basic compounds has been investigated employing this method [8–10,12,19]. However, not many papers in the literature are dealing with these six drugs. Among these substances levosulpiride is reported to be analyzed in HILIC as found in a paper that documents the development of HILIC–MS method for the analysis of levosulpiride in human plasma [20]. Clozapine is also used as one of the test substances in development of the two–dimensional separations using RP–HPLC and HILIC [21]. HILIC method was developed for the determination of lamotrigine in tablets and human plasma as well [22].

The aim of this study was a detailed assessment of retention behavior of 6 basic substances on 5 different columns by theoretical and empirical models in order to broaden the knowledge of chromatographic retention of basic substances on different types of column in HILIC. Theoretical models were applied for the investigation of organic modifier influence. Log *k* values of the analyzed substances were plotted versus the volume fraction of aqueous phase and versus the logarithm of the volume fraction of the aqueous phase in the mobile phase on each column. Additional statistical analysis of the fit of the experimental data into these two models was carried out with the intention to examine their predictive ability. This strategy enabled the possibility to evaluate the influence of the mobile phase composition on the analyte selectivity on each column. Next, broad range of mobile phase composition was investigated so as to assess dual RP–HILIC mechanism on bonded silica phases (diol and cyanopropyl). In the last stage of investigation, the simultaneous influence of the concentration of organic modifier and other chromatographic factors (pH of the aqueous phase and the concentration of ammonium acetate in the aqueous phase) on the retention behavior of the analyzed substances on Betasil Silica column was investigated by empirical models constructed with the aid of chemometrical tools.

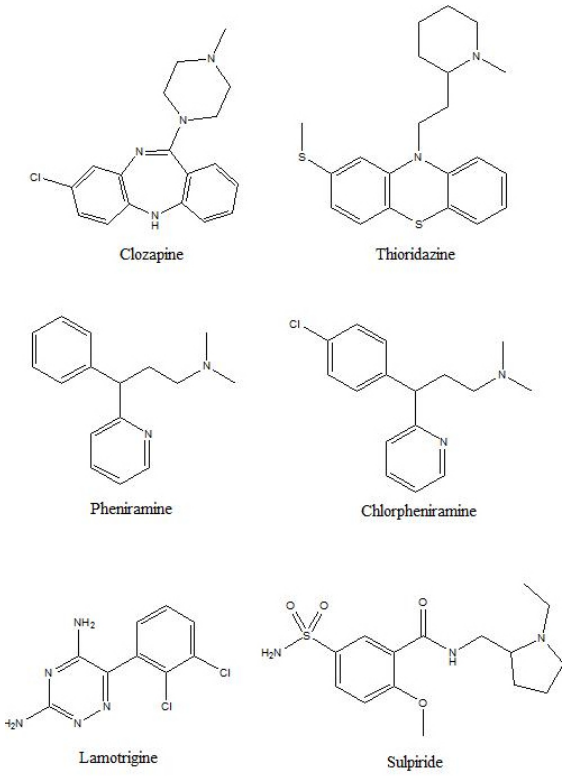

Figure 1. Chemical structures of the analyzed psychotropic drugs.

2. Experimental procedure

2.1. Chemicals

Working standards of clozapine, thioridazine, sulpiride, pheniramine, chlorpheniramine and lamotrigine were used for the preparation of the standard solutions. All reagents used were of 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 prepared mobile phases were filtered through a 0.45 μm membrane filter Alltech (Loceren, Belgium).

2.2. Chromatographic conditions

The chromatographic system Waters Breeze consisted of Waters 1525 Binary HPLC Pump, Waters 2487 UV/VIS dual absorbance detector, column heater 1500 Series and Breeze Software Windows XP for data collection. Separations were performed under HILIC mode on Betasil Silica–100 4.6×100 mm, 5 μm particle size column (Thermo Fisher Scientific Inc., Waltham, MA, USA), Betasil Diol–100 4.6×100 mm, 5 μm particle size column (Thermo Fisher Scientific Inc., Waltham, MA, USA), Betasil CN 4.6×100 mm, 5 μm particle size column (Thermo Fisher Scientific Inc., Waltham, MA, USA), Bakerbond Silica Gel 4.6×250 mm, 5 μm particle size column (J. T. Baker Inc., Phillipsburg, NJ, USA) and Bakerbond Cyanopropyl 4.6×250 mm, 5 μm particle size column (J. T. Baker Inc., Phillipsburg, NJ, USA). UV detection was performed at 254 nm. The samples were introduced through a Rheodyne injector valve with a 20 μL sample loop. Flow rate was 1 mL min^{-1} and the column temperature 30°C. The void volume on each column was determined with toluene in 95% acetonitrile.

2.3. Software

Experimental design and data analysis were performed using Design−Expert® 7.0.0. (Stat−Ease Inc., Minneapolis). Non–linear regression models were built using SPSS 18 (IBM Corp., New York).

2.4. Standard solutions

Stock solutions with concentrations 1 mg mL^{-1} were prepared by dissolving each clozapine, thioridazine, sulpiride, pheniramine, chlorpheniramine and lamotrigine in the mixture acetonitrile–water (85:15 v/v). The stock solutions were diluted up to the concentration of 100 μg mL^{-1} by the same mixture in order to obtain solutions that underwent the analysis. All the samples were stored at 4°C to prevent the degradation.

3. Theoretical details

The modeling of chromatographic responses can be generally performed by two approaches: theoretical modeling and empirical modeling [23–25]. Theoretical modeling connects some of the factors to the responses and can be very useful for the explanation of retention mechanisms. In this paper, equations describing the localized adsorption, non–localized adsorption and dual HILIC–RP retention were applied. On the other hand, the retention data obtained can be fitted into empirical models specially created for particular case. This can be useful when several factors are investigated simultaneously and they interact with each other [23–25]. Nevertheless, empirical models cannot be used for the elucidation of the retention mechanism. In this paper design of experiments and response surface methodology were applied for the creation of empirical models.

3.1. Equations describing the localized and non–localized retention models

In attempt to satisfactorily describe the effect of the stronger eluent in the mobile phase on the retention factor in HILIC it is useful to take into account equations describing other LC modes.

The models that describe RP–HPLC retention suggest the non–localized adsorption mechanism yielding the two parameter Eq. 1 [26,27]:

$$\log k = \log k_0 - m\,\varphi \quad (1)$$

where mobile phases consist of binary aqueous–organic solvents, k is the sample retention factor, φ is the volume fraction of the organic solvent in the binary mobile phase, $\log k_0$ is the extrapolated logarithm of the retention factor in pure water or aqueous buffer (at φ=1) and m is the solvent elution factor, showing the influence of the concentration of organic solvent on the rate of decrease in retention. Eq. 1 could be applied to HILIC separations in which non–localized adsorption controls the retention [4].

The non–aqueous NP–HPLC retention models that describe localized adsorption yield the two parameter Eq. 2 [28,29]:

$$\log k = \log k_0 - m \log \varphi \quad (2)$$

where mobile phases consist of non–polar, and a polar (non–aqueous) solvent, k is the sample retention factor, φ is the volume fraction of polar organic solvent in the mobile phase, $\log k_0$ is the extrapolated logarithm of the retention factor in pure polar (non–aqueous) solvent and m is the solvent elution factor, showing the influence of the concentration of polar solvent on the rate of decrease in retention. If Eq. 2 is applied to aqueous NP systems (HILIC systems), value φ represents the volume fraction of water (or aqueous buffer), the more polar solvent of the aqueous–organic mobile phase and $\log k_0$ is the

extrapolated logarithm of the retention factor in pure water (aqueous buffer) [5].

According to the presented equations, it was earlier suggested that the linearity of log *k vs*. *φ* plots points to the partitioning mechanism, the linearity of log *k vs*. log *φ* plots points to the adsorption mechanism, whereas the lack of fit of experimental data to both equations can be attributed to mixed–mode retention mechanism [4]. However, taking into account that various factors influence the chromatographic retention in HILIC (selection of analytes, the mobile phase composition range, *etc*.) and the approximations made in the derivation of the retention equations, there is no sound physical justification for this statement [4].

3.2. Equations describing dual HILIC–RP mechanism on polar columns and the determination of the transition point

The combination of RP and HILIC retention mechanisms is usually present on bonded silica phases [4]. RP mechanism predominates if the content of water or aqueous buffer is high, and with the increase of the concentration of organic solvent, the retention decreases until it reaches its minimum. With further increase of the concentration of the organic solvent, the retention starts to rise, as a consequence of the HILIC predomination. This dual mechanism is represented in the Eq. 3:

$$\log k = \Psi_1 \log k_1 + \Psi_2 \log k_2 \quad (3)$$

where Ψ_1 and Ψ_2 are the relative contributions of each mechanism to the retention. Eq. 3 can be transformed combining Eqs. 1 and 2 yielding the Eq. 4 [30]:

$$\log k = a_1 + m_{RP}\,\varphi(H_2O) - m_{HILIC} \log \varphi(H_2O) \quad (4)$$

where $\varphi(H_2O)$ is the volume fraction of the water (or of the aqueous buffer) in the mobile phase, m_{RP} is the parameter that characterizes the effect of the increasing concentration of water on the increasing RP contribution to the retention, m_{HILIC} is the measure of decreasing HILIC contribution by increasing the water content in the mobile phase and a_1 is an empirical constant. The parameters a_1, m_{RP} and m_{HILIC} of Eq. 4 can be determined by creating non–linear regression models through the calculation of retention factors measured at different volume fractions of water in the mobile phase. The obtained log $k/\varphi(H_2O)$ plot is parabolic and its minimum at the "U–turn" of volume fraction of water (or an aqueous buffer), φ_{min}, represents the amount of aqueous phase in the mobile phase at witch occurs the transition between RP and HILIC mechanism [4]:

$$\varphi_{min} = (0.434\, m_{HILIC})/m_{RP} \quad (5)$$

However, it should be noted that Eq. 4 fails to adequately describe the HILIC system at very low concentrations of water, lower than 2% ($\varphi_{min} < 0.02$) [29].

3.3. Empirical equations obtained by design of experiments methodology

For the investigation of simultaneous influence of several factors on the retention behavior, experimentally obtained data can be fitted to polynomial equation of adequate degree. The most frequently applied is second–order polynomial equation presented in the Eq. 6 [31]:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad (6)$$

where y is the response, x_1, x_2 and x_3 are the factors, b_0 is the intercept, b_1, b_2 and b_3 are the coefficients representing the linear single factor influence, b_{12}, b_{13} and b_{23} are the coefficients representing the factor interaction influence and b_{11}, b_{22} and b_{33} are the coefficients representing the quadratic single factor influence.

4. Results and discussion

4.1. Theoretical models

The preliminary studies represent the first and logical step in the process of in–depth assessment of the retention behavior. This phase is rather important and determines the direction of the following steps. During this particular research preliminary studies included the conducting of various experiments on different columns, using the different compositions of the mobile phase. The main purpose of this first step was to choose suitable columns on which the selected substances were to be analyzed. Regarding the data obtained, two silica, two cyanopropyl and one diol column (whose properties are given in the experimental section) were chosen for further research. It should be noted that two silica and two cyanopropyl columns differ from one another in their length and in the producer, however in this study, both pairs of columns were included to prove the consistency of the obtained results. The preliminary experiments were also rather useful in a rough estimation of the ranges of the acetonitrile concentration in the mobile phase, the concentration of the buffer (ammonium acetate) in the aqueous phase and pH of the aqueous phase. The main criterion in this process was the achievement of satisfactory separation of the analyzed substances on different columns within the reasonable

duration of chromatographic runs in order to enable the collecting of the retention data of all the substances analyzing the single mixture. This strategy led to substantially faster and doubtless more economic data collection.

In order to assess solely the influence of the concentration of organic modifier in the mobile phase on various stationary phases, in the next step, the change in the ion–exchange influence had to be eliminated. This was achieved by keeping the buffer concentration in the aqueous phase and pH of the aqueous phase constant at 20 mmol L^{-1} and at 3.5, respectively, whereas the volume fraction of the aqueous phase was varied in the range from 0.1 to 0.3 with the increment of 0.05 on each column. After the experiments had been carried out, the retention factors of the analyzed compounds were calculated and then transformed to logarithmic values (log *k*), which allowed the construction of log *k vs*. volume fraction of the aqueous phase (Fig. 2A) and log *k vs*. log volume fraction of the aqueous phase (Fig. 2B) plots that depict the influence of the concentration of the aqueous buffer on the overall retention on all five columns.

It should be noted that lamotrigine exhibited weak retention and eluted within the peak of the mobile phase, therefore the retention data of lamotrigine obtained on the examined columns was neither presented in Figs. 2A and 2B nor discussed in further text. Regarding the pKa values of the analyzed substances (pKa values of lamotrigine, thioridazine, clozapine, chlorpheniramine, pheniramine, and sulpiride are 3.4, 9.5, 7.6, 9.1, 9.3, and 10.2, respectively [32]) and the fact that pH of the overall mobile phase is definitely higher than 3.5, it appears that only lamotrigine is in its unionized state. This is the most probable explanation for its weak retention. The goodness of fit of the retention data to the Eq. 1 and Eq. 2 is presented in Table 1. Regarding the values of coefficients of determination, retention data on all the columns better fit to Eq. 2 in the range 10%–30% of the aqueous buffer content (HILIC range), implying that localized adsorption model is more suitable for retention prediction of the analyzed substances in the given range. On the other hand, due to generally low levels of coefficients of determination, on Bakerbond CN column none of the equations could satisfactorily describe the retention behavior of the analyzed substances. It should be noted that good fit of the experimental data in either of those two models cannot be a solid proof to distinguish which process is dominant in the retention mechanism: adsorption or partitioning. In particular case, because of the various factors influencing the retention, both of the processes are probably involved in the overall retention mechanism.

log *k* values of chlorpheniramine, clozapine and thioridazine on Betasil CN column, of pheniramine, chlorpheniramine, clozapine and thioridazine on Bakerbond Cyanopropyl column and of all the analyzed substances on diol column decreased with the increasing of the aqueous buffer content and then at some point started to rise implying the presence of dual HILIC–RP mechanism in the broad range of concentrations of the aqueous phase in the mobile phase (5–95%). HILIC–RP retention data is presented in Fig. 3. Table 2 shows the coefficients a_1, m_{RP} and m_{HILIC} of Eq. 4 determined utilizing non–linear regression, with the coefficients of determination in the range 0.95–0.99 implying that those models were acceptable. The obtained coefficients (m_{RP} and m_{HILIC}) were then used to calculate U–turn concentration of the aqueous buffer (φ_{min}). The transition point between RP and HILIC mechanism on three columns of the analyzed substances are given in Table 2. On Betasil Diol column φ_{min} were about 0.40, on Betasil CN column φ_{min} varied in the range 0.29–0.41, whereas on Bakerbond Cyanopropil column in the range 0.18–0.34. This undoubtedly led to the conclusion that the nature of the analyzed substances might have a substantial impact on the equation parameters and consequently on the position of the transition points.

All the chromatograms obtained during this phase of the research are given in Fig. 4.

On Betasil Silica column (lesser length) the lipophilicity of the analyzed substances was the major factor that determined their elution order (lamotrigine, thioridazine, clozapine, chlorpheniramine, pheniramine and sulpiride, respectively) (Fig. 4A). LogP values of lamotrigine, thioridazine, clozapine, chlorpheniramine, pheniramine and sulpiride are 2.5, 5.9, 3.7, 3.4, 3.2, and 0.57, respectively [32]. Obviously, more polar substances (chlorpheniramine, pheniramine and sulpiride) were retained more strongly, than the less polar (thioridazine and clozapine), which was particularly the case in highly organic mobile phases. The explanation for the unexpected behavior of lamotrigine was given previously. The retention data obtained on Bakerbond Silica column (greater length) was completely analogous to this column, except for the extended retention times, as a consequence of the column length (Fig. 4B). If the results on Betasil Silica column are compared with the results obtained on Betasil Diol column (Fig. 4E) of the same length, it can be easily noticed that the elution order remained the same. However, all the substances eluted earlier on diol column. This difference between retention times occurred as a result of the existence of ionizable silanol groups on the surface of the silica and the absence of potential ionizable groups on the surface of diol stationary phase [1,5]. Thus, the ion–exchange

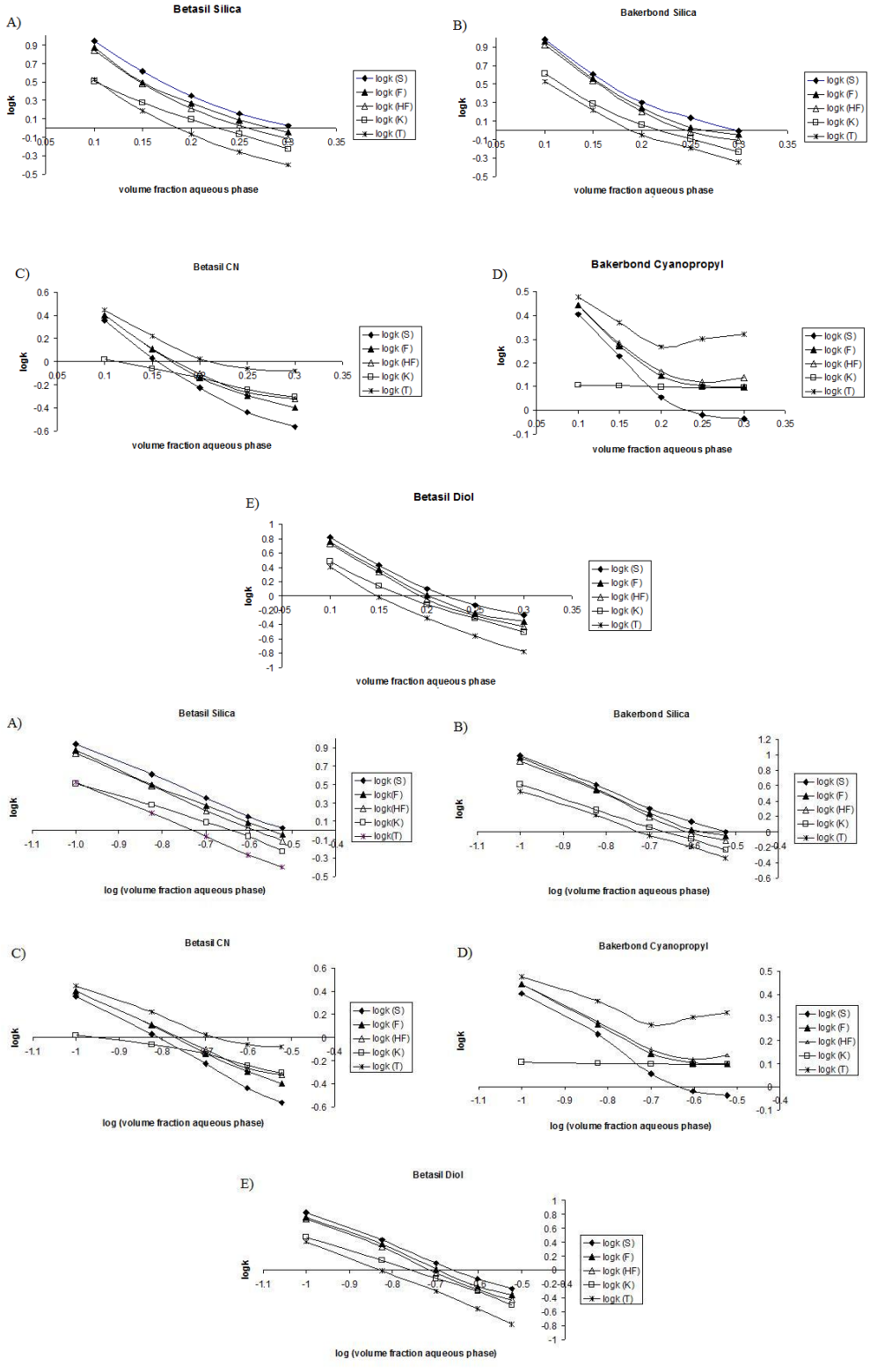

Figure 2. A) Plots of log *k* *vs*. volume fraction of the aqueous phase in the mobile phase and B) plots of log *k* *vs*. log volume fraction of the aqueous phase in the mobile phase. Solute identities: diamonds–sulpiride (S), full triangles–pheniramine (F), empty triangles–chlorpheniramine (HF), stars–clozapine (K), empty squares–thioridazine (T). Mobile phase: ACN–water in the range from (70:30 v/v) to (90:10 v/v) containing 20 mmol L^{-1} ammonium acetate pH 3.5. Chromatographic conditions: UV detection at 254 nm, column temperature 30°C, flow rate 1 mL min^{-1}. The column types are noted on top of each plot.

Table 1. The parameters log k_0 and m of the analyzed substances obtained using Eq. 1 and Eq. 2 in HILIC region of the mobile phase contents (10–30% of the aqueous phase).

Columns	Eq. 1			Eq. 2		
	log k_0	m	R^{2*}	log k_0	m	R^{2*}
Betasil Silica						
Sulpiride	1.335	–4.585	0.9722	–1.005	–1.950	0.9990
Pheniramine	1.238	–4.502	0.9587	–1.069	–1.928	0.9979
Chlorpheniramine	1.237	–4.727	0.9677	–1.179	–2.016	0.9992
Clozapine	0.842	–3.625	0.9825	–0.993	–1.552	0.9938
Thioridazine	0.955	–4.730	0.9707	–1.450	–2.001	0.9940
Bakerbond Silica						
Sulpiride	1.383	–4.834	0.9555	–1.093	–2.069	0.9937
Pheniramine	1.369	–5.101	0.9412	–1.253	–2.196	0.9907
Chlorpheniramine	1.355	–5.236	0.9492	–1.331	–2.246	0.9917
Clozapine	0.959	–4.163	0.9673	–1.170	–1.776	0.9992
Thioridazine	0.896	–4.320	0.9684	–1.311	–1.840	0.9978
Betasil CN						
Sulpiride	0.755	–4.618	0.9725	–1.601	–1.964	0.9988
Pheniramine	0.735	–3.993	0.9612	–1.310	–1.707	0.9975
Chlorpheniramine	0.694	–3.656	0.9422	–1.185	–1.573	0.9907
Clozapine	0.190	–1.681	0.9567	–0.654	–0.697	0.9720
Thioridazine	0.651	–2.699	0.9010	–0.747	–1.176	0.9708
Bakerbond Cyanopropyl						
Sulpiride	0.578	–2.256	0.9070	–0.588	–0.980	0.9717
Pheniramine	0.556	–1.718	0.8524	–0.342	–0.759	0.9441
Chlorpheniramine	0.539	–1.553	0.8145	–0.276	–0.692	0.9174
Clozapine	0.110	–0.051	0.9248	0.084	–0.022	0.9840
Thioridazine	0.504	–0.803	0.5597	0.085	–0.360	0.6891
Betasil Diol						
Sulpiride	1.287	–5.493	0.9658	–1.521	–2.343	0.9977
Pheniramine	1.258	–5.790	0.9533	–1.704	–2.473	0.9873
Chlorpheniramine	1.258	–5.955	0.9682	–1.780	–2.532	0.9936
Clozapine	0.928	–4.906	0.9870	–1.561	–2.066	0.9940
Thioridazine	0.976	–6.040	0.9884	–2.083	–2.537	0.9905

* R^2 – coefficient of determination

contribution plays an important role in chromatographic separation on silica columns in HILIC. As it was discussed above, on Betasil CN column (lesser length) different substances were influenced by a variety of interactions, which eventually led to continuous replacement of peak positions with increasing percentage of the organic solvent in mobile phase (Fig. 4C). When RP mechanism was dominant (lower acetonitrile concentration in mobile phase), the retention factors decreased with increasing percentage of acetonitrile and the elution order of the examined substances depended on their lipophilicity. In the mobile phases containing greater concentrations of acetonitrile (beyond the amount required for the transition between RP and HILIC mechanism), when HILIC interactions were dominant, the retention behavior was completely the opposite, which was usually in the range 70%–90% of acetonitrile concentration in the mobile phase. Similar situation with small variations

Table 2. Best–fit parameters a_1, m_{RP} and m_{HILIC} of Eq. 4, coefficients of determination (R^2) and U–turn concentrations calculated (φ_{min}) using Eq. 5 for sulpiride, pheniramine, chlorpheniramine, clozapine and thioridazine on Betasil diol, Betasil CN and Bakerbond Cyanopropyl columns in the composition range 5–95% of aqueous phase in the aqueous–organic mobile phase.

Compound	RP–HILIC				
	a_1	m_{RP}	m_{HILIC}	R^2	φ_{min}
Sulpiride					
Betasil diol	-2.123	2.664	2.468	0.9996	0.4021
Pheniramine					
Betasil diol	-3.088	3.624	3.446	0.9994	0.4127
Bakerbond Cyanopropyl	-1.364	2.022	1.604	0.9956	0.3443
Chlorpheniramine					
Betasil diol	-3.290	3.856	3.605	0.9993	0.4057
Betasil CN	-2.527	2.794	2.651	0.9997	0.4118
Bakerbond Cyanopropyl	-1.472	2.405	1.674	0.9941	0.3021
Clozapine					
Betasil diol	-2.325	2.104	1.933	0.9967	0.3987
Betasil CN	-1.168	1.151	1.080	0.9468	0.4072
Bakerbond Cyanopropyl	-0.628	1.420	0.599	0.9692	0.1831
Thioridazine					
Betasil diol	-3.407	3.610	3.413	0.9958	0.4103
Betasil CN	-2.884	4.346	2.901	0.9991	0.2897
Bakerbond Cyanopropyl	-1.783	3.703	1.889	0.9991	0.2214

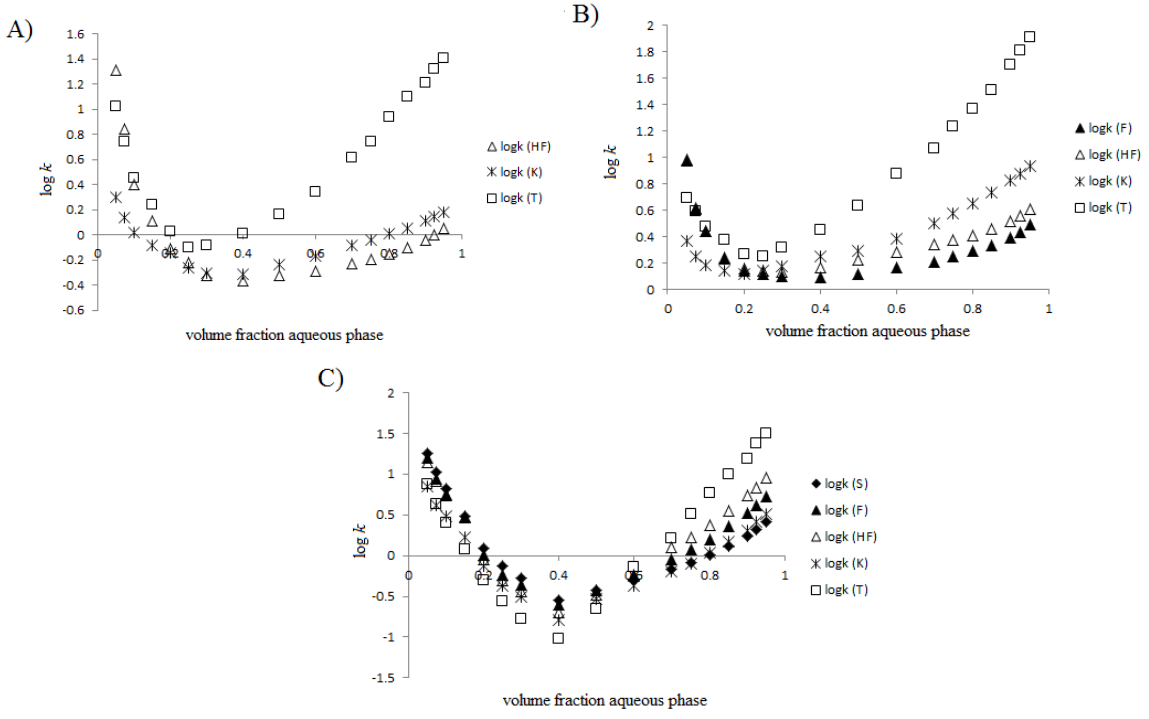

Figure 3. RP–HILIC data represented in log *k* *vs.* volume fraction of the aqueous phase plots in broad range of mobile phase compositions (5–95 %) on A) Betasil CN column, B) Bakerbond Cyanopropyl column and C) Betasil diol column. Solute identities: diamonds–sulpiride (S), full triangles–pheniramine (F), empty triangles–chlorpheniramine (HF), stars–clozapine (K), empty squares–thioridazine (T). Mobile phase: ACN–water in the range from (95:5 v/v) to (5:95 v/v) containing 20 mmol L^{-1} ammonium acetate pH 3.5. For other conditions see Fig. 2.

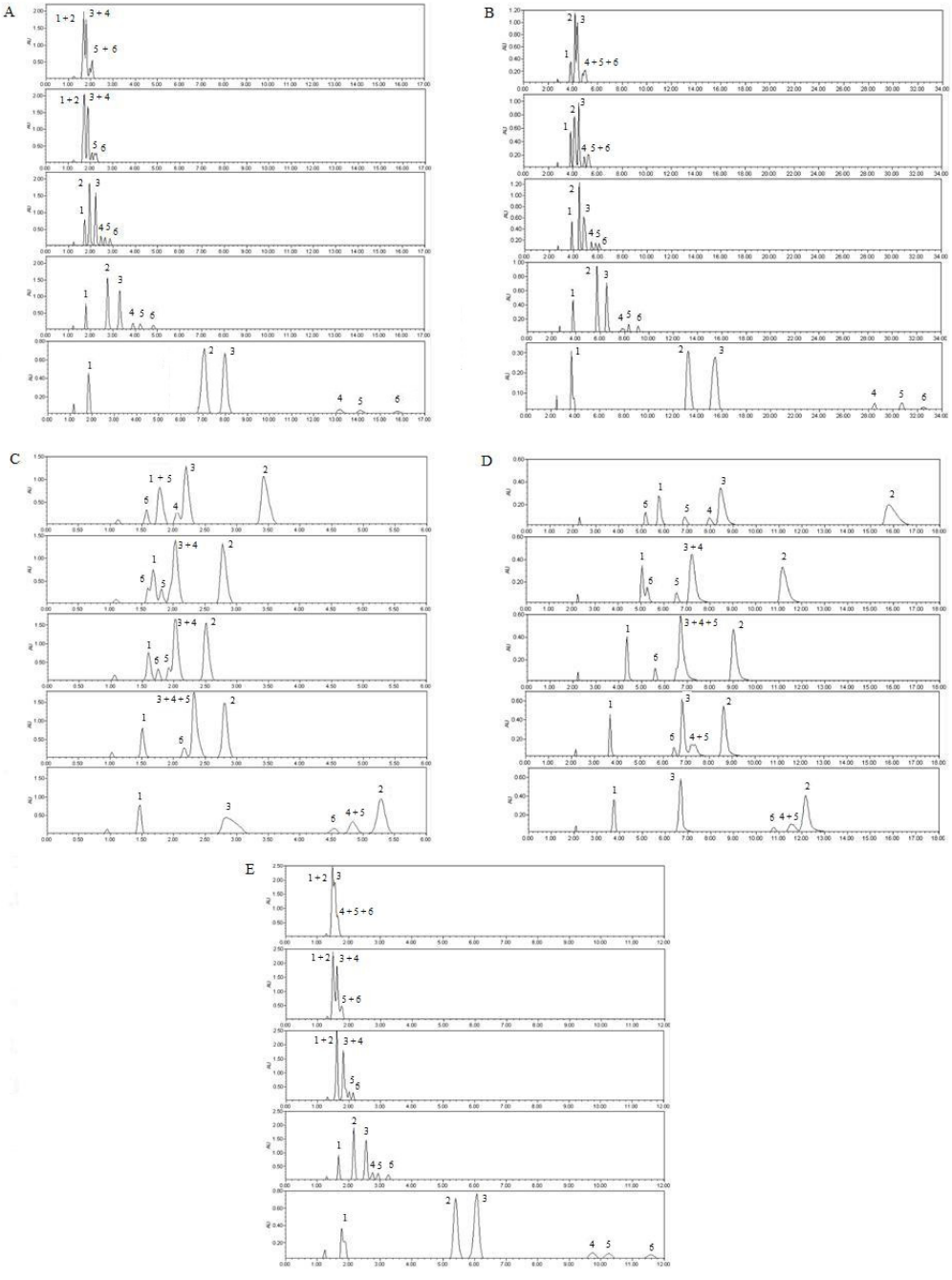

Figure 4. Chromatograms of the analyzed substances on A) Betasil Silica column, B) Bakerbond Silica column, C) Betasil CN column, D) Bakerbond Cyanopropyl column and E) Betasil diol column. Peak identities: 1–lamotrigine, 2–thioridazine, 3–clozapine, 4–chlorpheniramine, 5–pheniramine, 6–sulpiride. For other conditions see Fig. 2.

were observed on the Bakerbond Cyanopropyl column (greater length) (Fig. 4D), therefore the proposed mixed separation mechanism on this type of column was confirmed. As it was the case with silica columns, retention times were also extended due to the greater column length and some peaks were better separated under certain conditions.

4.2. Empirical models

Next step in this study was the investigation of simultaneous influence of organic modifier concentration, pH of the aqueous phase and ammonium acetate concentration in the aqueous phase on the retention of the analyzed substances on Betasil Silica column. This column was particularly selected due to the presence of

Table 3. Plan of experiments and the obtained results.

№	x_1	x_2	x_3	k_1	k_2	k_3	k_4	k_5	k_6
1	80 (-1)*	2.5 (-1)*	20 (0)*	0.659	0.987	1.431263	1.754	1.955	2.240
2	90 (+1)	2.5 (-1)	20 (0)	0.925	4.493	6.339	8.559	9.148	10.193
3	80 (-1)	4.5 (+1)	20 (0)	0.048	1.079	1.079	2.346	2.663	3.078
4	90 (+1)	4.5 (+1)	20 (0)	0.047	1.742	3.577	8.386	9.211	9.711
5	80 (-1)	3.5 (0)	5 (-1)	0.274	1.474	1.884	2.469	2.748	3.136
6	90 (+1)	3.5 (0)	5 (-1)	0.001	4.797	7.066	13.641	14.746	15.454
7	80 (-1)	3.5 (0)	35 (+1)	0.160	0.732	1.100	1.519	1.732	2.168
8	90 (+1)	3.5 (0)	35 (+1)	0.092	2.887	3.160	6.971	7.528	9.181
9	85 (0)	2.5 (-1)	5 (-1)	1.175	2.376	3.154	3.819	4.163	4.638
10	85 (0)	4.5 (+1)	5 (-1)	0.046	2.286	4.550	9.010	10.012	10.347
11	85 (0)	2.5 (-1)	35 (+1)	0.673	1.519	2.293	2.969	3.239	3.738
12	85 (0)	4.5 (+1)	35 (+1)	-0.045	1.121	1.456	3.543	3.956	4.689
13	85 (0)	3.5 (0)	20 (0)	0.115	1.830	2.295	3.571	3.934	4.724
14	85 (0)	3.5 (0)	20 (0)	0.144	1.598	2.052	3.097	3.431	4.111
15	85 (0)	3.5 (0)	20 (0)	0.146	1.579	2.032	3.083	3.414	4.106
16	85 (0)	3.5 (0)	20 (0)	0.156	1.783	2.255	3.477	3.833	4.600

x_1–concentration of acetonitrile (%); x_2–pH of the aqueous phase; x_3–concentration of ammonium acetate in the aqueous phase (mmol L^{-1}); k_1–retention factor of lamotrigine; k_2–retention factor of thioridazine; k_3–retention factor of clozapine; k_4–retention factor of chlorpheniramine; k_5–retention factor of pheniramine; k_6–retention factor of sulpiride
** in the brackets coded values for factor levels are given*

free silanol groups on its surface capable of ionization allowing the ion–exchange process with the ionized analytes [3,4,33]. Thus, it is logical to expect that apart from acetonitrile concentration in the mobile phase, pH of the mobile phase and buffer concentration will also influence their retention. As far as diol and cyanopropyl columns are concerned, there is a lack of charge on their surface (except a small amount of residual silanol groups) [3,4,33], therefore it is reasonable to assume the absence of influence of pH of the mobile phase and of buffer concentration on chromatographic retention. This was also confirmed during the preliminary phase of the research.

The empirical models were developed using the experimental design and response surface methodology. The most suitable choice in the present study was the application of Box–Behnken design as a tool that allowed the assessment of the influence of three factors on three levels [34,35]. It involved twelve experiments plus 4 central point replications (experimental plan is given in Table 3). The ranges of factor values are chosen according to the data obtained in the preliminary study. The obtained experimental values of the retention factors of the analyzed drugs are presented in Table 3. Quadratic models were suggested for all the outputs and their adequacy was examined with statistical analysis (ANOVA). Coefficients for coded factor levels of the proposed equations with corresponding *p*–values, coefficients of determination (R^2) and adjusted values of the coefficients of determination (adj. R^2) are presented in Table 4. All the obtained models had high values of R^2 (>0.94) and adj. R^2 (>0.85), which revealed that experimental data fitted well into the second–order polynomial equations.

The obtained models allowed the construction of 3D response surface plots in which the influence of single factors and their interactions can be visualized (Fig. 5).

The obtained coefficients of quadratic model (Table 4) allowed the interpretation of factors influence. The significant linear or quadratic terms indicate linear or quadratic dependence of the response on the single investigated factors. The significant interaction terms indicate that the influence of factors is not independent, but that the response changes differently when both factors are changed simultaneously. In this particular case, it is expected that the simultaneous change of acetonitrile content and pH value of water phase is not independent since the high acetonitrile concentration affects the overall pH of mobile phase. The same situation is expected between acetonitrile content and concentration of ammonium acetate in the aqueous phase.

Table 4. Coefficients of quadratic model.

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$

	k_1		k_2		k_3		k_4		k_5		k_6	
	coefficient	p-value	coefficient	p-value	coefficient	p-value	coefficient	p-value	coefficient	p-value	coefficient	p-value
b_0	0.17	0.0046*	1.66	0.0024*	2.07	0.0018*	3.18	0.0011*	3.95	0.0011*	4.74	0.0029*
b_1	-0.0095	0.8519	1.21	0.0001*	1.83	0.0001*	3.68	<0.0001*	3.94	<0.0001*	4.18	0.0001*
b_2	-0.42	0.0001*	-0.39	0.0314*	-0.32	0.1688	0.77	0.0746	0.92	0.0553	0.81	0.1295
b_3	-0.077	0.1668	-0.58	0.0060*	-1.08	0.0018*	-1.74	0.0028*	-1.9	0.0027*	-1.72	0.0099*
b_{12}	-0.067	0.3708	-0.71	0.0118*	-0.6	0.0819	-0.19	0.7190	-0.16	0.7777	-0.45	0.5139
b_{13}	0.051	0.4855	-0.29	0.1926	-0.78	0.0354*	-1.43	0.0304*	-1.55	0.0297*	-1.33	0.0496*
b_{23}	0.1	0.1877	-0.077	0.7121	-0.56	0.1013	-1.15	0.0632	-1.28	0.0573	-1.19	0.1197
b_{11}	-0.04	0.5848	0.53	0.0372*	0.74	0.0430*	1.7	0.0155*	1.57	0.0285*	1.54	0.0578
b_{22}	0.29	0.0057*	-0.12	0.5794	0.3	0.3357	0.38	0.4797	0.22	0.6981	-0.096	0.8880
b_{33}	0.0025	0.9721	0.28	0.2079	0.5	0.1370	1.27	0.0462*	1.17	0.0767	1.21	0.1151
R^2	0.9416		0.9535		0.9580		0.9649		0.9639		0.9503	
adj. R^2	0.8540		0.8837		0.8951		0.9122		0.9099		0.8758	

k_1–retention factor of lamotrigine; k_2–retention factor of thioridazine; k_3–retention factor of clozapine; k_4–retention factor of chlorpheniramine; k_5–retention factor of pheniramine; k_6–retention factor of sulpiride
** Coefficients significant for p–value < 0.05*

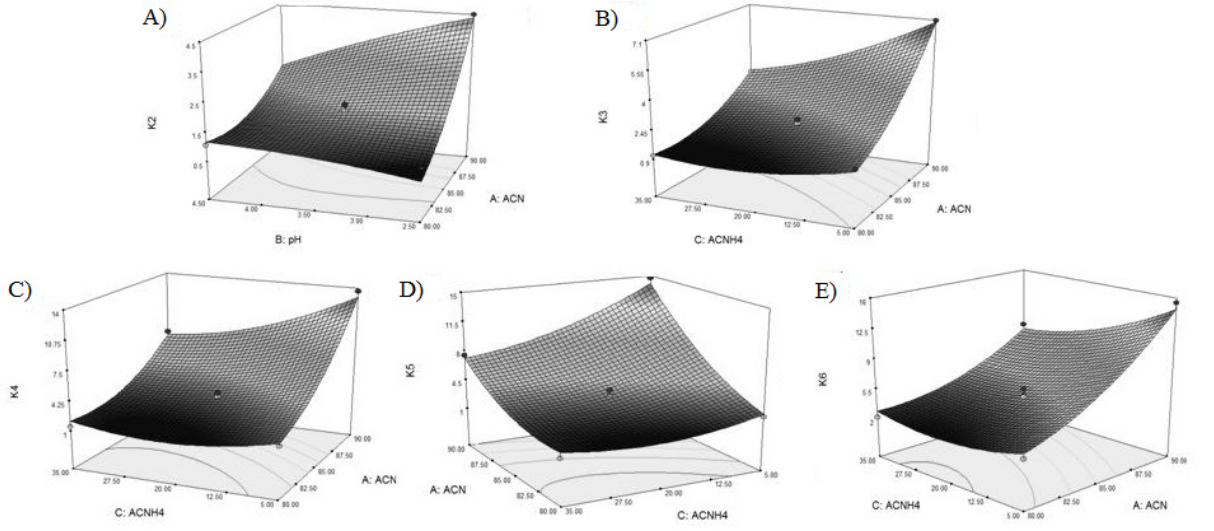

Figure 5. 3D response surface plots: A) k_2=f (acetonitrile concentration, pH of the aqueous phase); B) k_3= f (acetonitrile concentration, ammonium acetate concentration in the aqueous phase); C) k_4=f (acetonitrile concentration, ammonium acetate concentration concentration in the aqueous phase); D) k_5=f (acetonitrile concentration, ammonium acetate concentration concentration in the aqueous phase); E) k_6=f (acetonitrile concentration, ammonium acetate concentration concentration in the aqueous phase).

Lamotrigine was excluded from the discussion again due to its weak retention. Analyzing the Table 4 it can be noted that acetonitrile significantly influenced all the analyzed substances. Both linear (b_1) and quadratic (b_{11}) terms for this factor were significant for almost all substances. As it was noticed in the earlier phases of the research, with the increase of the acetonitrile concentration in the mobile phase, the retention factors of the examined substances increased. All the examined substances showed significant decrease in retention while increasing the concentration of ammonium acetate in the aqueous phase (the factor b_3 was significant for all the substances). As it is suggested in the literature, higher buffer concentrations lead to weaker retention due

to the competition of positively charged buffer molecules and ionized basic compounds for negatively charged silanol groups on the surface of bare silica [29].

The interaction coefficient b_{12} was significant for thioridazine referring to the fact that interaction of acetonitrile and pH affected this substance retention (Fig. 5A). For clozapine, chlorpheniramine, pheniramine and sulpiride the interaction coefficients b_{13} were significant implying that the combination of the high acetonitrile concentrations and low ammonium acetate concentrations caused these substances to retain stronger (Figs. 5B–5E). It should be noted that b_2 term is not significant in case of all substances, except thioridazine. Consequently, interaction b_{12} terms for those substances were also not significant; therefore the expected interaction between acetonitrile content and pH of the aqueous phase was not proved.

5. Conclusion

In this paper the thorough investigation of the retention behavior of the mixture of six basic drugs (lamotrigine, thioridazine, clozapine, chlorpheniramine, pheniramine and sulpiride) on five columns (two silica, two cyanopropyl and one diol) in hydrophilic interaction chromatography was presented. On the basis of the experimental data obtained on each column in the range 10%–30% of the aqueous buffer in the mobile phase, log *k vs*. volume fraction of the aqueous phase and log *k vs*. log volume fraction of the aqueous phase plots were constructed. The goodness of fit of the experimental data to localized and non–localized adsorption models was statistically evaluated. Due to higher values of coefficients of determination, localized adsorption models showed better predictive ability of the chromatographic retention of the analyzed compounds on all the columns. However, on Bakerbond Cyanopropyl column, the coefficients of determination were generally low for both models indicating their inability to predict the analytes' retention. On cyanopropyl and diol columns, analyzing the broad range of the aqueous phase in the mobile phase (5–95%), dual HILIC–RP mechanism was suggested and the transition points between these two mechanisms were determined for each analyte.

Finally, the examination of the influence of three factors simultaneously on the analytes' retention on Betasil Silica column using Box–Behnken design was carried out. The obtained second–order polynomial models were statistically analyzed using ANOVA and their good predictive ability was confirmed (R^2>0.94). Apart from lamotrigine, which showed weak retention, all the analyzed substances were most strongly influenced by acetonitrile concentration. The concentration of ammonium acetate in the aqueous phase showed substantial, but weaker influence. In case of thioridazine, factor interaction between acetonitrile and pH of the aqueous phase was significant. In case of clozapine, pheniramine, chlorpheniramine and sulpiride, factor interaction between acetonitrile and ammonium acetate concentration was significant. This was also illustrated in 3D response surface plots.

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