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Article

Chromatographic Analysis of Olopatadine in Hydrophilic Interaction Liquid Chromatography

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In this paper, chromatographic analysis of active substance olopatadine hydrochloride, which is used in eye drops as antihistaminic agent, and its impurity *E* isomer by hydrophilic interaction liquid chromatography (HILIC) and application of design of experiments (DoE) methodology are presented. In addition, benzalkonium chloride is very often used as a preservative in eye drops. Therefore, the evaluation of its chromatographic behavior in HILIC was carried out as well. In order to estimate chromatographic behavior and set optimal chromatographic conditions, DoE methodology was applied. After the selection of important chromatographic factors, Box–Behnken design was utilized, and on the basis of the obtained models factor effects were examined. Then, multi-objective robust optimization is performed aiming to obtain chromatographic conditions that comply with several quality criteria simultaneously: adequate and robust separation of critical peak pair and maximum retention of the first eluting peak. The optimal conditions are identified by using grid point search methodology. The experimental verification confirmed the adequacy of the defined optimal conditions. Finally, under optimal chromatographic conditions, the method was validated and applicability of the proposed method was confirmed.

Introduction

Poor retention of polar and/or ionic compounds or unsuitable peak shape of polar substances in reversed-phase liquid chromatography (RP-LC) in many cases was solved by application of hydrophilic interaction liquid chromatography (HILIC). Generally, HILIC separation is carried out on polar columns (such as bare silica) with mobile phases consisting of an aqueous–organic mixture (>50% of organic solvent, usually acetonitrile). Aqueous phase usually contains buffers such as ammonium acetate or ammonium formate. Such a mobile phase composition is compatible with mass detection, which could be considered an additional advantage of the HILIC method. Significance of the HILIC method could be confirmed through the increasing number of papers dealing with its application in drug analysis. Some of the most recently published are references (1–6). Taking into account analytes' characteristics, it was challenging to investigate behavior of geometric isomers *Z* and *E* of olopatadine in HILIC mode. Significance of this investigation is especially important due to the fact that one isomer is active substance and another is impurity. Because benzalkonium chloride is a typical additive in pharmaceutical formulations of this drug, it was included in the investigation as well. The chemical structures of the analyzed substances are presented in Figure 1.

Olopatadine IUPAC name is {(11*Z*)-11-[3-(dimethylamino)-propylidene]-6,11-dihydrodibenzo[*b,e*]oxepin-2-yl}acetic acid, and it is an antihistamine agent used in eye drops. A literature survey has shown that olopatadine has been determined by spectrophotometric methods (7) in eye drops, by high performance thin layer chromatographic (HPTLC) method (8) in bulk drug and eye drops and by stability indicating methods in tablets (9) and in bulk drug and tablets (10). Further on, for the analysis of olopatadine under International Conference on Harmonization (ICH) recommended stress conditions, liquid chromatography (LC) and LC coupled with mass spectroscopy (MS)/time of flight detector were used (11). For determination in biological samples, LC with MS detection is described (12, 13). Finally, olopatadine is official in The United States Pharmacopeia and The National Formulary (14), where the gradient RP-HPLC method on a C18 column for the determination of olopatadine and its impurities is suggested.

However, this study also covers investigation of chromatographic analysis of benzalkonium chloride in HILIC mode. Namely, commercially available eye drops contain benzalkonium chloride as preservative. Given that it is very often used a preservative in eye drops, many papers dealing with its determination by the LC method can be found (15–20). However, no papers dealing with its determination in HILIC mode are available in the literature.

Taking into account the literature survey, it is evident that there are no papers dealing with chromatographic analysis of olopatadine and its impurity, especially simultaneously with benzalkonium chloride.

Thus, the aim of this study was to investigate chromatographic behavior of geometric isomers of olopatadine and benzalkonium chloride in HILIC mode with the aim to define optimal chromatographic conditions suitable for their determination in eye drops. In order to get the most suitable chromatographic conditions, design of experiments (DoE) methodology was employed. On the basis of the obtained mathematical models, an appropriate interpretation of chromatographic behavior of investigated analytes in the mixture was carried out. The multi-objective robust optimization is performed aiming to achieve maximal retention of the first eluting peak, satisfactory separation of the critical peak pair and satisfactory robustness of the obtained separation criterion in terms of all investigated factors. The measurement of the robustness is performed calculating the partial derivatives of the response function with respect to the investigated factors. Grid point search methodology was applied for identification of the optimal chromatographic conditions.

Experimental

Chemicals

All used reagents were of the analytical grade. The mobile phase and the solvents were prepared from acetonitrile (Sigma,

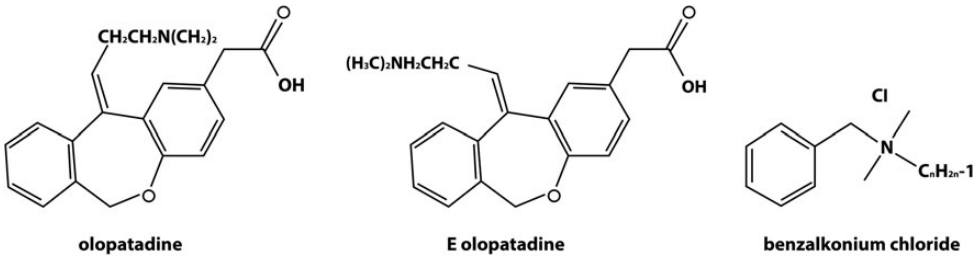

Figure 1. Chemical structure of analyzed substances.

St Louis, MO, USA), ammonium acetate (J. T. Baker, The Netherlands), glacial acetic acid (Zorka Pharma, Serbia) and HPLC grade water (Simplicity 185; Millipore, Germany). Working standards of olopatadine hydrochloride (STADA, Germany), its impurity *E* olopatadine (USV Limited, India) and benzalkonium chloride (Hemofarm AD, Serbia) were used.

Method's optimization

Standard solutions for method's optimization

Stock solutions were prepared by dissolving the substances into the acetonitrile–aqueous phase (15 mM ammonium acetate, pH 4.5) 82.50:17.50 v/v in order to obtain the concentration of 1 mg mL^{-1} for olopatadine hydrochloride and benzalkonium chloride and 0.1 mg mL^{-1} for *E* olopatadine. From the stock solution, the mixture that contained 0.5 mg mL^{-1} olopatadine hydrochloride, 5 μg mL^{-1} *E* olopatadine and 50 μg mL^{-1} benzalkonium chloride was prepared in 15 mM ammonium acetate. All the samples were stored at 4°C to prevent degradation.

Chromatographic conditions

The experiments were performed on the chromatographic system Finnigan Surveyor Thermo Scientific. ChromQuest was used for data collection. The analytical columns were Betasil Cyano (100 mm × 4.6 mm, 5 mm particle size), Betasil Silica (100 mm × 4.6 mm, 5 mm particle size), Betasil Diol (100 mm × 4.6 mm, 5 mm particle size) and Alltima Amino (250 mm × 4.6 mm, 5 mm particle size). The flow rate was 1 mL min^{-1} and the column temperature was 30°C. UV detection was carried out at 257 nm. The experimental plan was created according to Box–Behnken experimental design. The prepared mobile phases were filtered through a 0.45-μm membrane filter Althec (Lokeren, Belgium).

Mobile phase

The mobile phase composition was defined by the experimental plan given in Table I. The plan of experiments is defined by Box–Behnken design.

Software

Box–Behnken design was created by using DesignExpert 7.0.0 software. Three-dimensional (3D) response surfaces were obtained by using STATISTICA 7. For grid search point calculation, MATLAB was used.

Table I
Design of Experiments Created by Box–Behnken Design and Obtained Results

No.	*A*	*B*	*C*	k_1	k_2	k_3	$\alpha_{1,2}$
1	80 (−1)[a]	3.50 (−1)[a]	15 (0)[a]	0.828	1.091	2.131	1.318
2	85 (+1)	3.50 (−1)	15 (0)	1.370	1.944	2.511	1.419
3	80 (−1)	5.50 (+1)	15 (0)	0.622	1.113	4.290	1.790
4	85 (+1)	5.50 (+1)	15 (0)	1.378	2.633	5.464	1.911
5	80 (−1)	4.50 (0)	5 (−1)	0.855	1.776	6.347	2.077
6	85 (+1)	4.50 (0)	5 (−1)	1.591	3.527	8.374	2.217
7	80 (−1)	4.50 (0)	25 (+1)	0.594	0.932	2.249	1.569
8	85 (+1)	4.50 (0)	25 (+1)	1.202	2.079	2.911	1.730
9	82.50 (0)	3.50 (−1)	5 (−1)	1.731	2.562	4.373	1.480
10	82.50 (0)	5.50 (+1)	5 (−1)	1.042	2.089	10.726	2.005
11	82.50 (0)	3.50 (−1)	25 (+1)	0.816	1.040	1.854	1.274
12	82.50 (0)	5.50 (+1)	25 (+1)	0.893	1.500	3.374	1.680
13	82.50 (0)	4.50 (0)	15 (0)	0.940	1.670	3.450	1.777
14	82.50 (0)	4.50 (0)	15 (0)	0.906	1.607	3.392	1.774
15	82.50 (0)	4.50 (0)	15 (0)	0.915	1.620	3.432	1.770

A, concentration of acetonitrile (%); *B*, pH of the water phase; *C*, concentration of ammonium acetate (mmol L^{-1}); k_1, retention factor of olopatadine hydrochloride; k_2, retention factor of olopatadine hydrochloride, *E* isomer; k_3, retention factor of benzalkonium chloride; $\alpha_{1,2}$, selectivity factor.
[a]Coded values for factor levels are given in the brackets.

Method's validation

Solutions for linearity estimation

To evaluate the linearity of the developed method, eight solutions of olopatadine hydrochloride in the concentration range from 37.5 to 1000 μg mL^{-1}, eight solutions of benzalkonium chloride in the concentration range from 20 to 150 μg mL^{-1} and eight solutions of *E* isomer in the concentration range from 0.375 to 10 μg mL^{-1} were prepared in the optimal mobile phase.

Solution for accuracy estimation

The accuracy of the method was proved by preparing three series of solutions containing appropriate placebo (consists of all substances from eye drops except active substance and impurity), olopatadine hydrochloride, its impurity *E* isomer and benzalkonium chloride in the mobile phase. These mixtures were prepared on three levels: (i) low level 80%: containing 400 μg mL^{-1} of olopatadine hydrochloride and 4 μg mL^{-1} of the impurity, (ii) medium level 100%: containing 500 μg mL^{-1} of olopatadine hydrochloride and 5 μg mL^{-1} of the impurity and (iii) high level 120%: containing 600 μg mL^{-1} of olopatadine hydrochloride and 6 μg mL^{-1} of the impurity.

Furthermore, the accuracy of the preservative was demonstrated on three levels: (i) low level 80%: containing 80 μg mL^{-1} of benzalkonium chloride, (ii) medium level 100%: containing

100 μg mL^{-1} of benzalkonium chloride and (iii) high level 120%: containing 120 μg mL^{-1} of benzalkonium chloride.

Solutions for precision estimation

To attest the precision of the method, six solutions of eye drops in the mobile phase under optimal conditions containing 500 μg mL^{-1} of olopatadine hydrochloride and 50 μg mL^{-1} of benzalkonium chloride were prepared. The appropriate volume of impurity stock solution was added to all solutions in order to obtain the final concentration of 5 μg mL^{-1}.

Results

The results for retention factors of olopatadine (k_1), *E* isomer (k_2) and benzalkonium chloride (k_3) as well as for selectivity between geometric isomers were calculated and are presented in Table I.

Statistical analysis has shown that the most adequate mathematical model for responses k_2 and k_3 was the second-order polynomial model presented by the following equation:

$$Y = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC + b_{23}BC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2$$

where b_0 presents the intercept term, b_1, b_2, b_3 are the linear terms, b_{11}, b_{22}, b_{33} are the quadratic terms, b_{12}, b_{13}, b_{23} are the interaction terms, and *A*, *B* and *C* are investigated factors.

The response k_1 was modeled by the linear model with two factor interactions

$$Y = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC + b_{23}BC$$

where the meaning of the symbols is equivalent to the previous example.

The obtained results for coefficients of mathematical relationship and their statistical significance are presented in Table II. The results obtained for method's validation are presented in Table III.

Discussion

As it was noted in the 'Introduction' section, HILIC has become very popular for the analysis of polar and/or ionic compounds in recent years. Because olopatadine and its *E* isomer are amphoteric substances and possess ionic characteristics, HILIC mode was chosen for their analysis. Depending on pH of the mobile phase, olopatadine can be present in different ionic forms. Therefore, the pK_a values of ionizable groups are given. Namely, pK_a of the phenylacetic acid group is ∼2.58, whereas pK_a of the protonated tertiary amine group is ∼9.24. The third investigated substance, benzalkonium chloride, possesses a quaternary ammonium group in its structure and its determination in RP-LC system requires ion-pair reagents. However, HILIC mode could provide the adequate determination of benzalkonium chloride without ion-pair additives. In order to conduct in-depth chromatographic analysis of the presented mixture, a systematic approach including DoE methodology was employed. DoE was poorly used for HILIC method development, and only few papers can be found in the literature so far (21–24). In this paper, the Box–Behnken design was applied. The Box–Behnken design is based on three-level incomplete factorial designs where two factors are arranged in a full two-level design, while the level of the third factor is set at zero (25). If three factors are to be examined, 12 experiments plus central point replications are required. In addition, this design is nearly rotatable and especially useful when the points on one or more corners of the cube represent factor-level combinations that are unacceptably expensive or impossible to carry out because of physical constraints on the experimentation (26). DoE methodology enabled the construction of mathematical relationship between the selected factors and responses. On the basis of obtained models, influence of all investigated factors as well as their interactions on chromatographic responses could be easily defined. This is especially important in HILIC mode where factor interactions have a very important role in chromatographic behavior. Furthermore, the whole optimization procedure is automated and numerous simulated chromatograms under different chromatographic conditions can be created without performing experiments. In that way, the desired optimal conditions in terms of different quality criteria (adequate separation, minimal analysis duration, robustness, etc.) can be located.

Preliminary study

The experimental investigation started with preliminary experiments aiming to differentiate the most influential factors for the

Table II
Coefficients of Mathematical Models and Suitable Statistical Analysis

	k_1		k_2		k_3		$\alpha_{1,2}$	
	Coefficient	*P* value	Coefficient	*P* value	Coefficient	*P* value	Coefficient	*P* value
b_0	1.0446		+1.63		+3.42		+1.78	
b_1	0.3312	<0.0001*	+0.66	0.0001*	+0.53	0.0256*	+0.065	0.0593
b_2	−0.1025	0.0413	+0.087	0.1507	+1.62	0.0002*	+0.24	0.0003*
b_3	−0.2137	0.0010	−0.55	0.0001*	−2.43	0.0001*	−0.19	0.0008*
b_{12}	0.0550	0.3839	+0.17	0.0702	+0.20	0.4445	+0.005	0.9000
b_{13}	−0.0325	0.6010	−0.15	0.0950	−0.34	0.2126	+0.005	0.9000
b_{23}	0.1900	0.0130	+0.23	0.0244*	−1.21	0.0038*	−0.028	0.4996
b_{11}			+0.17	0.0743	+0.032	0.9020	+0.063	0.1708
b_{22}			−0.11	0.2014	+0.14	0.5912	−0.23	0.0021*
b_{33}			+0.28	0.0150*	+1.51	0.0017*	+0.060	0.1853
R^2	0.9288		0.9846		0.9869		0.9726	
Adj. R^2	0.8754		0.9570		0.9634		0.9232	

k_1, retention factor of olopatadine hydrochloride; k_2, retention factor of olopatadine hydrochloride, *E* isomer; k_3, retention factor of benzalkonium chloride; $\alpha_{1,2}$, selectivity factor.
*Coefficients significant for $P < 0.05$.

Table III
Important Data for Method Validation

Parameter	Olopatadine hydrochloride	*E* isomer	Benzalkonium chloride
Linearity			
Linearity range ($\mu g\ mL^{-1}$)	37.5–1,000	0.375–10	20–150
Slope (*a*)	22.91	25.799	1.103
Intercept (*b*)	233.81	−1.695	0.074
Correlation coefficient (*r*)	0.9992	0.9994	0.9990
Accuracy given as recovery (%)			
Low level (80%)	101.5	83.15	100.34
Medium level (100%)	101.18	83.90	98.91
High level (120%)	101.32	90.65	101.28
Precision			
Precision RSD (%)	1.87	5.77	1.99
LOD and LOQ			
LOD ($\mu g\ mL^{-1}$)		0.100	6
LOQ ($\mu g\ mL^{-1}$)		0.375	20

chromatographic analysis of the selected mixture. This stage included the rough examination of the mixture behavior on different polar columns such as silica, cyano, diol and amino columns using different mobile phases.

The acetonitrile concentration was varied in the range from 75 to 90% in the mobile phase. It was concluded that increasing the percentage of organic solvent leads to stronger retention of all three substances on all four columns, which is in accordance with the findings in the literature (1). The reason is that a decrease in polarity of the mobile phase decreases its eluting strength, therefore polar analytes retain stronger on the surface of polar columns. Increasing the buffer concentration generally leads to a moderate increase in retention of polar analytes on silica, diol and other neutral columns as a consequence of the thickening of the adsorbed aqueous layer on the column surface (27, 28). In the case of olopatadine at the pH of the aqueous phase 3.5, both the basic and acidic groups were in their ionized forms. In other words, a zwitterionic form of the molecule was dominant. In this study, increasing the ammonium acetate concentration (5–60 mM) led to the moderate increase of retention of olopatadine and its *E* isomer. Logically, this influence was greater on the investigated silica and diol columns in comparison with the cyano column because of their greater polarity. Because ionic interactions between the ionized analytes and columns' surface were not involved in the separation mechanism on these columns, olopatadine and its *E* isomer, present in their zwitterionic forms, as well as benzalkonium chloride, present in its cationic form, exhibited similar retention behavior. On the other hand, the amino column, that is positively charged under the applied chromatographic conditions, acts as an anion exchange column. Namely, positively charged analytes are involved in electrostatic repulsive interactions with the column surface (6). Thus, the increase in retention of the analyzed compounds with the increase in ammonium acetate concentration on the amino column in the current research was rather expected and it was the result of the increasing degree of neutralization of the column surface with the buffer counterions. However, due to the presence of electrostatic repulsion, all the analytes weakly retained on the amino column; therefore, their satisfactory retention behavior was impossible to achieve. On silica and diol columns, benzalkonium chloride showed significantly different retention behavior in comparison with the other analytes. Namely it eluted significantly later, whereas the peaks of other analytes eluted close to the peak of the mobile phase; therefore, the analysis lasted unreasonably long. This was probably due to a considerable difference in their polarity, because the ionic interactions did not play an important part in the retention mechanism on these columns. Furthermore, the peak of benzalkonium chloride on these columns had tailing. The most acceptable analysis duration, as well as peak shape, was achieved on the cyano column; therefore, this column underwent the next stages of the research. In addition, in this stage isocratic elution was chosen because of the slow equilibration of HILIC columns and gradient elution was not considered.

Design of experiments methodology and retention behavior

After the preliminary investigation as factors that could have significant influence on chromatographic behavior of selected analytes acetonitrile, pH of the aqueous phase and concentration of ammonium acetate in aqueous phase were selected. Further on, levels of factors were defined: for acetonitrile 80% was set as lower level and 85% as upper level; for pH of the aqueous phase lower level was 3.5 and upper was 5.5 pH units and for ammonium acetate concentration 5 mM was lower and 25 mM was upper level. After definition of experimental space, the plan of experiments was created by applying the Box–Behnken experimental design and is presented in Table I. Following the defined experimental plan, the experiments were conducted and retention factors of olopatadine (k_1), *E* isomer (k_2) and benzalkonium chloride (k_3) were selected as responses. Also, selectivity between geometric isomers was calculated. The obtained results are presented in Table I.

The obtained results for coefficients and their statistical significance are presented in Table II. Namely, *P* values corresponding to coefficients related to acetonitrile (factor A) were <0.05 for all followed retention factors. In that way, a very strong influence of acetonitrile on retention factors was confirmed. The retention factor of *E* olopatadine was also under strong influence of ammonium acetate concentration. However, two factor interaction (b_{23} term) corresponding to ammonium acetate concentration and pH of the aqueous phase was also influential in the case of *E* olopatadine. Furthermore, the retention factor of the last eluted substance (benzalkonium chloride) was under influence of all the analyzed factors. Especially important was the concentration of ammonium acetate in the aqueous phase whose influence could be seen through quadratic coefficient (b_{22}) for which *P* values was <0.05. Regarding the absolute values of linear coefficients in the case of olopatadine isomers, the acetonitrile content exhibited the strongest, whereas the pH exhibited the weakest influence on their retention. Nevertheless, benzalkonium chloride retention was most strongly influenced by ammonium acetate concentration and least influenced by the pH value of the aqueous phase. In order to visualize influence of important factors and significance 3D graphs were constructed. Therefore, the dependence of k_2 from the acetonitrile content and ammonium acetate concentration is given in Figure 2A and the dependence of k_3 from the ammonium acetate concentration and pH of the aqueous phase is given in Figure 2B.

In Figure 2A, a very strong influence of acetonitrile on retention factor k_2 can be seen. Increasing content of acetonitrile in the mobile phase increased the analyte's retention factor. Positive influence is confirmed with the positive sign for coefficient b_1. On

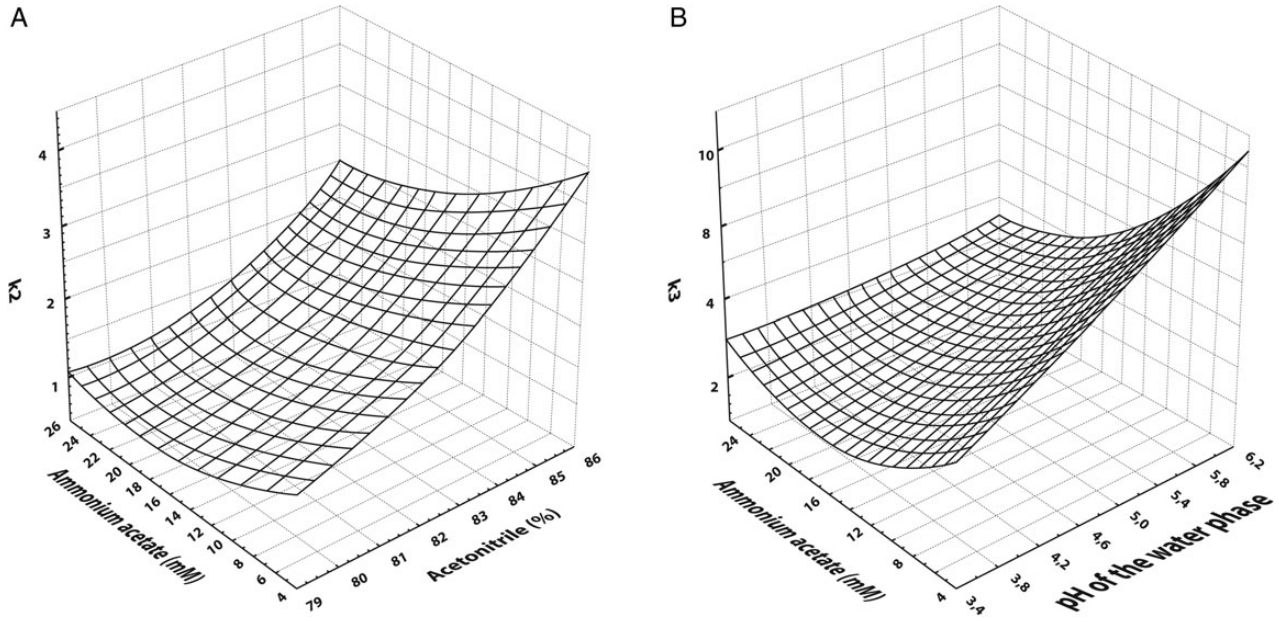

Figure 2. 3D graphs: (A) k_2 = f (acetonitrile content, ammonium acetate concentration) and (B) k_3 = f (ammonium acetate concentration, pH of the aqueous phase).

the other hand, lower concentration of ammonium acetate led to increasing of retention factor (negative sign for b_3).

Next, in Figure 2B longer retention of benzalkonium chloride is obtained with increasing pH of the aqueous phase while decreasing the concentration of ammonium acetate led to merely a slight increase of retention factor. However, 3D graphs confirmed results obtained by mathematical models and allowed deeper elucidation of chromatographic behavior of investigated substances.

Multi-objective optimization

Once the chromatographic behavior of individual substances was fully described, the optimization of chromatographic separation of the investigated mixture was performed. This stage of the research started by definition of the optimization goals. As the first goal, adequate retention of olopatadine (measured as k_1 value) is set because this substance showed poor retention in the investigated experimental domain. The second optimization goal was the maximal separation of the critical peak pair (geometric isomers of olopatadine) measured as selectivity factor. The experimentally obtained k_1 and k_2 values for all performed experiments are applied for calculation of the selectivity factor ($\alpha_{1,2}$) value, and the obtained results are given in Table I. Furthermore, the second-order polynomial model describing the dependence of $\alpha_{1,2}$ on investigated chromatographic factors is created and its adequacy is confirmed through statistical analysis. The created model and relevant parameters are presented in Table II. It could be seen that this response is under significant influence of concentration of ammonium acetate and pH of the aqueous phase. Next, in any chromatographic analysis it is very important to check robustness of important responses, which is a selectivity factor in this study.

Therefore, the adequate selectivity factor value is not sufficient if its robustness is not provided. Therefore, as the final goal, the maximal robustness of critical peak pair separation is set. The assessment of the selectivity factor robustness was performed by calculation of partial derivatives of obtained response function with respect to the investigated factors in order to evaluate the impact of small variations in factors on the response (29, 30).

The created models for $\mathrm{d}\alpha/\mathrm{d}A$, $\mathrm{d}\alpha/\mathrm{d}B$ and $\mathrm{d}\alpha/\mathrm{d}C$ were as follows:

$$\mathrm{d}\alpha/\mathrm{d}A = -1.647 + 0.002 * B + 0.0002 * C + A * 0.020 * A$$
$$\mathrm{d}\alpha/\mathrm{d}B = 2.178 + 0.002 * A - 0.003 * C - 0.459 * B$$
$$\mathrm{d}\alpha/\mathrm{d}C = -0.041 + 0.0002 * C - 0.003 * B + 0.001 * C$$

The created mathematical models for k_1, $\alpha_{1,2}$, $\mathrm{d}\alpha_{1,2}/\mathrm{d}A$, $\mathrm{d}\alpha_{1,2}/\mathrm{d}B$ and $d\alpha_{1,2}/\mathrm{d}C$ enable theoretical examination of the experimental space without performing of additional experiments.

The next step was multi-objective robust optimization aiming to achieve maximal k_1 and $\alpha_{1,2}$ and minimal $\mathrm{d}\alpha_{1,2}/\mathrm{d}A$, $\mathrm{d}\alpha_{1,2}/\mathrm{d}B$ and $\mathrm{d}\alpha_{1,2}/\mathrm{d}C$. The optimization is performed by grid point search (28). The grid density was defined in the following way: the increment for acetonitrile was 1%, for pH value 0.2 pH units and for ammonium acetate concentration 2 mM. In that way 726 ($6 \times 11 \times 11$) simulated chromatograms are constructed and k_1, $\alpha_{1,2}$, $\mathrm{d}\alpha_{1,2}/\mathrm{d}A$, $\mathrm{d}\alpha_{1,2}/\mathrm{d}B$ and $d\alpha_{1,2}/\mathrm{d}C$ are calculated for each of them. The optimal points were located aiming to achieve $k_1 > 1.5$, $\alpha_{1,2} > 1.2$ and minimal possible $\mathrm{d}\alpha_{1,2}/\mathrm{d}A$, $\mathrm{d}\alpha_{1,2}/\mathrm{d}B$ and $\mathrm{d}\alpha_{1,2}/\mathrm{d}C$ values. The finally selected working point was $A = 85\%$, $B = 4.5$ and $C = 5$ mM. These conditions are experimentally verified and the obtained chromatogram is presented in Figure 3. Under optimal chromatographic conditions analysis was repeated five times and repeatability of retention times was confirmed.

Eventually, the presented systematic approach gives possibility to perceive all possible changes of chromatographic behavior of the analyzed substances under defined experimental space. If

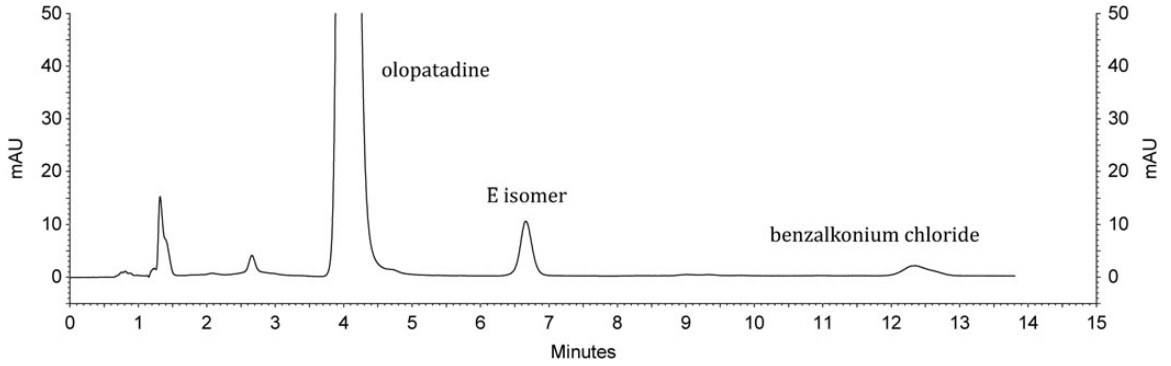

Figure 3. Chromatogram obtained under optimal chromatographic conditions: Betasil Cyano (100 mm × 4.6 mm, 5 mm particle size) with mobile phase consisted of 85% acetonitrile and 15% aqueous phase that contains 5 mM ammonium acetate. The pH was adjusted to 4.5 with glacial acetic acid, the flow rate was 1 mL min^{-1}, the column temperature was 30°C, and UV detection 257 nm.

this step is performed by a well-known tool such as experimental design methodology, then the next step that led toward routine analysis of the method could be done routinely without any surprise.

Method's validation

Finally, after setting up optimal chromatographic conditions by using the proposed methodology, which is experimentally verified, the method was fully validated. Method validation represents detailed investigation of the analytical method and contributes to the fact that the method, when correctly applied, promises results that fit to the aim. This process shows performance characteristics and limitations of a method, as well as influences that may change these characteristics and to which extent during its regular use. In this paper, validation scope includes linearity, accuracy and precision testing, as well as the determination of limit of detection (LOD) and limit of quantification (LOQ) for the impurity and preservative. Obtained results are presented in Table III.

Linear relationships of the peak areas versus concentrations for the concentration ranges mentioned above were obtained for olopatadine hydrochloride, its impurity *E* isomer and benzalkonium chloride. As the correlation coefficient (r) for the calibration curves was >0.998, it can be concluded that the calibration curves were within the linearity acceptance criteria.

To estimate the method accuracy, the recovery values for laboratory mixtures were calculated. The obtained values for all conducted tests were within the required values (98–102% for olopatadine hydrochloride and benzalkonium chloride, 80–120% for *E* isomer). The relative standard deviation (RSD) values for precision were <2% for olopatadine hydrochloride and benzalkonium chloride and <10% for impurity *E* isomer (not more than 1% in eye drops).

For the quantitative analysis of impurity and preservative, it was important to determine the values of LOD and LOQ. The signal-to-noise ratios of 3.3:1 and 10:1 were taken as LOD and LOQ, respectively, and further confirmed by taking dissolutions from the secondary stock solution till the peak area obtained was 3.3 times (for LOD) and 10 times (for LOQ) bigger than the standard deviation of blank solution after six injections. The obtained values for benzalkonium chloride and *E* isomer are presented in Table III and the obtained values for RSD were 7.14% and 3.02% ($n = 5$), respectively.

Conclusion

In this paper, the chromatographic behavior of olopatadine and its *E* isomer as its impurity was investigated in HILIC mode. Additionally, benzalkonium chloride was also analyzed, usually utilized as a preservative in eye drops. Using DoE methodology, systematic investigation was conducted and obtained results enabled complete evaluation of chromatographic behavior of investigated substances in HILIC mode. This is the first time that such a mixture was tracked in HILIC mode that can be considered very important. In addition, by multi-objective robust optimization and grid search methodology, optimal chromatographic conditions were defined and experimentally verified. This methodology gave a reliable and practical method. Eventually, the method was fully validated and the applicability of the method in routine analysis was confirmed.

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