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ORIGINAL RESEARCH PAPER

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Design of experiments in optimization and validation of hydrophilic interaction liquid chromatography method for determination of amlodipine besylate and its impurities

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ABSTRACT

A new and rapid hydrophilic interaction liquid chromatographic method has been developed for the quantitative analysis of amlodipine besylate and its specific impurities (D, E, and F). For development of this method, a systematic approach which includes Design of Experiments methodology was applied. For the method optimization, Box–Behnken design and specific way Derringer's desirability function were applied. They provided identification of the optimal chromatographic conditions on the basis of obtained mathematical models and graphical procedures (three D graphs). The optimal chromatographic conditions were the analytical column ZORBAX NH₂ (250 × 4.6 mm, 5 μm particle size); mobile phase consisted of acetonitrile–water phase (50 mM ammonium acetate, pH adjusted to 4.0 with glacial acetic acid) (90.5:9.5 v/v); column temperature 30 °C, mobile phase flow rate 1 mL min^{−1}, wavelength of detection 230 nm. As other validation parameters were also found to be suitable, the possibility to apply the proposed method for the determination of amlodipine besylate and its impurities in any laboratory under different circumstances has been proven.

KEYWORDS

amlodipine, HILIC, design of experiments, Derringer's desirability function

INTRODUCTION

Chemometrics observes each process as a system consisting of certain elements. By applying Design of Experiments (DoE) it is possible to change several factors simultaneously, performing a relatively small number of experiments, with the possibility to assess the influence of factors (chromatographic conditions) on selected responses (chromatographic parameters) and the effects of interactions between individual factors. In this way, obtaining the real/true optimum of the experiment (*global optimum*) is achieved [1–3].

The goal of method optimization is to find the conditions under which the examined system gives the maximal or minimal response, as well as to define the mathematical model that establishes the relationship between the change of factors and the system response in the best way possible [4]. Defining optimal conditions is achieved by applying optimization types of design, in which factors that have a significant impact on the response of the system are examined at several levels. These designs are designated as *response surface methodology* [5, 6]. The most commonly used response surface designs include: full factorial design on three levels, central composite design, Box–Behnken design and Doehlert design. They enable the establishment of a quadratic functional dependence between the examined factors and the system response. These designs examine factors at three levels, as this is the minimum number of points required to obtain a quadratic model. This model contains linear terms, interaction terms, and quadratic terms, which is why it can describe linear influence,

interaction influence, and nonlinear relationships between factors and system responses. The general form of quadratic response model is given by the Eq. (1):

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + \dots + b_Nx_N + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + \dots + b_{(N-1)N}x_{(N-1)}x_N + b_{11}x_1^2 + b_{22}x_2^2 + \dots + b_{NN}x_N^2 + \varepsilon \quad (1)$$

where N is the total number of variables, ε error (*lack of fit and pure error*), b_0 section, a b_1x_1 , b_2x_2 , b_3x_3 , ..., $b_{12}x_1x_2$, $b_{13}x_1x_3$, $b_{23}x_2x_3$, ..., $b_{(N-1)N}x_{(N-1)}x_N$ interaction members, b_k x_k are linear members, $b_{11}x_1^2$, $b_{22}x_2^2$, $b_{NN}x_N^2$ quadratic members. This model, unlike the interaction model, also contains quadratic members, which is why it can describe nonlinear relationships between factors and system responses [1, 7].

In chromatography, it is usually necessary to achieve a good separation of all tested compounds with the shortest possible duration of a chromatographic analysis time. If the optimal values of the examined factors are located in different parts of the experimental region and do not overlap, it is a big problem to find conditions that simultaneously satisfy several chromatographic goals. In such cases, a *multicriteria methodology* is used to achieve an appropriate compromise between the given chromatographic objectives [5].

Derringer's desirability function is the most important methodology of multicriteria decision making when it comes to optimization of chromatographic methods. It is applicable to linear and nonlinear mathematical models and does not require the selection of a priority system response. During the method optimization, it allows finding the most desirable combination of chromatographic factors [8–10]. This application of chemometric approach, in addition to finding the most optimal conditions for chromatographic separation, also enables a robust method designing and the quality incorporation directly during the process of method development [9].

The *Derringer's desirability function* methodology is based on constructing a desirable response function for each individual system response (d_i) in which the requirements/objectives that each system response needs to be met are entered. The relative importance of that answer is also entered. The scale for each individual desired response function ranges from $d = 0$ (for a completely undesirable response) to $d = 1$ (for a perfectly desirable response) [11].

As well as individual, the global function of desirable answers can have values from $D = 0$ when all system responses have an undesirable value, to $D = 1$ when all system responses have a desirable value. The closer the D value is to the value of 1, the closer the system is to the global optimum, i.e., all system responses are closer to the target value. If the value is $D = 1$, all system responses are met (global optimum is reached). The global function of the desired answers can be displayed in the form of a three-dimensional space (3D chart) for easier observation of the global optimum [10, 12]. The only drawback of this optimization method was that the achieved global optimum is hardly applicable in

practice (eg acetonitrile content in mobile phase 91.38, pH value of aqueous buffer solution 2.73, and buffer concentration 33.31 mM). Therefore, by rounding the numbers to the integer values of the test factors, the value of D for the global optimum (D less than 1) is also changed. This paper presents for the first time a new way of using the Derringer function of desirable responses where the global optimum is examined in integer values of critical factors.

Hydrophilic interaction liquid chromatography (HILIC) is a new chromatographic technique that has become very popular in the last 5–7 years. HILIC is used for the analysis of hydrophilic compounds, amphiphilic compounds with a high degree of polarity, as well as compounds that do not have a strong enough charge (ions) to be examined by ion-exchange chromatography. HILIC enables the analysis of charged compounds (compounds in ionized form), where the separation mechanism is similar to ion-exchange chromatography (IEC). This method has the characteristics of three types of chromatography: NP-HPLC, RP-HPLC and IEC. HILIC is a very fast chromatographic method that provides separation of very complex mixtures in short analysis time. The mechanisms of separation have not yet been fully understood, but it is known that, individually or in combination, three mechanisms are present so far: partitioning, adsorption, and ion exchange. Retention in the HILIC system is still being intensively investigated [13].

Amlodipine besylate (AB) is a significant and frequently used drug from group of calcium blockers. In combination with other drugs (diuretics, beta blockers, ...) it is used to treat cardiovascular diseases: hypertension, angina pectoris, etc [14]. On the market, this drug is quite common, most often in the form of tablets in doses of 5 mg and 10 mg. Due to the great popularity of its application, a large number of pharmaceutical industries produce it. Amlodipine besylate is official in the European Pharmacopoeia 9.0 where for its assay and for impurities determination RP-HPLC on C18 column with isocratic elution method for AB (run time ~25 min) is used [15].

A literature review describes various methods for AB testing, such as LC methods for determining AB from tablets and biological material in combination with ramipril, valsartan, hydrochlorothiazide, atorvastatin, perindopril, indapamide and other drugs [16–20]. So far, no HPLC method has been published in the modern literature for the analysis of AB and its specified impurities (D, E, and F) that is shorter than 20 min. Also, there is a UPLC method for the analysis of AB and its impurities in combination with other drugs (run time greater than 10 min) [21]. Chemical structures and chemical names of the analyzed substances are given in Fig. 1. Impurities D, E and F are process-related impurities of AB, and impurity D is also a degradation impurity of AB. Because of that, these impurities are analyzed during shelf life, according to manufacturers specification.

The aim of this study was to investigate the chromatographic behavior of AB and its impurities in HILIC mode in order to define the optimum chromatographic conditions suitable for their determination in tablets. To get more

Fig. 1. The chemical structures and name of investigated substance from Ph. Eur. 9.0

desirable chromatographic conditions and to successfully develop a new HILIC method, DoE methodology was used. This is the first time that the HILIC method for the qualitative and quantitative analysis of amlodipine besylate and its impurities D, E and F has been developed.

EXPERIMENTAL

Chemicals and reagents

The analyzed substance: amlodipine besylate working standard (Lek, Ljubljana) and impurities: 3-ethyl 5-methyl 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methylpyridine-3,5-dicarboxylate CRS European Pharmacopoeia reference standard (amlodipine impurity D) Sigma-Aldrich, Germany; diethyl-amlodipine CRS European Pharmacopoeia reference standard (amlodipine impurity E) Sigma-Aldrich, Germany; dimethyl-amlodipine European Pharmacopoeia reference standard (amlodipine impurity F) Sigma-Aldrich, Germany. The mobile phase and the solvents were prepared from acetonitrile (Fisher Scientific, England),

ammonium acetate (Lachner, Czech Republic), glacial acetic acid (Lachner, Czech Republic), and HPLC grade water. Vazotal[®] 5 mg tablets (Hemofarm, Serbia) were used for analysis. All the reagents utilized in this study were of the analytical grade.

Chromatographic conditions

The experiments were performed on chromatographic system Agilent 1200 series consisting of HPLC Pump, Quaternary Pump, ALS Autosampler, and UV/VIS DAD detector. Agilent ChemStation software was used for data collection. The analytical column was ZORBAX NH₂ column (250 × 4.6 mm, 5 μm particle size) Agilent Technologies, USA. Throughout the complete experimental procedure, the following instrumental chromatographic conditions were maintained: flow rate of the mobile phase 1 mL min⁻¹, column temperature 30 °C and UV detection at 230 nm. The experimental plan was created according to Box–Behnken design for optimization and fractional factorial design (FFD 2⁴⁻¹) for robustness. The prepared mobile

phases were filtered through a 0.45 μm nylon membrane filter Whatman, England.

Mobile phase

The mobile phase consisted of acetonitrile and water phase (with added ammonium acetate and glacial acetic acid), where the amount of organic solvent, ammonium acetate concentration in the water phase and pH of the water phase were varied according to the experimental plan. The mobile phase under optimal chromatographic conditions was as follows: acetonitrile–ammonium acetate (50 mmol L⁻¹) in water, pH 4.0 adjusted with glacial acetic acid (90.5:9.5, v/v).

Standard solutions

Stock solutions for the method optimization and robustness testing contained 100 $\mu\text{g mL}^{-1}$ of amlodipine besylate, and 10 $\mu\text{g mL}^{-1}$ of all impurities in the mixture of acetonitrile–ammonium acetate (50 mmol L⁻¹) in water, adjusted with glacial acetic acid to pH 4.0 (90:10, v/v). Placebo mixture (siliconized microcrystalline cellulose, pregelatinized starch and magnesium stearate – was donated by the pharmaceutical industry Hemofarm d.o.o. Banja Luka, Republic of Srpska) for selectivity estimation was prepared in a concentration ratio corresponding to the content in the pharmaceutical dosage form (Vazotal® 5 mg tablets). A standard solution containing 100 $\mu\text{g mL}^{-1}$ of amlodipine besylate and 10 $\mu\text{g mL}^{-1}$ of each impurity was used to prove the selectivity. For linearity estimation five solutions containing amlodipine besylate (from 50 to 200 $\mu\text{g mL}^{-1}$) and its impurities D, E and F (from LOQ to 15.0 $\mu\text{g mL}^{-1}$) were prepared in the mobile phase. The accuracy estimation is performed using three series of three solutions containing placebo, amlodipine besylate in concentrations 111.2 $\mu\text{g mL}^{-1}$ (80%), 139 $\mu\text{g mL}^{-1}$ (100%) and 166.8 $\mu\text{g mL}^{-1}$ (120%), as well as its impurities D, E and F in concentrations 8.0, 10.0 and 12.0 $\mu\text{g mL}^{-1}$ respectively. All solutions were prepared in the optimal mobile phase. The precision estimation was performed on real samples using Vazotal® 5 mg tablets which contain 6.934 mg of amlodipine besylate per tablet. Six samples contained 100 $\mu\text{g mL}^{-1}$ of amlodipine besylate were prepared in mobile phase and spiked with impurities (D, E and F) in concentration of 10 $\mu\text{g mL}^{-1}$.

Solutions for estimating limit of detection (LOD) and limit of quantification (LOQ) were prepared individually for each compound (impurities D, E and F) in concentrations 1.0, 0.5, 0.25, 0.10, 0.05, 0.025, 0.01 $\mu\text{g mL}^{-1}$. All solutions were prepared in mobile phase.

Software

Experimental plan and data analysis, according to Box–Behnken design and FFD 2⁴⁻¹ were created in Design-Expert 7.0.0 software (Stat-Ease Inc., Minneapolis, MN, USA). Three-dimensional response surface was obtained by Design-Expert 7.0.0 software. Method optimization, Deringer's desirability function and global optimum have been studied and defined in Design-Expert 7.0.0 software. The

values of partition coefficient ($\log P$), distribution coefficient ($\log D$) and the value of pKa of the analyzed compounds were estimated in MarvinSketch 15.4.13 (ChemAxon Kft, Budapest, Hungary).

RESULTS AND DISCUSSION

In quality control of API and finished product (e.g. tablets, capsules, etc.), especially during shelf-life, it is necessary to develop a reliable and sensitive chromatographic method, which will allow complete separation of API and its impurities in a short time, as well as qualitative and quantitative analysis. In this paper, using the chemometric approach, the HILIC method for quality control of AB in tablets was developed, as well as monitoring the presence and determination of specific impurities D, E and F.

Preliminary experiments and identification of critical parameters of the chromatographic system

In this study, good separation of all analyzed compounds in a short time of the analysis was set as a goal. Before performing preliminary analyzes, pKa, $\log P$ and $\log D$ values were determined based on the chemical structure of tested compounds. For this analysis, MarvinSketch® program was used. This procedure is very important in HILIC chromatography in order to quickly make the best selection of the stationary phase, the mobile phase composition and the mobile phase pH value. By studying these values it can be ensured that all tested compounds during the analysis will be either 100% in ionic or 100% in molecular form.

Based on the obtained pKa values of the analyzed compounds (AB pKa = 9.40; impurity D, impurity E and impurity F pKa = 9.45) the most suitable pH value of the mobile phase was selected. The selected pH value of 4.0 will ensure that AB and impurities D, E and F are ~100% dissociated (ionic form).

By analyzing the obtained $\log P$ and $\log D$ values for AB ($\log P$ = 1.64; $\log D$ = -1.17) and its impurity D ($\log P$ = 2.66; $\log D$ = -0.37), impurity E ($\log P$ = 1.99; $\log D$ = -1.04) and impurity F ($\log P$ = 1.28; $\log D$ = -1.75), the order of elution in HILIC system should be impurity D → impurity E → AB → impurity F. Elution order is followed by the pattern of the increasing hydrophilicity. This means, impurity F has the lowest $\log P$ and $\log D$ values and as most hydrophilic compounds will be eluted last in HILIC system.

Preliminary tests were performed on several different HILIC columns: silica, cyano, amino and diol. The amino column was selected as the most suitable stationary phase for further analysis. In preliminary studies, acetonitrile was selected as the organic component of the mobile phase, the content of which varied in the range of 80–96%. Ammonium acetate was selected as a buffer and its concentration in the aqueous phase was tested in the range of 10 – 70 mmol L⁻¹. Since AB, as well as its impurities D, E and F are of an alkaline character, it is necessary to perform the experiments at low pH values in order to maintain them in ionized form

(as cations). In this sense, the pH of the aqueous phase was adjusted with glacial acetic acid in the range of 3.5–5.0, and then analyzes were performed. The results obtained during the preliminary research made it possible to select a set of critical responses of the chromatographic system. These responses that will be monitored in the further method development are:

1. $Rs_{impD,impE}$ – the resolution between impurity D and impurity E;
2. $Rs_{impE,impAB}$ – the resolution between impurity D and amlodipine besylate;
3. $Rs_{AB,impF}$ – the resolution between amlodipine besylate and impurity F;
4. As_{AB} – the asymmetry factor of amlodipine besylate chromatographic peak;
5. tR_{impF} – the retention time of impurity F (analysis time).

In the second step of the HILIC method development, through the performance of preliminary experiments, the critical parameters of the chromatographic system were identified. The ones that will be monitored during optimization phase of the method are:

1. acetonitrile content in mobile phase
2. ammonium acetate concentration in water phase (buffer concentration), and
3. pH value of water phase.

Their influence on the monitored responses of system proved to be very significant. With small changes in all three selected factors, large changes in all monitored system responses are caused (resolution factors, the asymmetry factor of AB and analysis time). In these studies, the behavior of examined responses to changes in selected factors was concluded – by increasing the acetonitrile content in mobile phase and pH value of water phase, and decreasing the

ammonium acetate concentration in water phase leads to better resolution of all tested compounds, better asymmetry factor of AB, and longer analysis time.

During the preliminary studies, the influence of column temperature (25–40 °C), mobile phase flow (0.5–1.5 mL min⁻¹), and wavelength of absorption maximum (190–400 nm) for all four tested compounds were also examined. It was concluded that increasing the value of mobile phase flow and column temperature, leads to irrelevant shortening of the analysis time. These factors do not affect the critical pairs resolution, nor the asymmetry factor of AB. Given the quite predictable influence of these factors on the HILIC system behavior, they were defined as uncritical and were maintained at a constant level in the continuation of the test. It was concluded that the best values of all monitored system responses were obtained at a column temperature of 30 °C, a mobile phase flow rate of 1 mL min⁻¹, and a determination wavelength of 230 nm.

Optimization – multi-criteria decision making

In the HILIC method optimization phase, for creating the Box–Behnken design, the intervals of the examined factors were selected and the final ranges were:

89–92% for acetonitrile content in the mobile phase,
4.0 – 5.0 for pH value of water phase, and
40 – 60 mM for ammonium acetate concentration in water phase.

In the process of the method optimization, Box–Behnken design was used (Table 1). That enabled a clear definition of individual and joint influences of the examined factors on the observed system responses. Response surface methodology was used in the optimization process – enables definition of quadratic response models that accurately describes the

Table 1. Plan of Box–Behnken design experiments and the obtained data

No.	Factors			Responses				
	Acetonitrile (%)	pH value	Buffer concentration (mM)	$Rs_{impD,impE}$	$Rs_{impE,AB}$	$Rs_{AB,impF}$	tR_{impF}	As_{AB}
1	89.00	3.00	50.00	1.39	2.92	3.37	6.04	1.04
2	92.00	3.00	50.00	1.89	3.22	4.10	9.84	0.98
3	89.00	5.00	50.00	2.13	2.87	3.41	6.30	1.05
4	92.00	5.00	50.00	4.34	3.34	4.01	11.26	1.04
5	89.00	4.00	40.00	0.20	3.26	0.15	3.16	5.02
6	92.00	4.00	40.00	3.32	4.29	0.65	6.06	7.96
7	89.00	4.00	60.00	4.19	0.34	0.18	3.50	4.19
8	92.00	4.00	60.00	5.91	3.33	3.55	9.66	1.66
9	90.50	3.00	40.00	2.27	3.07	3.72	7.22	1.03
10	90.50	5.00	40.00	3.88	3.19	3.78	7.80	1.07
11	90.50	3.00	60.00	1.12	3.26	3.93	7.81	0.98
12	90.50	5.00	60.00	2.70	3.18	3.77	8.50	0.97
13	90.50	4.00	50.00	3.60	3.02	3.69	8.86	1.05
14	90.50	4.00	50.00	3.45	3.18	3.81	7.94	1.04
15	90.50	4.00	50.00	3.50	3.23	3.87	7.91	1.04

$Rs_{impD,impE}$ – resolution factor between impurity D and impurity E; $Rs_{impE,AB}$ – resolution factor between impurity E and amlodipine besylate; $Rs_{AB,impF}$ – resolution factor between amlodipine besylate and impurity F; tR_{impF} – retention time of impurity F; As_{AB} – factor asymmetry of amlodipine besylate.

response for all values of the chromatographic conditions in the experimental region. To calculate quadratic regression model coefficients, each design variable must be studied at three distinct levels at least, and, consequently, a Box–Behnken design was used in this optimization study [9].

From the obtained results (Table 1) it can be seen that the system is quite sensitive to small changes in acetonitrile content, but also to other two examined factors – this is a specific characteristic of HILIC system. The monitored responses of the system show that during optimization process, the values for all three resolution factors (Rs) vary from a value of 0.15–0.34 (significant overlap of the critical chromatographic pair: $Rs_{impD,impE}$, $Rs_{impE,AB}$ and $Rs_{AB,impF}$) to values significantly greater than 1.2 (very good separation of the critical chromatographic pair). The asymmetry factor AB values vary in the range 0.971–5.022, which indicates a high level of all three factors influence on the asymmetry of this chromatographic peak (normal as value is $1.0 < As < 1.2$). The retention time of impurity F varies in the range of 3.16–11.26 min. This response is monitored due to the duration of the analysis, but also in the optimization process, it shows how sensitive the HILIC system to the slightest changes of all three examined factors is.

Using DoE in the method optimization process, in order to clearly define how each individual factor and their two-factor interactions affect the observed system responses, statistical analysis yielded ANOVA test parameters for a model describing the chromatographic behavior of AB and its impurities D, E and F. The obtained coefficients are shown in Table 2.

The obtained results for the ANOVA test parameters show that a very high value was calculated for the mathematical model for coefficients of determination R^2 and for adjusted R^2 . The lowest values obtained are $R^2 = 0.8978$ and

$R^2_{adj} = 0.8839$, which indicates a good correlation between experimentally obtained and model-calculated values of responses ($Rs_{impD,impE}$, $Rs_{impE,AB}$, $Rs_{AB,impF}$, As_{AB} , tR_{impF}). In this study, the adjusted R^2 was well within acceptable limits of $R^2 > 0.80$ which revealed that the experimental data were a good fit for the quadratic model equations [9, 12]. Also, the model reliability was additionally confirmed with small RSD values ($RSD < 1.0\%$) [22].

Based on the obtained results (Table 2), the factors and factor interactions that are relevant for each examined response are precisely defined (the one that meets the requirement that the p -value is < 0.05).

Quadratic response model obtained on the basis of ANOVA test parameters was created by inserting only those values obtained for factors and factor interactions that proved to be significant (p -value < 0.05 and it is given by Eqs. (2) and (6):

$$Rs_{impD,impE} = 3.52 + 1.21A + 0.80B - 0.58C + 0.43AB - 0.35AC - 1.00B^2 \quad (2)$$

$$Rs_{impE,AB} = 3.15 + 1.09A + 0.58AC - 0.25A^2 \quad (3)$$

$$Rs_{AB,impF} = 3.79 + 0.67A + 0.76AC - 1.39A^2 + 1.32B^2 - 1.31C^2 \quad (4)$$

$$tR_{impF} = 8.24 + 2.23A + 0.65C + 0.82AC - 1.06A^2 + 1.18B^2 - 1.58C^2 \quad (5)$$

$$As_{AB} = 1.04 - 0.91C - 1.37AC + 1.84A^2 - 1.86B^2 + 1.83C^2 \quad (6)$$

A – acetonitrile content in the mobile phase; B – pH value of water phase; C – ammonium acetate concentration in water phase (buffer concentration).

Table 2. Coefficients of the quadratic response model

	$Rs_{impD,impE}$		$Rs_{impE,AB}$		$Rs_{AB,impF}$		tR_{impF}		As_{AB}	
	Coeff.	P-value	Coeff.	P-value	Coeff.	P-value	Coeff.	P-value	Coeff.	P-value
b_0	3.52	0.0023*	3.15	0.0125*	3.79	0.0131*	8.24	0.0012*	1.04	0.0478*
b_1	1.21	0.0010*	1.09	0.0025*	0.67	0.0233*	2.23	<0.0001*	0.04	0.9176
b_2	0.80	0.0022*	0.01	0.8624	-0.02	0.9316	0.37	0.1134	0.14	0.9734
b_3	-0.58	0.0042*	0.04	0.4870	0.37	0.1378	0.65	0.0193*	-0.91	0.0467*
b_{12}	0.43	0.0077*	0.04	0.5131	-0.03	0.9132	0.29	0.3383	0.01	0.9846
b_{13}	-0.35	0.0115*	0.58	0.0087*	0.76	0.0490*	0.82	0.0302*	-1.37	0.0456*
b_{23}	-0.01	0.8741	-0.05	0.4536	-0.05	0.8679	0.03	0.9235	-0.01	0.9832
b_{11}	-0.08	0.1656	-0.25	0.0458*	-1.39	0.0062*	-1.06	0.0135*	1.84	0.0242*
b_{22}	-1.0	0.0016*	0.20	0.0727	1.32	0.0072*	1.18	0.0089*	-1.86	0.0235*
b_{33}	-0.03	0.5653	-0.17	0.0936	-1.31	0.0078*	-1.58	0.0025*	1.83	0.0249*
R^2	0.9996		0.9979		0.9419		0.9783		0.8978	
R^2_{adj}	0.9973		0.9854		0.8373		0.9391		0.8839	
RSD (%)	0.0160		0.11001		0.0135		0.5234		0.3841	

$Rs_{impD,impE}$ – resolution factor between impurity D and impurity E; $Rs_{impE,AB}$ – resolution factor between impurity E and amlodipine besylate; $Rs_{AB,impF}$ – resolution factor between amlodipine besylate and impurity F; tR_{impF} – retention time of impurity F; As_{AB} – factor asymmetry of amlodipine besylate; R^2 – coefficient of determination; R^2_{adj} – adjusted R^2 values which represent R^2 values adapted to the total number of experiments; RSD (%) – relative standard deviation for model reliability ($RSD < 1.0$); b_0 – section; b_1 – linear member of factor A; b_2 – linear member of factor B; b_3 – linear member of factor C; b_{12} – interaction member of factors A and B; b_{13} – interaction member of factors A and C; b_{23} – interaction member of factors B and C; b_{11} – quadratic member of factor A; b_{22} – quadratic member of factor B; b_{33} – quadratic member of factor C.

*Significant coefficient for p -value ≤ 0.05 .

Based on the coefficients of the quadratic response model, as well as their signs (Table 2), it is possible to estimate which factors and which factor interactions have an impact on the examined responses. From the obtained results of the ANOVA test, factors A, B and C have a significant influence on the response of $Rs_{impD,impE}$ (Eq. (2)), as well as factor interactions AB and AC – by increasing acetonitrile content and pH value of water phase, and by decreasing buffer concentration in the mobile phase, the resolution between critical pair of impurities D and E increases. Since there is a significant influence of buffers in the two-factor interaction AC, additional analysis is necessary in order to find the best value for this response.

The response of $Rs_{impE,AB}$ (Eq. (3)) and the response of $Rs_{AB,impF}$ (Eq. (4)) are significantly influenced by factor A, and factor interactions AC – by increasing the content of acetonitrile and buffer concentration, increases the resolution between the chromatogram pair of impurities E and AB. Also, the resolution between the critical pair AB and impurity F increases.

On tR_{impF} response (Eq. (5)), factors A and C, and factor interaction AC have a significant effect – by increasing the content of acetonitrile and buffer concentration, the retention time of impurity F prolongs.

On the As_{AB} response (Eq. (6)) factor C and factor interaction AC have a significant effect – by decreasing the content of acetonitrile and buffer concentration, As_{AB} response is increasing. This change in the As_{AB} response is the opposite of all other responses, and because of that research is required to find the global optimum.

Factor interactions A^2 , B^2 , C^2 have a significant effect – by increasing the content of acetonitrile, the pH-value of the buffer aqueous solution increases while lowering the buffer concentration achieves better symmetry of the AB peak.

Quadratic members of factors A^2 , B^2 , C^2 (Eqs. (2)–(6)) indicate the presence of a nonlinear relationship between A, B and C, and the analyzed system responses. These factors with the (+) sign indicate that this change has a concave shape (extreme value is the minimum of function), while factors with the (–) sign indicate that this change has a convex shape (extreme value is the maximum of function). Quadratic members of factors A^2 and C^2 with the (–) sign indicate that the response values will decrease with increasing the content of acetonitrile and buffer concentration to a certain threshold, after which the response value starts to increase. Quadratic member of factors B^2 with the (+) sign indicates that the response value will increase with decreasing the pH value of water phase to a certain threshold, after which the response value starts to decrease.

From all of the above, it is concluded that the most significant influence on this chromatographic system has the acetonitrile content in the mobile phase (factor A), followed by the buffer concentration (factor C), as well as their interactions (AC). The pH value of the water phase (factor B) had the least influence on the examined responses of the chromatographic system, of the three most significant factors selected. However, this factor influence is also very

important for the $Rs_{impD,impE}$, tR_{impF} and As_{AB} responses. Respectively, as the value of factor B increases, the value of the $Rs_{impD,impE}$, and tR_{impF} responses increases, and the As_{AB} response value decreases. This means that a better resolution of the critical pair of chromatographic peaks of impurities D and E is provided, but the analysis duration is extended and the asymmetry factor value for AB is reduced.

Since the system is quite complex and that there are significant two-factor interactions between all three examined factors, 3D graphs were constructed. In order to describe the analyzed system better, the response surface methodology was used. Based on the obtained results of the ANOVA test, 3D graphs, that represent the dependence of the examined system responses from the observed factors, were constructed. These graphs represent a very convenient way to visually study the behavior of analytes in the examined system. The obtained graphs are shown in Fig. 2.

From the obtained 3D-graphs for each observed response, it can be concluded that significant two-factor interactions are present. The best $Rs_{impD,impE}$, $Rs_{impE,AB}$, $Rs_{AB,impF}$ responses are obtained at acetonitrile content values of 90.5–91.5%, buffer concentration of 45–50 mM and pH value of 4.0. Increasing or decreasing the values of these factors decreases the examined resolutions, but also decreases the value of the As_{AB} response. Increasing the acetonitrile content and the buffer concentration in the mobile phase prolongs the analysis time (tR_{impF}).

In order to define optimal chromatographic conditions for pharmaceutical analysis of test compounds, it is necessary to monitor multiple responses that have opposite goals simultaneously, i.e., to provide good resolution of three critical pairs ($Rs_{impD,impE}$, $Rs_{impE,AB}$, $Rs_{AB,impF}$) for relatively fast analysis time, but also a good AB asymmetry peak.

Combinations of examined factors must be such that the analyzed responses have satisfactory values. For simultaneous optimization of all examined factors, and in order to obtain a global optimum (space in which all set goals are met), the method of multicriteria optimization was applied – Derringer's desirability function. The acceptable ranges for examined factors, also for the selected answers, sets the goals to be achieved by Derringer's desirability function in order to achieve the global optimum, and are given in Table 3.

By the selected values of the weighting coefficients, the preferred response function shape for each of the observed system responses is defined. Afterward, the significance coefficients values, which are used to calculate the global optimum, are determined.

As chromatographic system observed responses optimization as a goal, certain ranges are set, and shown in Table 3. All values within the set ranges are considered acceptable. The weighting coefficients are set to $s = t = 1$, which means that the desired answers function will linearly reach the value in the defined range. Value for coefficient of significance: for resolution factors ($Rs_{impD,impE}$, $Rs_{impE,AB}$, $Rs_{AB,impF}$) the highest coefficient of significance was set and was 5 (priority of the first place), for answer As_{AB} the coefficient

Fig. 2. 3-D graph for critical responses in function of the two critical parameters of chromatographic system (content of acetonitrile in the mobile phase [%] and concentration of ammonium acetate in water phase [mmol L⁻¹] or pH-value of water phase); A) $Rs_{impD,impE}$ – resolution factor between impurity D and impurity E; B) $Rs_{impE,AB}$ – resolution factor between impurity E and amlodipine besylate; C) $Rs_{AB,impF}$ – resolution factor between amlodipine besylate and impurity F; D) As_{AB} – factor asymmetry of amlodipine besylate; E) tR_{impF} – retention time of impurity F

Table 3. Derringer's desirability function – limit values and goals for determining the global optimum

	Variables	Range		Weight	Goal	Relative importance
		Lower limit	Upper limit			
Factors (inputs)	Acetonitrile (%)	89	92	1	in range	3
	pH value	3	5	1	in range	3
	Buffer concentration (mM)	40	60	1	in range	3
Responses (outputs)	$Rs_{impD,impE}$	0.20	5.91	1	>1.2	5
	$Rs_{impE,AB}$	0.34	4.29	1	>1.2	5
	$Rs_{AB,impF}$	0.15	4.10	1	>1.2	5
	As_{AB}	0.97	7.96	1	1.0–1.2	4
	tR_{impF}	3.16	11.26	1	in range	2

$Rs_{impD,impE}$ – resolution factor between impurity D and impurity E; $Rs_{impE,AB}$ – resolution factor between impurity E and amlodipine besylate; $Rs_{AB,impF}$ – resolution factor between amlodipine besylate and impurity F; As_{AB} – factor asymmetry of amlodipine besylate; tR_{impF} – retention time of impurity F.

of significance was set to 4 (priority of the second place), and for tR_{impF} the significance coefficient was 2 (not priority, but still significant). Value of coefficient significance for responses was set to 3 (important to keep in range). All stated values are of $p_i > 1$. This means that in order to achieve the

global optimum (D) all factors and all answers are significant. The assigned value of the coefficient of significance, the importance of reaching the individual optimal value of the answer (d_i) in achieving the global optimum (D) was emphasized [23, 24].

The defined limits, weighting coefficients and significance coefficients for the examined factors and the observed system responses were processed in the Design Expert® software program and thus the combination of factors that gives the global optimum ($D = 1$) was determined. From many combinations of factors in which the global optimum was achieved ($D = 1$), the values of these factors were expressed as decimal numbers (i.e., 90.62% acetonitrile, pH = 3.65, buffer concentration 47.49 mM et cetera). However, a global optimum study was performed with the integer values of the examined factors (predicted decimal numbers are rounded to the nearest integer value). Based on the parameters of the ANOVA test, 3D graph and Derringer's desirability function, the global optimum ($D = 1$) was approved and the optimal chromatographic conditions were selected: 90.5% (V/V) acetonitrile, pH = 4.0, and ammonium acetate buffer concentration 50 mM. The predicted response values corresponding to the latter value of D were: $Rs_{impD,impE} = 3.52$, $Rs_{impE,AB} = 3.15$, $Rs_{AB,impF} = 3.79$, $tR_{impF} = 8.24$ min, $As_{AB} = 1.04$ (Fig. 3). The response surface obtained for the global desirability function (global optimum) and the optimal chromatographic conditions are presented in Fig. 4.

Validation of the HILIC method for testing amlodipine and its impurities

After defining the optimal conditions, the robustness of the method was evaluated. The influence of four factors on five system responses was investigated, and a FFD 2^{4-1} to create the experiments was chosen. The experimental design is shown in Table 4. In this phase, the variations of acetonitrile content in the mobile phase and aqueous phase pH-value were followed, as well as ammonium acetate concentration in the aqueous phase and the mobile phase flow rate. The mobile phase flow rate was chosen as the fourth factor (factor D) to determine, in addition to three significant factors, its influence on the robustness of the method. This is especially important to follow changes in resolution factors.

Factor intervals were chosen to correspond to the expected factors variation (acetonitrile content $\pm 0.5\%$, the aqueous phase pH-value ± 0.2 pH units, ammonium acetate concentration in the aqueous phase ± 2 mmol L⁻¹ and mobile phase flow rate ± 0.1 mL min⁻¹).

The obtained results for the critical pair resolution factor ($Rs_{impD,impE}$, $Rs_{impE,AB}$, $Rs_{AB,impF}$) show that in all experiments, $Rs > 1.2$ values were obtained, as well as that the As_{AB} values are satisfactory (1.0–1.2). This confirms that a small change in all examined factors does not significantly affect the separation between these analyzed compounds. Special attention should be paid to the tR_{impF} response whose value varies from 6.99 to 10.80 min, ie with small changes in the examined factors there are significant changes in the duration of the analysis. From the obtained results for robustness assessment (Table 4) it can be concluded that all values of the monitored system responses are satisfactory and acceptable.

The next phase of the method robustness testing was a statistical interpretation of factors influence using a t -test and Dong's algorithm. Based on the data obtained for tracked system responses, the effect of four investigated factors was estimated. The results obtained for responses (Table 5) – statistical estimation of effects shows that all responses behave robustly within the examined range, ie all effects values have a lower value than the $E_{critical}$ ($Rs_{impD,impE} = 2.737$; $Rs_{impE,AB} = 0.643$; $Rs_{AB,impF} = 0.949$; $As_{AB} = 0.371$; $tR_{impF} = 4.670$) for the probability 0.05 [25].

From the results obtained for the robustness assessment (Tables 4 and 5), it can be concluded that all the values of the tracked responses are satisfactory and acceptable, which confirms that the method is robust.

The selectivity of the method was confirmed by comparing the chromatograms of tested compounds with the placebo chromatogram (Fig. 5). It can be seen that at the retention times corresponding to AB and impurities D, E and F, there are no chromatographic peaks of substances that interfere, and that originate from placebo. It can be concluded that the method is selective.

Fig. 3. The graphical presentation of the optimal mobile phase composition, as well as the predicted responses and corresponding adopted constraints

Fig. 4. A) The 2D-graph of Desirability space in function of two critical parameters of the chromatographic process (content of acetonitrile in the mobile phase [%] and pH-value in the water phase; B) the experimentally obtained chromatogram under the optimal conditions

Table 4. Plan of FFD 2^{4-1} experiments and obtained data

No.	Factors				Responses				
	Acetonitrile (%)	Buffer concentration (mM)	pH value	flow rate	$Rs_{impD,impE}$	$Rs_{impE,AB}$	$Rs_{AB,impF}$	As_{AB}	tR_{impF}
1	90.00	48.00	3.80	0.90	2.59	3.25	3.94	1.03	8.60
2	91.00	48.00	3.80	1.10	3.31	3.29	4.05	1.03	8.59
3	90.00	52.00	3.80	1.10	3.89	2.75	3.31	1.02	7.19
4	91.00	52.00	3.80	0.90	6.11	3.46	4.12	1.20	10.80
5	90.00	48.00	4.20	1.10	2.42	3.07	3.72	1.02	6.99
6	91.00	48.00	4.20	0.90	3.01	3.42	4.21	1.02	10.54
7	90.00	52.00	4.20	0.90	2.71	3.05	3.54	1.10	8.83
8	91.00	52.00	4.20	1.10	3.50	2.96	3.88	1.02	8.89
9	90.50	50.00	4.00	1.00	2.82	3.27	3.99	1.02	8.65
10	90.50	50.00	4.00	1.00	2.78	3.27	3.98	1.03	8.57
11	90.50	50.00	4.00	1.00	2.80	3.32	4.01	1.03	8.56

$Rs_{impD,impE}$ – resolution factor between impurity D and impurity E; $Rs_{impE,AB}$ – resolution factor between impurity E and amlodipine besylate; $Rs_{AB,impF}$ – resolution factor between amlodipine besylate and impurity F; As_{AB} – factor asymmetry of amlodipine besylate; tR_{impF} – retention time of impurity F.

Table 5. Factors effects and results for the $E_{critical}$

Factors	$Rs_{impD,impE}$	$Rs_{impE,AB}$	$Rs_{AB,impF}$	As_{AB}	tR_{impF}
A	1.08	0.25	0.44	0.11	1.80
B	1.22	-0.20	-0.27	0.15	0.24
C	-1.06	-0.06	-0.02	-0.12	0.02
D	-0.33	-0.28	-0.21	-0.15	-1.78
$E_{critical}$ ($\alpha = 0.05$)	2.737	0.643	0.949	0.371	4.670

$Rs_{impD,impE}$ – resolution factor between impurity D and impurity E; $Rs_{impE,AB}$ – resolution factor between impurity E and amlodipine besylate; $Rs_{AB,impF}$ – resolution factor between amlodipine besylate and impurity F; As_{AB} – factor asymmetry of amlodipine besylate; tR_{impF} – retention time of impurity F; $E_{critical}$ – critical value of the effect.

Linearity, accuracy and precision testing, as well as the determination of LOD and LOQ for the impurities D, E and F and amlodipine were performed and obtained results are presented in Table 6. Finally, the developed method was applied for the analysis of Vazotal® 5 mg tablets containing 6.934 mg AB. The method proved to be sensitive, specific, accurate, precise and applicable for the analysis of AB tablets.

CONCLUSION

This study presents a detailed step-by-step development of a new HILIC method for amlodipine besylate and its specific

Fig. 5. Chromatogram that confirms the selectivity of the HILIC method

Table 6. Validation parameters

	AB	Impurity D	Impurity E	Impurity F
LOD ($\mu\text{g mL}^{-1}$)	–	0.010	0.010	0.010
LOQ ($\mu\text{g mL}^{-1}$)	–	0.025	0.025	0.025
<i>Linearity</i>				
Concentration range ($\mu\text{g mL}^{-1}$)	50–200.0	0.025–15.0	0.025–15.0	0.025–15.0
$y = ax + b$	$y = 27.01x - 0.19$	$y = 25.94x + 9.46$	$y = 26.54x + 9.80$	$y = 26.56x + 22.32$
Sa	0.0032	0.5625	0.1812	0.3300
Sb	0.4318	6.9177	2.2281	4.0578
tb	–0.4430	1.3679	4.3975	5.5004
r	1.0000	0.9993	0.9999	0.9998
<i>Accuracy</i>				
80% (80 $\mu\text{g mL}^{-1}$)				
Recovery (%)	101.2	98.55	100.65	99.87
RSD (%) ^a	0.18	0.25	0.56	0.39
100% (100 $\mu\text{g mL}^{-1}$)				
Recovery (%)	98.5	97.56	97.40	100.88
RSD (%) ^a	0.90	0.37	0.42	0.87
120% (120 $\mu\text{g mL}^{-1}$)				
Recovery (%)	98.1	98.30	96.39	97.71
RSD (%) ^a	0.51	0.91	0.19	0.76
<i>Precision</i>				
Concentration ($\mu\text{g mL}^{-1}$)	100.0	10.0	10.0	10.0
RSD (%) ^b	0.66	1.70	0.93	2.14

AB – amlodipine besylate; LOD – limit of detection; LOQ – limit of quantification; Sa – standard deviation of the slope; Sb standard deviation of the intercept; tb – intercept value; r – correlation coefficient (>0.99 for active ingredients, >0.98 for related compounds [22]);

^a RSD – relative standard deviation for accuracy ($<2\%$ for active ingredients, $<15\%$ for related compounds); ^b RSD – relative standard deviation for precision ($\leq 1.0\%$ for active ingredients, $<10\%$ for related compounds) [22].

impurities analysis, using a chemometric approach. Using response surface methodology and new way for Derringer's desirability function, the method was optimized successfully. The robustness of the method, as well as all other validation parameters, were examined. This is the first time that the HILIC method for the analysis of amlodipine besylate and its specific impurities has been developed, but also the first time that a method that lasts quite a short time (10 min) has been developed. The method proved to be selective, accurate, precise and sensitive, which was confirmed in the routine analysis of Vazotal[®] 5 mg tablets.

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