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Development and validation of a stability-indicating RP-HPLC method for determination of aripiprazole and its degradation products

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Abstract

The goal of this study was the optimization of chromatographic conditions and validation of the isocratic RP-HPLC method for monitoring the stability of aripiprazole, identification and quantitative analysis of aripiprazole and its degradation products in tablets.

In addition, robustness was tested by applying the methodology of experimental design.

The forced degradation study of aripiprazole was conducted in accordance with the ICH guidelines. The stability of the active pharmaceutical substance was tested under the conditions of hydrolysis in acidic, neutral and basic environments, thermal degradation, oxidation and photolysis. The active pharmaceutical ingredient was degraded under oxidation conditions, and the identity of the resulting degradation product, N-oxide, was confirmed. Under the other conditions tested, the active pharmaceutical substance was found to be stable. The developed method RP-HPLC allowed the separation of degradation products and aripiprazole and was defined as a stability-indicating method. The proposed method was validated for qualitative and quantitative analysis of aripiprazole and its degradation products. Accordingly, selectivity, linearity, precision, accuracy, limit of detection, limit of quantification, and robustness of the method were tested. The Box-Behnken experimental design was used in robustness testing.

Key words: aripiprazole, liquid chromatography, Box-Behnken experimental design, forced degradation study, degradation products

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Introduction

Aripiprazole is a piperazine analog, an arylpiperidine-quinolinone derivative. It is chemically described as 7-[4-[4-(2,3-dichlorophenyl)-1-piperazinyl] butoxy]-3,4-dihydro-2(1H)-quinolinone (Figure 1).

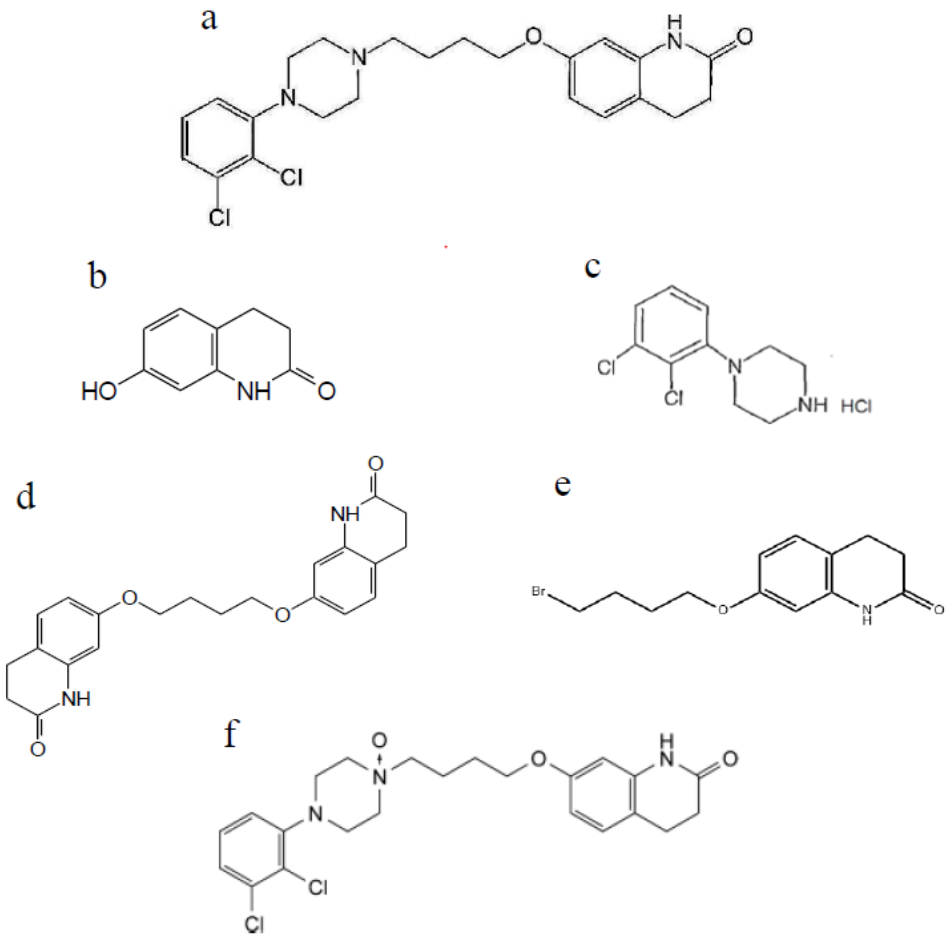

Figure 1. Structural formula of aripiprazole (a), Impurity A (b), Impurity B (c), Impurity C (d), Impurity D (e) and Impurity E (f)

Slika 1. Strukturna formula aripiprazola (a), Nečistoće A (b), Nečistoće B (c), Nečistoće C (d), Nečistoće D (e) i Nečistoće E (f)

Aripiprazole belongs to the newer generation of antipsychotics, which are also called atypical antipsychotics or second-generation antipsychotics. However, due to its specific mechanism of action and a different GPCR binding profile compared to other atypical antipsychotics, some authors classify it as a third-generation antipsychotic.

Aripiprazole achieves its antipsychotic effect through dopamine D_2 and serotonin 5-HT_{1A} and 5-HT_{2A} receptors. Unlike earlier atypical antipsychotics that generally function as D_2 antagonists, aripiprazole is a partial D_2 receptor agonist. In addition, it acts as an agonist of 5-HT_{1A} receptors and an antagonist of 5-HT_{2A} receptors (1).

Aripiprazole does not have an asymmetric center and occurs in an optically inactive form. It is an achiral molecule, that implies the absence of stereoselectivity and configurationally instability, possibilities of inversion – formation of stereoisomeric impurities (2, 3).

Three impurities may occur during the synthesis of aripiprazole: impurities A, B and D. The above-mentioned impurities are also potential degradation products. Impurity A, chemically described as 7-hydroxy-3,4-dihydroquinolin-2(1H)-one, and impurity B, 1-(2,3-dichlorophenyl)-piperazine hydrochloride, are the starting materials in the aripiprazole synthesis process. In contrast, impurity D, 7,7'-[butane-1,4-diybis(oxy)] bis(3,4-dihydroquinolin-2(1H)-one, is an intermediate in the synthesis of aripiprazole.

Impurity C, 7,7'-[butane-1,4-diybis(oxy)] bis(3,4-dihydroquinolin-2(1H)-one, is a dimer of impurity D and can be formed by its dimerization. Impurity E, 7-[4-[4-(2,3-dichlorophenyl)-1-oxidopiperazin-1-yl] butoxy]-3,4-dihydro-1H-quinolin-2-one, is the N-oxide of aripiprazole and a degradation product formed in the oxidation process. Manufacturers' impurities A, B and E are official in the monograph of the active substance of the European Pharmacopoeia under the name impurities A, B and Imp E (Ph. Eur. impurities F) was confirmed as a degradation product in the stability study. The chemical structures of impurities A, B, C, D, and E and aripiprazole are shown in Figure 1.

The literature survey revealed several reported simple, fast, isocratic RP-HPLC and UPLC methods for the quantification of aripiprazole or simultaneously with its impurities in the pharmaceutical dosage forms with ultraviolet detection (4-10).

Aripiprazole and certain impurities were separated by isocratic methods with shorter elution (9-10). However, HPLC methods with gradient elution were required for successful separation of aripiprazole from a larger number of impurities (11-13). Another, simple and simultaneous RP-HPLC method for quantitative analysis of aripiprazole, degradation products of aripiprazole, methylparaben and propylparaben, in oral solution was developed (14).

One of the last developed RP-HPLC methods with a new optimization strategy based on the mixed quantitative structure–retention relationship (QSRR) model was created for improving the RP-HPLC separation of aripiprazole and its impurities (IMP A-E) (15).

Aripiprazole is official in the European Pharmacopoeia monograph 2617 (16), the British Pharmacopoeia (17) and the US Pharmacopoeia (18). The methods for determining the content and testing the purity of aripiprazole according to Ph. Eur, BP and USP are identical. The test is performed by the method of reverse-phase liquid chromatography with gradient elution. The US Pharmacopoeia also has monographs for the dosage form, aripiprazole tablets and aripiprazole orally disintegrating tablets.

The developed method, compared with the previously published analytical protocols, as well as in relation to the pharmacopoeial method, represents a significant improvement since it is: (1) a method with simple sample preparation (2), a simple and fast isocratic method that enables the separation and quantitative analysis of aripiprazole and its degradation products in a short time interval (12 min), and since, (3) due to these benefits (speed, simplicity and economy), it is suitable for routine use.

Experimental

Chemicals and materials

All standard substances, aripiprazole and its impurities (impurity A, B, C, D and E) were obtained from Orchid Chemicals & Pharmaceuticals Ltd, India. Solvents and chemicals used are: Acetonitrile gradient grade (Baker, USA), Water HPLC grade, Potassium dihydrogen phosphate, p.a. (Sigma Aldrich, Germany), Triethylamine - HPLC grade (Fisher Chemical, USA), Ortho-phosphoric acid 85%, p.a. (Baker, USA).

The following solvents and reagents were additionally used for the forced degradation study: Hydrochloric acid conc. (Zorka Pharma, Serbia), Sodium hydroxide, p.a. (Zorka Pharma, Serbia) and Hydrogen peroxide 30%, p.a. (Sigma Aldrich, Germany).

Equipment

The chromatographic system included an Agilent 1200 Series configuration with a PDA detector.

Solutions for validation testing

Aripiprazole stock solution was prepared by accurately weighing 40 mg of aripiprazole standard substance into a 20 ml volumetric flask and making up to the mark with the mobile phase. The solution thus obtained had a concentration of 20 mg mL^{-1}. A stock solution of each impurity was prepared individually, by accurately weighing and transferring 5 mg of standard substances to a 50 ml measuring vessel and filling up to the mark with the mobile phase. The solutions thus obtained had a concentration of 0.1 mg mL^{-1}.

The concentrations of the standard solutions for the linearity test were 140, 170, 200, 232 and 260 μg mL^{-1} for aripiprazole, and 0.20, 0.30, 0.40, 1.0 and 2.0 μg mL^{-1} for the impurities.

The concentrations range for aripiprazole was from 70 to 130%, and for its impurities it was 0.1–1%, calculated relative to the working concentration of aripiprazole.

Standard solutions for precision and accuracy contained 140, 200 and 260 μg mL^{-1} of aripiprazole and 0.20, 0.40 and 2.0 μg mL^{-1} for impurities. The solutions were prepared by spiking the placebo mixture with an exact amount of aripiprazole and its impurities. Three solutions of each concentration were prepared and each solution was injected three times.

Solutions for sensitivity testing were prepared by diluting concentrated stock solutions.

LOQ and LOD test solution concentrations are shown in Table I.

Table I Limits of detection (LOD) and limits of quantification (LOQ)

Tabela I Limiti detekcije (LOD) i limiti kvantifikacije (LOQ)

	LOQ (μg/mL)	LOD (μg/mL)
Aripiprazole	0,20	0,10
Impurity A	0,07	0,03
Impurity B	0,10	0,05
Impurity C	0,20	0,12
Impurity D	0,20	0,12
Impurity E	0,20	0,12

A working solution of the selectivity test was prepared by diluting standard stock solutions to final concentrations of 2.0 μg mL^{-1} for impurities and 200 μg mL^{-1} for aripiprazole. At the same time, for the purpose of comparison, a placebo solution was also prepared. The placebo solution is prepared so that it contains the same excipients as the finished product, except for the active substance.

The preparation of solutions for testing the robustness of the method involved the preparation of the buffer solution: 20mM KH_2PO_4 containing 1% TEA, and the pH of the buffer solution was at first 3.20, then 3.00, and finally 2.80 pH units. Buffer solution and acetonitrile were mixed in three volume ratios: 65:35, V/V; 67:33, V/V and 69:31, V/V.

Preparation of stress agents for the study of forced degradation

As stress agents for the forced degradation study, the following agents were used: hydrochloric acid solution 1mol L^{-1}, sodium hydroxide solution 2 mol L^{-1}, and hydrogen peroxide solution 1%. Hydrochloric acid solution 1mol L^{-1} was obtained by diluting commercial concentrated 37% hydrochloric acid, sodium hydroxide solution 2mol L^{-1} was obtained by dissolving the sodium hydroxide substance in the appropriate ratio in demineralized water, while hydrogen peroxide solution was obtained by diluting a 30% hydrogen peroxide solution with demineralized water in the appropriate ratio to obtain a 1% solution of hydrogen peroxide.

Preparation of standard solution for the study of forced degradation

Aripiprazole standard solution for the forced degradation study had a concentration of 1.0 mg mL^{-1}. After exposure to the stress agent, all solutions were diluted with a mobile phase so that the concentration of aripiprazole was 200 μg mL^{-1}.

Preparation of control samples for the study of forced degradation

During the forced degradation study, several types of control samples were analyzed comparatively with stressed samples: control samples at time zero, dark control and blank test. Control samples at time zero were prepared in the same way as stressed samples, but were injected fresh, immediately after preparation. A dark control was used for comparison with samples exposed to photo degradation. These samples were prepared in the same way and analyzed at the same time points as the stressed samples, but were stored protected from light in the meantime, to prevent degradation. The blank contained the same components as the stressed sample, except for the active substance itself. The blank was prepared in the same manner and analyzed at the same time points as the stressed samples.

Preparation of the sample solution

Zolprix film tablets containing 5mg of aripiprazole were weighed on an analytical balance and the average tablet mass was calculated. The tablets were crushed in a mortar and the tablet mass was homogenized by mixing. The amount of tablet mass corresponding to two average tablet masses was accurately measured on an analytical balance and quantitatively transferred into a 50 ml measuring vessel. About 35 ml of solvent was added to the dish, the dish was treated in an ultrasonic bath for 15 minutes, and then made up to the mark with the mobile phase and filtered through a membrane filter (0.45 μm). The expected concentration of aripiprazole was 200 μg mL^{-1}.

Results and discussion

Development of the analytical method

The first parameter that had to be chosen was the detection wavelength of the examined compounds. Given that aripiprazole shows two maxima at the wavelengths of 217 nm and 252 nm, those two wavelengths were chosen for preliminary testing. At a lower wavelength, there was a better response, but the higher wavelength was chosen due to better chromatographic parameters and the elimination of placebo and solvent interference. During the development of the analytical method, many analytical columns were used. In the further selection of analytical columns, it was concluded that the criterion for the selection of the stationary phase could be the value of carbon load. A high percent of carbon implies a greater lipophilicity of the column, which should enable greater retention and better separation of components. In this way, Discovery HS C_{18} column, 100x4.6mm; 5μm (Supelco), whose carbon content is 20, was chosen. Given that the solubility of aripiprazole is best at an acidic pH and that the solubility increases with a decrease in the pH value of the solution, the development went in the direction of using a pH range of 3-5 pH units. Optimum separation was achieved at pH 3.0, where peak resolutions and symmetries showed the best results.

After a series of experiments, the optimal conditions that enabled the complete separation of aripiprazole and its five impurities were defined by changing the key

parameters of the method: working flow rate, pH, proportion of acetonitrile and column temperature.

Reducing the flow rate increases the degree of separation of the critical pairs, impurities A and B on the one hand, and aripiprazole and impurity E on the other hand. Based on the obtained results, the optimal flow rate of 1ml/min was defined. This flow rate provides a satisfactory resolution of the critical pairs (Rs>2), with the shortest run time. Longer elution of components negatively affected the shape of the peaks that are last eluted (impurities C and D), which significantly reduces the sensitivity of the method.

The proportion of acetonitrile proved to be the factor with the greatest influence on the resolution of critical pairs and duration of the analysis. As with the change in the flow rate, with the decrease in the percentage of acetonitrile, the resolutions between the critical pairs were better, but due to the longer duration of the analysis, the economy of the method was reduced. Increasing the percentage of acetonitrile resulted in an unacceptable decrease in the resolution, especially of the second critical pair of aripiprazole and impurity E. A significant decrease in the percentage of acetonitrile led to a complete overlapping of the peaks of aripiprazole and impurity E. Based on the results obtained, it was found that the optimal percentage of acetonitrile was 33%.

The next parameter considered was the temperature. The test showed that increasing the temperature of the column leads to a decrease in the total duration of the analysis, because the retention times of all components are shortened. The optimal temperature was 40ºC. Higher temperatures were not suitable due to the stability of the columns, and lower temperatures (35°C, 25°C), in addition to increasing the duration of the analysis, also negatively affected the separation, especially the first critical pair of impurities A and B.

Optimal chromatographic conditions were achieved on column Discovery HS C_{18}, 100 x 4.6 mm, 5 μm (Supelco), with the mobile phase consisting of buffer pH 3.0 (20mM KH_2PO_4, 1% triethylamine, pH adjusted with 85% ortho-phosphoric acid): acetonitrile (67:33, V/V). The column temperature was 40 ºC, flow rate 1 mL min^{-1} and the detection was performed at 252 nm. The injection volume was 20 μL and the mobile phase was used as solvent.

The forced degradation study

The forced degradation study of the active pharmaceutical substance aripiprazole was conducted according to the ICH guidelines (19). The experimental conditions must include testing the drug's sensitivity to hydrolysis, oxidation, thermal degradation, moisture and light. The exact conditions are left to the analyst's choice.

During the forced degradation study it is necessary to achieve a suitable level of degradation (5–20%). It is considered that this percentage of degradation provides a representative picture that corresponds to the degradation that would occur under realistic storage conditions. A degradation of less than 5% makes it difficult to identify primary impurities, because they are present in a small amount, while a high percentage of

degradation increases the possibility of creating secondary and tertiary degradation products, which unnecessarily complicates the interpretation of results.

The hydrolysis test in a neutral environment was performed with the active pharmaceutical substance in an aqueous solution at a temperature of 70 ºC. After 4 hours at an elevated temperature, one peak with RRt 0.82 (Impurity 1) is observed on the chromatogram of the tested sample. However, since total degradation of at least 5% was not achieved, this degradation product was not considered a key degradation product (Table II), so it was discarded, and the sample was declared stable to hydrolysis conditions in a neutral medium.

Table II Summary results of the forced degradation study of aripiprazole

Tabela II Sumarni rezultati studije forsirane degradacije aripiprazola

	1M HCl 70 °C 4h	**2M NaOH 70°C 4h**	**H_2O 70 °C 4h**	**1% H_2O_2 40 °C 1h**	**105°C 24h**	**Sunlight 360h**
Aripiprazole	100%	100%	99,846%	95%	100%	100%
Purity factor	999,966	999,938	999,965	999,974	999,880	999,835
Degradation	0%	0%	0,15%	5%	0%	0%
N-oxide	/	/	/	5%	/	/
Impurity 1	/	/	0,15%	/	/	/

As was assumed at the very beginning based on literature data, the active pharmaceutical ingredient was degraded under oxidation conditions, and the identity of the resulting degradation product, N-oxide, was confirmed (Figure 2). Under the other tested conditions, the active pharmaceutical substance was found to be stable.

The summary results of the study of the forced degradation of the active pharmaceutical substance aripiprazole, as well as the conditions, are shown in Table II.

During the tests, mass balance was monitored for all stress samples to ensure that all degradation products were detected. The mass balance expressed in percentages is calculated as the sum of the percentage representation of the non-degraded substance and the percentage representation of all degradation products in the stressed sample. This sum should ideally be as close as possible to 100%, in order to detect all degradation products. The mass balance value for all samples was greater than 99%, which indicated the suitability of the method proposed, ensuring the detection of all the resulting degradation products. In addition to mass balance, peak purity was also monitored for all stress samples. Peak purity value for all samples was greater than 990, which confirmed the high purity of the peak. Related substances and excipients do not co-elute and do not interfere in the process of quantifying the tested component.

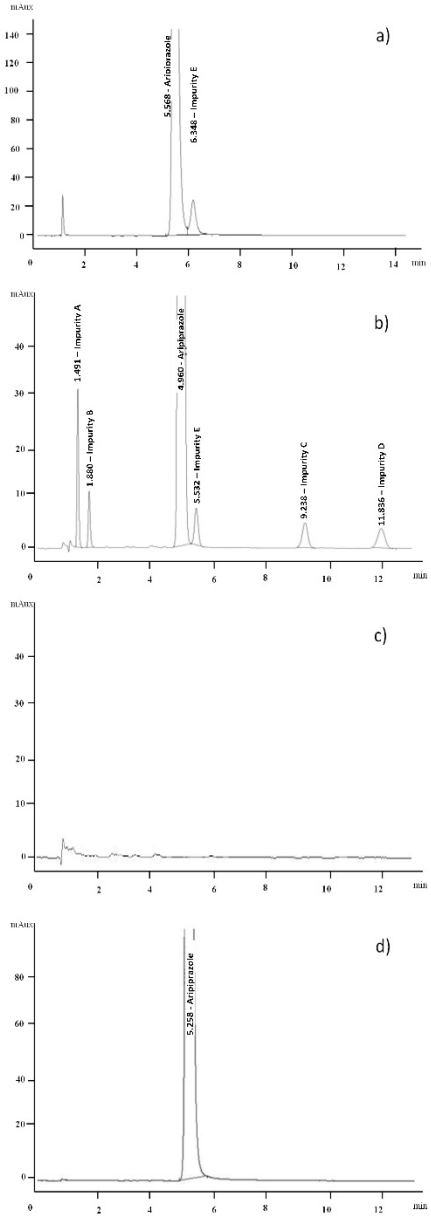

Figure 2. Chromatograms of the stressed sample of aripiprazole solution treated with 1% hydrogen peroxide at 40 ºC after 1h (a), working solution of the standard for the selectivity (b), placebo solution (c) and sample solution (d) (column Discovery HS C_{18}, 100 x 4.6 mm, 5 µm; Mobile phase: buffer pH 3.0 (20mM KH_2PO_4, 1% triethylamine, pH adjusted with 85% ortho-phosphoric acid): acetonitrile (67:33, V/V))

Slika 2. Hromatogram stresiranog uzorka rastvora aripiprazola tretiranog 1% vodonik peroksidom na 40 ºC posle 1h (a), radnog rastvora standarda za ispitivanje selektivnosti (b), rastvora placeba (c) i rastvora uzorka (d) (kolona Discovery HS C_{18}, 100 x 4.6 mm, 5 µm; mobilna faza: pufer pH 3.0 (20mM KH_2PO_4, 1% triethylamine, pH podešen sa 85% orto-fosfornom kis.) / acetonitril (67:33, V/V))

Validation of the analytical method

In order to validate the stability-indicating RP-HPLC method for the determination of aripiprazole and its impurities, the following parameters were examined: selectivity, linearity and range, precision, accuracy, limit of detection, limit of quantification and robustness.

Selectivity

Under the specified chromatographic conditions, a placebo solution, a sample solution, and a working standard solution for selectivity testing were injected. Working standard solution for selectivity testing contains aripiprazole in the amount of 100% and its degradation products in the amount of 1% in relation to the working concentration of aripiprazole in the sample solution.

The retention times of the chromatographic peaks from the chromatogram of the placebo solution were compared with the retention times of the peaks from the chromatogram of the working solution of the selectivity test standard and the sample solution.

Selectivity was confirmed, considering that there are no peaks on the chromatogram of the placebo solution with retention times corresponding to the retention times of aripiprazole and its impurities A, B, C, D and E.

Chromatograms of the placebo solution and the working standard solution for selectivity testing are shown in Figure 2.

The chromatographic parameters of the RP-HPLC analysis are shown in Table III.

Table III Chromatographic parameters for RP-HPLC analysis of aripiprazole and impurities

Tabela III Hromatografski parametri za RP-HPLC analizu aripiprazola i nečistoća

	Impurity A	**Impurity B**	**Aripiprazole**	**Impurity E**	**Impurity C**	**Impurity D**
t_r	1,441	1,725	4,444	4,911	8,831	11,763
A_s	1,157	1,210	1,328	0,936	1,005	1,078
N	3197	3444	6541	8327	8927	8908
R_s	/	2,577	16,098	2,142	13,288	6,710
rrt	0,324	0,388	1	1,105	1,987	2,647

t_r retention time, A_s symmetry factor, N Number of theoretical plates, R_s resolution, rrt relative retention time

t_r retenciono vreme, A_s faktor simetrije, N broj teoretskih platoa, R_s rezolucija, rrt relativno retenciono vreme

Linearity

For the linearity test, five standard solutions of different concentrations for aripiprazole and impurities A, B, C, D and E were prepared. The solutions were injected into the chromatographic system. The concentration range for aripiprazole was from 70 to 130%, and for impurities from 0.1 to 1%, calculated relative to the working concentration of aripiprazole in the sample solution. The dependence of the peak area (*y*) of the tested substances as a function of concentration (*x*) was analyzed. The calibration curves were constructed by the method of least squares, and each point was obtained as a result of three injections of the corresponding solutions. Regression analysis of the obtained data showed that there is a linear dependence between the area of the peaks and the concentrations of the tested compounds in the concentration range of 140-260 μg mL^{-1} for aripiprazole and 0.2-2 μg mL^{-1} for impurities, because the correlation coefficients for all obtained calibration curves were greater than 0.999. The results of the regression analysis for the tested substances are shown in Table VII.

Precision (repeatability) and accuracy

The precision (repeatability) of the method and the accuracy were tested by analyzing a laboratory mixture of standards containing known amount of impurities (concentration 0.20; 0.40 and 2.0 μg mL^{-1}), aripiprazole (concentration 140; 200 and 260 μg mL^{-1}), as well as placebo components in a ratio corresponding to the composition of auxiliary components in the sample. Three solutions of each concentration were prepared. Based on the obtained results of standard deviation and relative standard deviation, which for aripiprazole ranged from 0.68-1.96%, and for impurities from 0.00-3.51%, the good precision of the method was confirmed. Due to satisfactory recovery values for all concentrations levels of each analyte, which for aripiprazole ranged from 98.5-100.7%, and for impurities from 96.5-104.6%, the method accuracy was proven.

The results for the precision and accuracy tests are shown in Table VII.

Determining the LOQ and LOD was done by a combination of experimental work and evaluation of the signal/noise ratio. Standard solutions for determination LOQ and LOD were injected three times. The analysis of the obtained results confirmed a good precision, and also in all three repetitions the defined requirements for the limits were met. The limit of detection (LOD) is the concentration that has a signal-to-noise ratio of at least 3:1 (S/N>3), while the limit of quantification is the concentration that has a signal-to-noise ratio of at least 10:1 (S/N >10). The obtained results are shown in Table I.

Robustness

For robustness testing, the Plackett Burman design, where a significant number of factors can be tested with a relatively small number of experiments to obtain the best estimate, is most frequently used. During development and optimization, the method proved to be robust in terms of most factors. It was only noticed that the percentage of ACN in the mobile phase and the temperature of the column represent factors that significantly affect the robustness of the system. In order to examine and precisely define

the influence of these two factors (chromatographic conditions) on the resolution of critical pairs, the robustness of the method was tested using the Box-Behnken experimental design. In relation to the fact that Box-Behnken with two factors does not exist, a third factor was also selected. The chromatographic conditions (factors) whose influence on the robustness of the chromatographic system was examined were: the percentage of acetonitrile in the mobile phase (F1), the pH value of the buffer (F2) and the column temperature (F3). The chromatographic parameters whose response was monitored during the experiment were the resolution factor between impurity A/B (Rs_1) and resolution factor between aripiprazole/impurity E (Rs_2). The obtained resolution values of the critical pairs of tested substances during fifteen experiments in the Box-Behnken experimental design are shown in Table IV. The Design Expert 7.0.0 program was used to process the obtained results. After processing the obtained results, for the first critical pair, impurities A and B, there was no obvious correlation between the response Rs_1 (resolution) and the given variables. For this reason, the program suggested the *Square root* of the response. In further processing, it was assessed whether the examined dependence of the analyzed response on the factor fits best into linear dependence, the dependence described by two-factor interactions or a second-order polynomial. By analyzing the parameters of the model, a linear f (x) model was selected.

Analyzing the *Lack of Fit* test determined the adequacy of the selected model. After the appropriate model was chosen, the obtained experimental results were analyzed using the *Response Surface Methodology*. The results of the analysis of variance (ANOVA) for the response factor, linear model, for the first critical pair, impurity A and impurity B, are shown in Table V. Factors with a p-value of less than 0.05 are considered significant.

This method enables a graphical representation of the system's response as a function of one or more factors. A 3D plot of the dependence of Rs_1 of the first critical par (impurities A and B) on the proportion of acetonitrile and the pH value of the buffer, and a 3D plot of the dependence of Rs_1 of the first critical par on the proportion of acetonitrile and temperature of the column are shown in Figure 3.

The response of the system can also be represented by a linear equation. The resulting 3D diagram for the first critical pair corresponds to the following equation for the coded factors:

$$y = 1{,}67 – 0{,}32x_1 - 7{,}859E\text{-}003x_2 + 7{,}503E\text{-}003x_3$$

where y represents the resolution of the critical pair impurities A and B, x_1 the percentage of acetonitrile in the mobile phase, x_2 the pH of buffer, and x_3 the temperature of the column.

Based on the obtained results, it was concluded that only the percentage of acetonitrile in the mobile phase has a significant influence on the separation of impurities A and B. Since the resolution of the critical pair was greater than 1.5 in all experiments, the robustness of the chromatographic system was confirmed.

The analysis of the second response factor Rs_2, resolution of the second critical pair of aripiprazole and impurity E were examined by *Two-factor interactions* (2FI).

Table IV Resolution values of critical pairs of test substances obtained by the Box-Behnken experimental design

Tabela IV Vrednosti rezolucije kritičnih parova ispitivanih supstanci dobijene u Box-Behnken eksperimentalnom dizajnu

Number of experiments	Order of examination	% ACN	pH	t° C	Rs_1	Rs_2
1	4	31	2,8	40	4,07	2,90
2	7	35	2,8	40	1,79	1,35
3	14	31	3,2	40	4,00	2,99
4	11	35	3,2	40	1,78	0,70
5	3	31	3,0	30	4,03	3,13
6	6	35	3,0	30	1,84	0,60
7	5	31	3,0	50	3,83	2,71
8	8	35	3,0	50	1,98	0,60
9	9	33	2,8	30	2,73	2,30
10	15	33	3,2	30	2,72	2,18
11	13	33	2,8	50	2,89	1,87
12	2	33	3,2	50	2,76	1,93
13	1	33	3,0	40	2,77	2,02
14	12	33	3,0	40	2,73	2,05
15	10	33	3,0	40	2,94	2,20

Rs_1-resolution between impurities A and B

Rs_2-resolution between aripiprazole and impurity E

Rs_1-rezolucija između nečistoća A i B

Rs_2- rezolucija između aripiprazola i nečistoće E

Analyzing the *Lack of Fit* test determined the adequacy of the 2FI model. The results of an analysis of variance (ANOVA) for the response factor, 2FI model, for the second critical pair, aripiprazole and impurity E, are shown in Table VI.

A 3D plot of Rs_2 (resolution between aripiprazole and impurity E) versus the percentage of acetonitrile, pH, and temperature is shown in Figure 3.

Table V Analysis of variance to examine the influence of acetonitrile content, pH of mobile phase and column temperature on the resolution of impurities A and B

Tabela V Analiza varijanse za ispitivanje uticaja udela acetonitrila, pH mobilne faze i temperature kolone na rezoluciju nečistoće A i B

Factor	Sum of squares	Number of degrees of freedom	Mean sum of squares	F-value	p-value
Model	0,81	3	0.27	338,68	<0.0001
% ACN	0,81	1	0.81	1014,86	<0.0001
pH	4,941E-004	1	4,941E-004	0,62	0,4481
Temperature	4,503E-004	1	4,503E-004	0,56	0,4684
Lack of fit	6,59E-003	9	7,328E-004	0,67	0,7246
Pure error	2,189E-003	2	1,094E-003		
Sum	0,82	14			

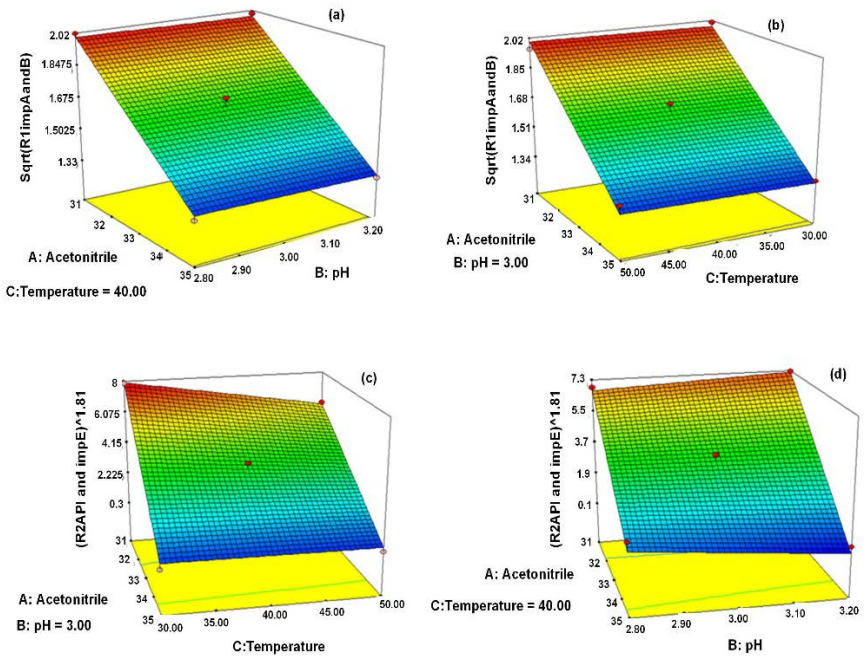

Figure 3. 3D diagram of the dependence of Rs_1 (resolution between impurities A and B) on the percentage of acetonitrile in the mobile phase and the pH value of buffer (a), Rs_1 on the percentage of acetonitrile in the mobile phase and temperature of the column (b), Rs_2 (resolution between aripiprazole and impurity E) on the percentage of acetonitrile in the mobile phase and temperature of the column (c) and Rs_2 on the percentage of acetonitrile in the mobile phase and pH of the buffer (d)

Slika 3. 3D dijagram zavisnosti Rs_1 (rezolucije između nečistoće A i B) od procenta acetonitrila u mobilnoj fazi i pH vrednosti pufera (a), Rs_1 od procenta acetonitrila u mobilnoj fazi i temperature kolone (b), Rs_2 (rezolucije između aripiprazola i nečistoće E) od procenta acetonitrila u mobilnoj fazi i temperature kolone (c) i Rs_2 od procenta acetonitrila u mobilnoj fazi i pH pufera (d)

Table VI Analysis of variance to examine the effects of acetonitrile fraction, mobile phase pH and column temperature on the resolution of aripiprazole and impurity E

Tabela VI Analiza varijanse za ispitivanje uticaja udela acetonitrila, pH mobilne faze i temperature kolone na rezoluciju aripiprazola i nečistoće E

Factor	Sum of squares	Number of degrees of freedom	Mean sum of squares	F-value	p-value
Model	82,18	6	13,7	127,66	<0.0001
A: % ACN	78,47	1	78,47	731,42	<0.0001
B: pH	0,14	1	0,14	1,26	0,2937
C: Temperature	2,03	1	2,03	18,95	0,0024
AB	0,63	1	0,63	5,88	0,0416
AC	0,82	1	0,82	7,64	0,0244
BC	0,090	1	0,090	0,84	0,3865
Lack of fit	0,86	8	0,11	1,06	0,5591
Pure error	0,65	6	0,11		
	0,21	2	0,10		
Sum	83,04	14			

The response of the system can also be represented by a linear equation. The resulting 3D diagram for the first critical pair corresponds to the following equation for the coded factors:

$$y = 3,84 – 3,13x_1 -0,13x_2 -0,50x_3 -0,40x_1x_2+0,45x_1x_3+0,15x_2x_3$$

where y represents the resolution of the critical pair of aripiprazole and impurity E, x_1 percentage of acetonitrile, x_2 the pH of buffer and x_3- the temperature of the column.

The obtained results indicate that the proportion of acetonitrile and column temperature are factors that significantly affect the resolution of aripiprazole and impurity E, while the change in the pH of the mobile phase has no significant effect. It can be concluded that the optimal conditions must be kept under control in order to maintain a satisfactory resolution.

For a quantitative determination of aripiprazole and its impurities A, B, C, D and E by the validated stability-indicating RP-HPLC method in Zolprix tablets 5 mg tbl^{-1}, sample solutions at a concentration of 200 μg mL^{-1} were prepared and injected into the

Table VII Results for precision, accuracy and linearity tests for the tested substances

Tabela VII Rezultati ispitivanja preciznosti, tačnosti i linearnosti za ispitivane supstance

	Weigh (µg/mL)	Determined (µg/mL)	RSD (%)	Recovery (%)	y=ax+b	r
Aripiprazole	140	141,04 ± 2,76*	1,96	100,7	y=41259.14x-110.99	0,9992
	200	196,90 ± 1,64*	0,68	98,5		
	260	259,30 ± 5,21*	1,95	99,7		
Impurity A	0,2	0,2385 ± 0,0017*	0,84	101,9	y=58225.68x-0.0694	0,9999
	0,4	0,4185 ± 0,0030*	0,71	104,6		
	2	2,0776 ± 0,0096*	0,46	103,9		
Impurity B	0,2	0,2092± 0,0021*	1,02	104,6	y=27026.69x-0.1208	0,9999
	0,4	0,4090 ± 0,0021*	0,52	102,2		
	2	2,0481 ± 0,0560*	2,74	102,4		
Impurity C	0,2	0,2071 ± 0,0020*	0,97	103,5	y=42986.06x-1.4691	0,9999
	0,4	0,4126 ± 0,0013*	0,33	103,1		
	2	1,9301 ± 0,0374*	1,94	96,5		
Impurity D	0,2	0,2095 ± 0,0015*	0,71	103,9	y=39272.54-0.5193	0,9998
	0,4	0,4126 ± 0,0144*	3,51	103,2		
	2	2,0365 ± 0,0459*	2,25	101,8		
Impurity E	0,2	0,1976 ± 0,0000*	0,00	98,8	y=40024.91x-0.4072	0,9999
	0,4	0,3941 ± 0,0072*	1,83	98,5		
	2	1,9690 ± 0,0564*	2,87	98,5		

x – concentration of the solution (µg/mL)

y – peak area

a – the slope of the calibration curve

b – intercept on the y axis

r – correlation coefficient

*Sd – Standard deviation (n = 9)

RSD-relative standard deviation

chromatographic system. The sample solutions were prepared in triplicate. Determination of the content of aripiprazole was done using the constructed calibration curve method. The identification was carried out through the retention times and the UV-spectrum recorded on the PDA detector. The result achieved for aripiprazole was 5,06 ± 0.07 mg tbl^{-1} (mean ± SD; n = 3). The degradation products were not detected. The low RSD value for aripiprazole (1.29 %) indicates an adequate and respectable repeatability of the method. The results of the content of aripiprazole and its degradation products in Zolprix tablets are shown in Table VIII, and represent the mean value of three independent determinations, each of which was injected three times.

Table VIII Results of the content of aripiprazole and its degradation products in Zolprix tablets

Tabela VIII Rezultati određivanja sadržaja aripiprazola i njegovih degradacionih proizvoda u Zolprix tabletama

	Result	Specification limit	RSD (%)	Recovery (%)
Aripiprazole	5,06 ± 0,07 mg/tbl.	4,75-5,25 mg/tbl.	1,29	100,32
Impurity A	below the LOD	Max 0,2%	/	
Impurity B	below the LOD	Max 0,2%	/	
Impurity C	below the LOD	Max 0,2%	/	
Impurity D	below the LOD	Max 0,2%	/	
Impurity E	below the LOD	Max 0,2%	/	

*Sd – Standard deviation (n = 3x3)
RSD-relative standard deviation

Conclusion

A new reverse-phase liquid chromatography method for simultaneous qualitative and quantitative analysis of aripiprazole and its impurities A, B, C, D and E was optimized and developed. The study of forced degradation of the active pharmaceutical substance aripiprazole, which was conducted under controlled conditions, showed degradation only under oxidation conditions. As the proposed RP-HPLC method enabled the efficient separation of the active pharmaceutical substance aripiprazole and the resulting degradation product (N-oxide), but also Impurities A, B, C and D, which are also potential degradation products of aripiprazole, the RP-HPLC method was defined as a method for monitoring the stability of a pharmaceutical product (stability-indicating method).

The validation of the stability-indicating RP-HPLC method for simultaneous qualitative and quantitative analysis of aripiprazole and its impurities was carried out. Based on the obtained results, it was confirmed that the developed method is selective, accurate, sensitive and precise.

Using the Box-Behnken experimental design, the robustness of the method was examined. The influence of three factors (the percentage of acetonitrile in the mobile phase, pH of the mobile phase, and column temperature) on the chromatographic system was investigated. The stability of the chromatographic system in the case of the first critical pair, impurities A and B, was proven with the allowed variations in the proportion

of acetonitrile, the pH of the buffer, and the temperature of the column. In the case of the second critical pair, aripiprazole and impurity E, optimal conditions must be kept under control in order to maintain a satisfactory resolution, because the proportion of acetonitrile and temperature have a significant influence on the resolution.

The validated method was applied to determine the content of aripiprazole and its impurities A, B, C, D and E in Zolprix tablets. The results obtained for aripiprazole, 5.06 ± 0.07 mg tbl^{-1}, were in accordance with the specification limits set by the manufacturer. The degradation products were not detected. The chromatograms of the sample and placebo solutions are shown in Figure 2.

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Razvoj i validacija RP-HPLC metode za praćenje stabilnosti u cilju određivanja sadržaja aripiprazola i njegovih degradacionih proizvoda

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Kratak sadržaj

Cilj rada bio je optimizacija hromatografskih uslova i validacija izokratske HPLC metode za praćenje stabilnosti aripiprazola, identifikacija i kvantitativna analiza aripiprazola i njegovih degradacionih proizvoda u tabletama, kao i ispitivanje robusnosti predložene analitičke metode primenom eksperimentalnog dizajna.

Studija forsirane degradacije aktivne farmaceutske supstance aripiprazola sprovedena je u skladu sa ICH smernicama. Ispitivana je stabilnost aktivne supstance u uslovima hidrolize u kiseloj, neutralnoj i baznoj sredini, termalne degradacije, oksidacije i fotolize. Aktivna supstanca je pokazala degradaciju u uslovima oksidacije, pri čemu je potvrđen identitet nastalog proizvoda, N-oksida, Pri ostalim ispitivanim uslovima, aktivna farmaceutska supstanca je pokazala stabilnost. Kako je predložena RP-HPLC metoda omogućila efikasno razdvajanje degradacionih proizvoda i aripiprazola, definisana je kao metoda za praćenje stabilnosti farmaceutskog proizvoda.

Sprovedena je validacija predložene metode za istovremenu identifikaciju i kvantifikaciju aripiprazola i njegovih degradacionih proizvoda. Tom prilikom su ispitani selektivnost, linearnost, preciznost, tačnost, limit detekcije i limit kvantifikacije, a primenom Box-Behnkenovog eksperimentalnog dizajna ispitana je i robusnost metode.

Ključne reči: aripiprazol, tečna hromatografija, Box-Behnken eksperimentalni dizajn, forsirana degradacija, degradacioni proizvodi