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Green RP-HPLC method for impurity profile of amlodipine in tablets

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Abstract

Increased awareness of nature preservation has encouraged the introduction of the green analytical chemistry (GAC) practice concepts concerning several important aspects, including sustainable development, environmental impact, and minimum waste. The aim of this research was to contribute to the implementation of this approach for the pharmaceutical industry while retaining the crucial aspects and strict requirements of quality control of medicines. Therefore, an ethanol-based, green and robust high-performance liquid chromatography (HPLC) method for the determination of related substances of amlodipine (AML) in film-coated tablets was developed and optimized using the Design of Experiments (DoE). The chromatographic separation was performed on an RP-select B column (250 x 4.0 mm, 5 μm), using a mixture of 0.04 M sodium dihydrogen phosphate monohydrate (pH 4.0) and ethanol (60:40 *% v/v*) as a mobile phase. The optimized conditions provided the separation of two specified impurities (impurity D and impurity F). The selectivity of the method was confirmed using forced degradation studies. The Analytical Eco-scale approach and AGREE metrics confirmed that the method conforms to the GAC principles. The validated method was successfully applied for the determination of related substances in three samples from the market, demonstrating the applicability of the method in routine analysis.

Key words: amlodipine, impurity profile, green method, HPLC, greenness evaluation

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Introduction

In the last decades, the world has faced ever-growing issues in the areas of public health, energy consumption, and environmental preservation. To address these concerns, the green analytical chemistry (GAC) practice has been introduced in relation to several important concepts such as sustainable development, environmental impact and minimum waste (1). In the context of pharmaceutical quality control, the intent to replace traditional, non-environmentally friendly processes with more sustainable ones is evident. Since reverse-phase high-performance liquid chromatographic (RP-HPLC) methods are the most widely used for pharmaceutical quality control, environmentally friendly approaches have been highly valued by the pharmaceutical sector, which has prioritized the removal of mobile phases containing dangerous organic solvents (2, 3).

Amlodipine (AML) is a synthetic dihydropyridine calcium channel blocker with antihypertensive and anti-anginal effects, listed as an essential antihypertensive medication by the World Health Organization. Over the last two decades, several generic amlodipine besylate tablets have been launched by different manufacturers, currently resulting in one of the highest volume sales of medicines on the market (4).

Impurities of the active pharmaceutical ingredients (APIs) are a crucial aspect of quality control in the pharmaceutical industry. Impurities could arise from starting materials, reagents, intermediates, or degradation of the API during manufacturing, storage, or transportation (5). By implementing a comprehensive monitoring strategy for the impurity profile of the medicines, the pharmaceutical industry can ensure the quality, safety, and efficacy of their medicines, meet regulatory standards, and protect patient health.

The method for determination of related substances of AML active substance is described in the European Pharmacopoeia monograph for Amlodipine besylate (04/2016:1491) (6), and in the USP monograph for Amlodipine besylate tablets (7). A review of the literature revealed that several analytical techniques have been reported for the determination of the content of AML and its impurities, mainly based on LC techniques. The compendial LC methods for related substances determination of AML (6, 7), as well as the reported HPLC methods in the literature (8–14), are based mainly on mobile phases containing hazardous solvents such as acetonitrile (ACN) or methanol (MeOH), with relatively long elution times of 40 minutes on average.

According to the authors' knowledge, only one green RP-HPLC method for the simultaneous quantitative determination of amlodipine, hydrochlorothiazide, telmisartan, and their related substances in the pharmaceutical dosage form has been reported. However, even though the method was evaluated as green, toxic organic solvents (ACN and MeOH) are still used in the mobile phase and for sample preparation (15).

The aim of this study was to develop a green, sustainable and robust ethanol-based RP-HPLC method for the determination of the impurity profile of AML in film-coated tablets, which could be used in routine analysis for quality control of medicinal products in the pharmaceutical industry, as well as for market surveillance.

Experimental materials and method

Reagents and chemicals

AML besylate CRS and AML for peak identification CRS (containing AML and EP impurities D, F and E) were purchased from EDQM, Council of Europe. AML-related compound A USP RS (EP impurity D) was obtained from the U.S. Pharmacopoeia (USP). AML EP impurity F (amlodipine dimethyl ester) was supplied from Veeprho Laboratories Pvt. Ltd. Ethanol 96% (for analysis) (Emsure, Ph. Eur), Ethanol (HPLC gradient grade), Sodium dihydrogen phosphate monohydrate, orto-Phosphoric acid (99,0 %) and Hydrogen peroxide 30% (Perhydrol™ for analysis Emsure ISO) were purchased from Merck, Germany. Sodium hydroxide (for analysis, pellets), as well as Hydrochloric acid (37%, analytical reagent grade), were purchased from Fisher Scientific, UK. Water (highly purified) was obtained using a TKA-LAB Reinstwasser system (Niederelbert, Germany). Regenerated cellulose membrane syringe filters (RC), pore size 0.45 µm, were purchased from Agilent Technologies (California, USA). Film-coated tablets containing 10 mg AML from three different manufacturers, purchased from local pharmacies, were used to test the applicability of the method.

Chromatographic conditions

The chromatographic analysis was performed on an Agilent HPLC 1100 series (Agilent Technologies, USA) with a photo-diode array detector, using a LiChrospher® 60 RP-select B column (250 x 4.0 mm, 5 µm) (Merck KGaA, Darmstadt, Germany). The Chem Station for LC3D software was used for instrument control, data acquisition and processing. The mobile phase was composed of a mixture of 0.04 M sodium dihydrogen phosphate monohydrate (pH 4.0 adjusted with orto-phosphoric acid) and ethanol (60:40 *%, v/v*) with isocratic elution and a flow rate of 1.0 mL/min. The chromatographic run time was set to 15 minutes for standard solutions and 40 minutes for sample solutions. The column temperature was set to 40ºC and an injection volume of 25 µL was used. The detection wavelength used was 237 nm.

Preparation of standard solution and sample solution

For the preparation of AML besylate stock standard solution, AML besylate CRS (accurate weight, equivalent to 4.0 mg AML) was dissolved in a 25 mL volumetric flask with a mobile phase. The solution was treated in an ultrasonic bath until fully dissolved, then diluted to the mark with the same solvent. Stock solutions of EP imp. D and imp. F were prepared in the same manner, by dissolving 4.0 mg imp. D in a 10 mL, and 2.0 mg imp. F in a 25 mL volumetric flask. Stock solutions were further diluted with the same solvent to obtain a final concentration of 4.0 µg/mL for imp. D (1.0% of the test solution), and 0.8 µg/mL for imp. F and for AML (0.2% of the test solution), corresponding to the specified limits in the USP for amlodipine-related compound A (= EP impurity D; maximum 1.0%) and unspecified degradation products (maximum 0.2%) given in the USP monograph for Amlodipine tablets. The standard solution for

peak identification was prepared by dissolving 2.5 mg of the reference standard in 5 mL of the mobile phase.

For the preparation of the sample solutions, twenty tablets containing 10 mg AML were weighed and powdered in a dry and clean mortar. The powdered tablet mass corresponding to 10 mg AML was transferred to a 25 mL volumetric flask, the mobile phase was added, and the solution was treated on an ultrasonic bath for 15 min. The solution was filled up to the mark with the same solvent.

Before the injection, all solutions were filtered through a 0.45 µm regenerated cellulose (RC) membrane filter. All of the analyses were carried out protected from light.

Preparation of samples for forced degradation studies

Standard solutions of AML besylate CRS were exposed to thermal degradation, photolytic degradation, oxidative degradation, and acid and alkaline hydrolysis. For acid and alkaline hydrolysis, standard solutions of AML (0.8 mg/mL) were prepared in 1 M HCl and 1 M NaOH, accordingly. After 15 minutes, the solutions were neutralized by diluting each solution with 1 M NaOH and 1 M HCl, respectively. The neutralized degraded solutions were diluted with mobile phase (in the ratio 1:2, *v/v*). Thermal degradation was performed by exposing a standard solution of AML (0.4 mg/mL) to a temperature of 80ºC for 24h, while photolytic degradation was performed by the exposure of the standard solution of AML (0.4 mg/mL) to daylight for 24 hours. For oxidative degradation, the standard solution of AML (0.8 mg/mL) was prepared in 1% H_2O_2 and further diluted with a mobile phase (in the ratio 1:2, v/v).

Design of experiments (DoE)

MODDE 10.0 Software (Umetrics, Umea, Sweden) was used for the optimization of chromatographic conditions (17 experiments of 2^3 Central Composite Face Centered DoE) and for robustness testing (11 experiments of 2^2 Central Composite Face Centered DoE), enclosed in Table II and Table VI, respectively.

Results and discussion

Method development

An eco-friendly, ethanol-based RP-HPLC method for the determination of AML-related substances in film-coated tablets was developed. According to the Ph. Eur. monograph for amlodipine besylate (Ph. Eur.), four specified impurities (A, D, E and F) are described, while three impurities (imp. A or Ph. Eur. imp. D, lactose adduct, and glucose/galactose adduct) are listed in the USP monograph for amlodipine besylate tablets (USP). Ph. Eur impurity D (or USP impurity A) is a degradation product, and it is needed for demonstrating the suitability requirements of the method, while the other impurities are process-related. The forced degradation showed that impurity F also arises as a degradation product of AML during oxidative, thermal and photolytic degradation

(Table I). Forced degradation studies also revealed that the most significant increase of the degradation products occurs during thermal degradation, where unknown impurities 3 and 4 increase by a considerable amount (0.66% and 0.98%, respectively). The most significant increase of imp. D was observed during oxidative degradation, which is in line with the findings presented by Ahmed et al. (15).

Table I Results obtained from the forced degradation studies of AML besylate

Tabela I Rezultati dobijeni iz studija prinudne degradacije amlodipin-besilata

Conditions/ compound	Acidic degradation (1M HCl, 15 min)		Alkaline degradation (1M NaOH, 15 min)		Oxidative degradation (1% H_2O_2, 15 min)		Photolytic degradation (24 h exposure on daylight)		Thermal degradation (24h at 80ºC)	
	RRT	%	RRT	%	RRT	%	RRT	%	RRT	%
Impurity D	0.51	0.32	0.51	0.07	0.52	0.34	0.52	0.06	0.51	0.21
Impurity F	/	/	/	/	0.71	< 0.05	0.71	< 0.05	0.70	< 0.05
Unknown 1	/	/	0.39	< 0.05	/	/	/	/	/	/
Unknown 2	0.60	0.05	/	/	/	/	/	/	/	/
Unknown 3	/	/	/	/	/	/	/	/	0.76	0.66
Unknown 4	/	/	/	/	/	/	/	/	3.31	0.98

Considering the need for reduction of the environmental footprint during the method development process, the Design of Experiment (DoE) approach was applied. The initial chromatographic conditions were chosen based on our previous research focused on developing a sustainable RP-HPLC method for the simultaneous determination of amlodipine and atorvastatin in film-coated tablets (16). The analysis performed in the previously reported study (16) indicated that the C8 stationary phase and acidic mobile phase lead to lower retention time and better peak symmetry for AML. Central Composite Face (CCF) quadratic experimental design was used to assess the effects of three chromatographic factors (percentage of EtOH in the mobile phase, pH of the buffer and ionic strength of the buffer) on desired chromatographic responses: resolution (*Rs*) between imp. D and imp. F (*Rs* imp. D/imp. F), *Rs* imp. F/ AML, the capacity factor of the earliest eluting peak (*k'* imp. D) and *k'* of AML. To identify the optimal chromatographic conditions necessary to obtain the desired method performance, seventeen experiments were performed (Table II).

Table II Critical factors and chromatographic responses for optimization of the green RP-HPLC method for related substances quantification of AML in tablets using full factorial CCF DoE

Table II Ključni faktori i hromatografski odgovori za optimizaciju zelene RP-HPLC metode za kvantifikaciju srodnih supstanci amlodipina u tabletama korišćenjem potpunog faktorskog CCF DoE

	Experimental factors			Responses			
Exp.	EtOH content (*%, v/v*)	pH of the buffer	Ion strength of the buffer	*Rs* (AML imp. D/ AML imp. F)	*Rs* (AML imp. F/ AML)	*k'* imp. D	*k'* AML
N1	40	2.8	30	3.42	4.55	2.98	6.83
N2	46	2.8	30	2.14	2.94	1.81	3.40
N3	40	4.2	30	3.60	4.73	3.11	7.17
N4	46	4.2	30	2.18	3.02	1.89	3.60
N5	40	2.8	50	3.43	4.51	2.99	6.90
N6	46	2.8	50	2.16	2.92	1.82	3.48
N7	40	4.2	50	3.43	4.57	3.05	6.99
N8	46	4.2	50	1.49	1.55	3.06	7.03
N9	40	3.5	40	4.44	5.60	3.02	6.95
N10	46	3.5	40	2.10	2.95	1.85	3.51
N11	43	2.8	40	2.99	3.79	2.30	4.85
N12	43	4.2	40	2.92	3.71	2.37	4.94
N13	43	3.5	30	3.59	4.55	2.34	4.88
N14	43	3.5	50	3.57	4.57	2.34	4.89
N15	43	3.5	40	3.55	4.51	2.32	4.83
N16	43	3.5	40	3.60	4.57	2.33	4.85
N17	43	3.5	40	3.58	4.53	2.33	4.86

The calculated factorial coefficients of the full factorial CCF DoE illustrate the connection between the measured response variables and the chromatographic factors under investigation. The obtained coefficient plots of the model (Figure 1) indicated that the percentage of EtOH (% EtOH) in the mobile phase had the most significant negative impact on the evaluated chromatographic responses. The pH value of the buffer had a slightly negative effect on the resolution between the critical pair of peaks; and, as anticipated, this factor had a positive effect on the *k'* values of the analytes. The effect of the ionic strength of the buffer was comparable to the effect observed for the pH value of the buffer as a factor.

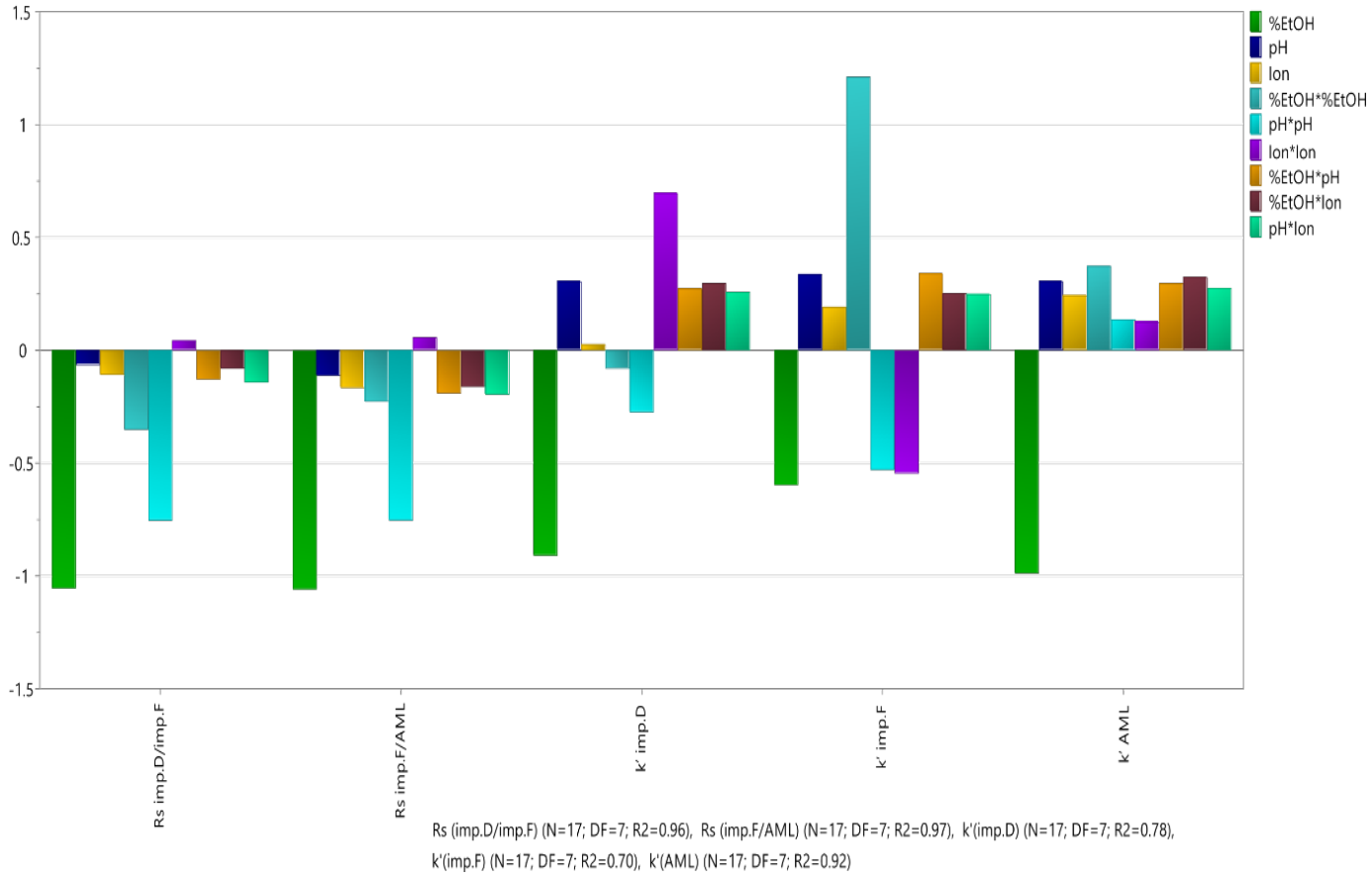


Figure 1. Regression coefficient plot (% EtOH: percentage of EtOH in the mobile phase; pH: pH value of the buffer, ion: ionic strength of the buffer)

Slika 1. Grafikon koeficijenata regresije (% EtOH: procenat EtOH-a u mobilnoj fazi; pH: pH vrednost pufera, jon: jonska jačina pufera)

To determine the optimal range for the studied chromatographic factors, a contour diagram was created for each chromatographic response based on the % EtOH and pH value of the buffer in the mobile phase, while ionic strength was set as constant at 40 mM (Figure 2). The ionic strength was kept constant at its mid-value due to the lowest impact of this factor on the chromatographic responses being investigated (Figure 1). The results obtained using the Response Surface Methodology showed that the resolution between imp. D/imp. F and imp. F /AML was above 2.0 in the whole tested range, while satisfactory retention of imp. D ($k' > 2$) could be obtained if the % EtOH was below 43%. Given the possibility that, except for the evaluated specified impurities of AML, unspecified impurities could emerge during the shelf-life period of the medicinal product, the optimal value for % EtOH was chosen to be 40%. The evaluation of the contour plots (Figure 2a to Figure 2c) showed that, at the defined percentage of EtOH (40%), better resolution and retention time of imp. D could be obtained if the pH value of the buffer

was around 4.0. The contour plot obtained for *k'* of AML (Figure 2d) shows that the retention of AML is mostly dependent on the % EtOH.

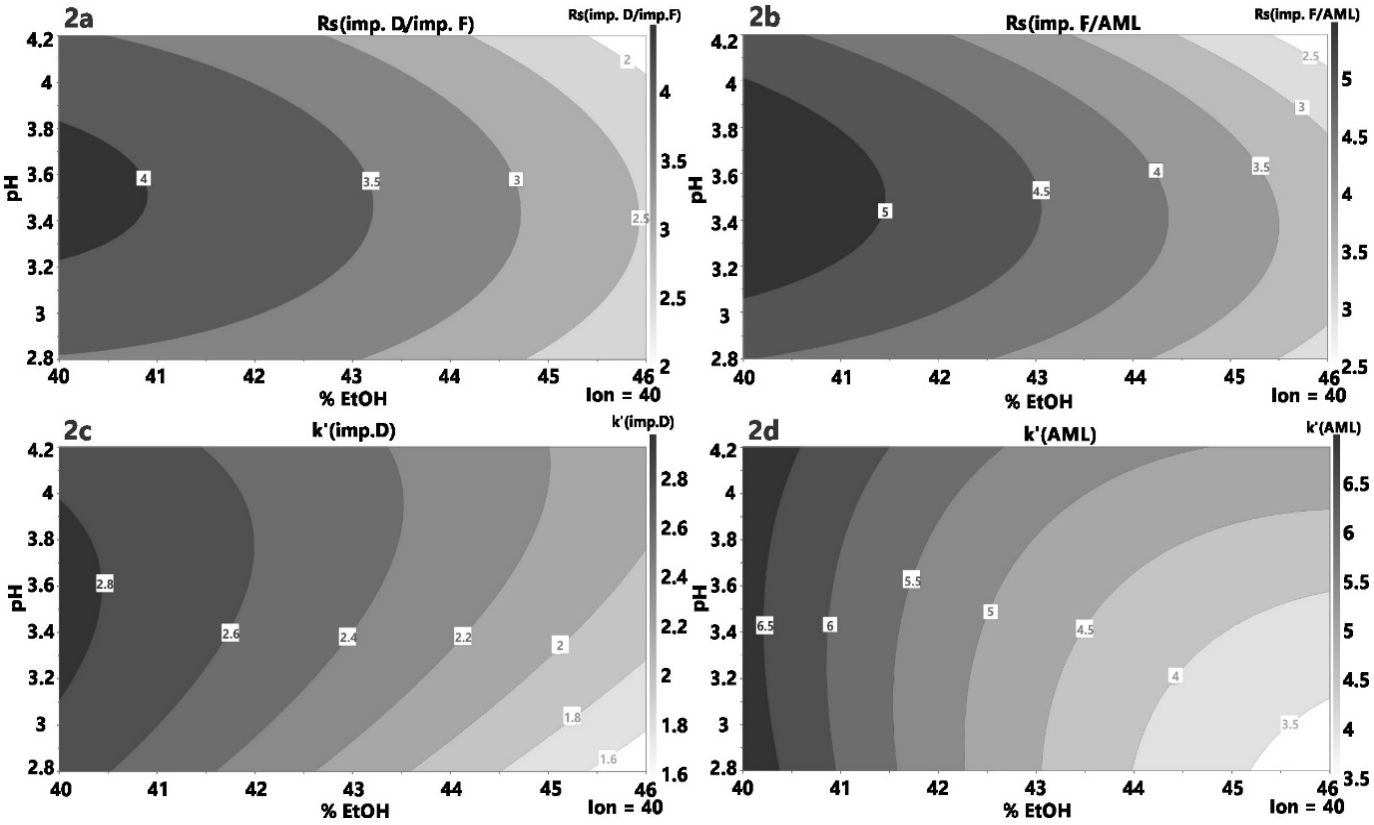


Figure 2. Response contour diagram of the evaluated chromatographic response: a) Resolution imp. D/imp. F; b) Resolution imp. F/AML; c) *k'* of imp. D; d) *k'* of AML

Slika 2. Dijagram kontura odgovora hromatografskih odgovora koji su procenjivani: a) Rezolucija nečistoće D/nečistoće F; b) Rezolucija nečistoće F/AML; c) *k'* nečistoće D; d) *k'* amlodipine

The optimal chromatographic conditions for impurity profiling of AML, providing satisfactory value for *k'* of imp. D and resolution between the evaluated impurities and AML, were achieved using 40 mM buffer at pH 4.0 and EtOH (60:40 *% v/v*) (Figure 3).

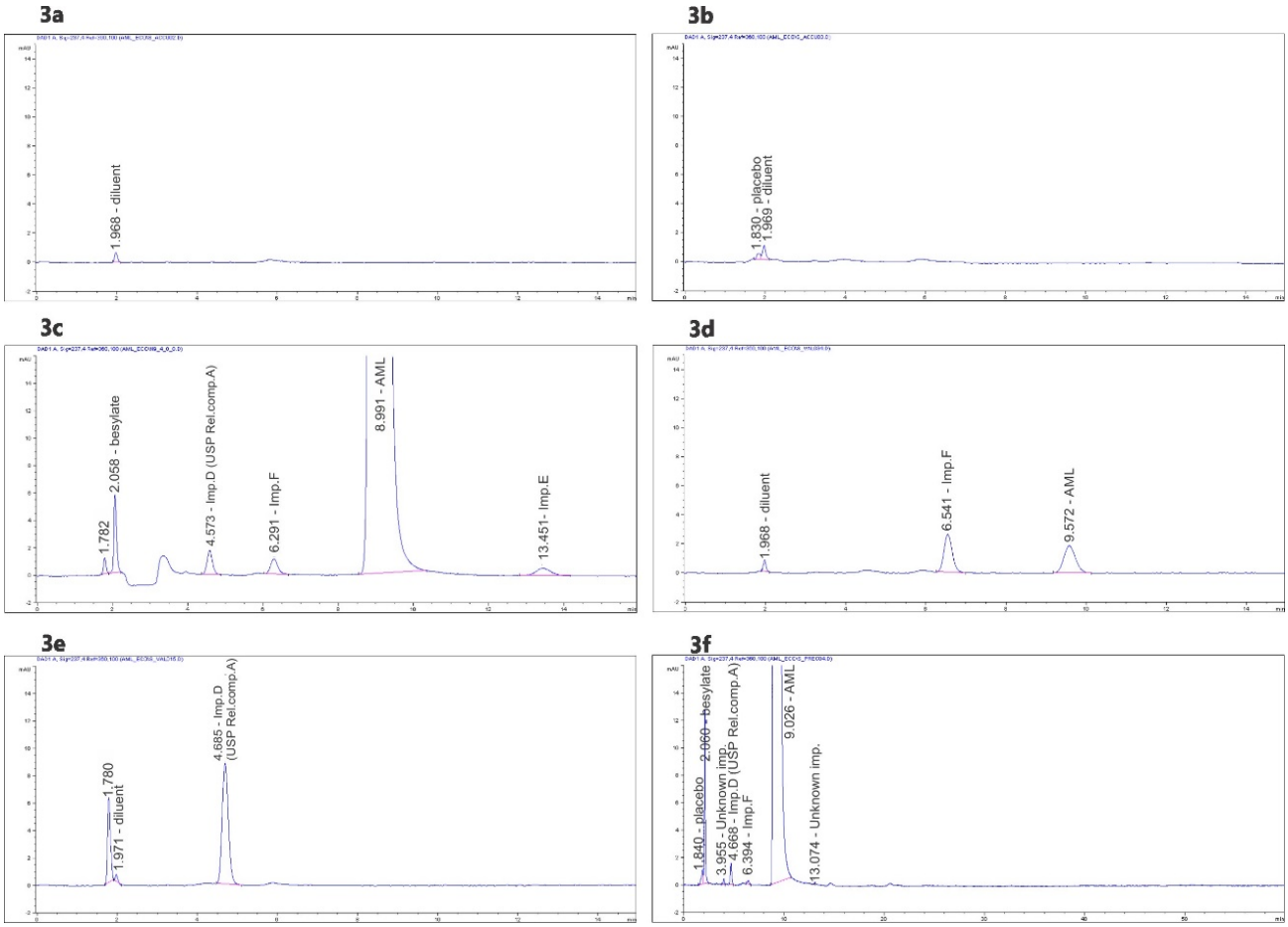


Figure 3. Chromatograms obtained at optimal chromatographic conditions: a) Solvent; b) Placebo solution, c) Standard solution for peak identification, d) Standard solution containing AML and imp. F, e) Standard solution containing imp. D, and f) Sample solution

Slika 3. Hromatogrami dobijeni pri optimalnim hromatografskim uslovima: a) Rastvarač; b) Placebo rastvor, c) Standardni rastvor za identifikaciju analita, d) Standardni rastvor koji sadrži AML i nečistoću F, e) Standardni rastvor koji sadrži nečistoću D, f) Rastvor uzorka

Method validation

Validation of the analytical method included testing of the method specificity, linearity, limit of detection, limit of quantification, accuracy, precision, and robustness, according to the ICH guideline (17).

Specificity/selectivity and system suitability: The system suitability was evaluated by injection of Amlodipine for peak identification CRS solution, as well as six replicate injections of standard solutions of AML, imp. D and imp. F at their working concentrations (0.8 μg/mL, 4 μg/mL and 0.8 μg/mL, accordingly). Table III summarizes the obtained results for system suitability parameters under the optimized

chromatographic conditions. The obtained results indicate the system suitability criteria are met, thus confirming the suitability of the proposed method. The specificity of the method was assessed by injection of diluent (mobile phase), placebo solution, solution of AML for peak identification CRS (containing AML and EP impurities D, F and E), test solution, standard solution of AML, as well as separate standard solutions of its impurities (imp. D and imp. F) (Figure 3). The evaluation of the specificity/selectivity was complemented by the performed forced degradation studies (Figure 4). In the chromatograms (Figure 3 and Figure 4), there is a slight elevation of the baseline around 5.9 min, originating from the diluent. This elevation is not integrated as a peak, because it is below the limit of detection. This elevation does not interfere with any other peak. No other interfering peaks from the diluent or placebo were detected, and all impurities were separated from each other as well as from AML. Thus, the specificity of the method was confirmed.

Table III Results for systems suitability parameters obtained under the optimized chromatographic conditions

Tabela III Rezultati parametara pogodnosti Sistema dobijeni pod optimalnim hromatografskim uslovima

Chromatographic parameters	Acceptance criteria	Impurity D	Impurity F	AML
Relative retention time (RRT)	~ 0.5 for imp. D ~ 0.8 for imp. F	0.52	0.71	1.0
Peak symmetry (*As*)	≤ 2.0	1.17	1.12	1.12
Number of theoretical plates (*N*)	≥ 2000	4652	4800	5282
Resolution imp. D/imp. F	≥ 2.0	5.63		/
Resolution imp. F/AML	≥ 2.0	/	6.14	
RSD of Rt (n=6)	≤ 2.0	0.1%	0.1%	0.2%
RSD of peak areas (n=6)	≤ 5.0	1.3%	0.2%	0.6%

Linearity: The method linearity was estimated from five standard solutions of AML and EP impurity F prepared at concentration levels ranging from the reporting threshold (0.05%) corresponding to 25% of the working concentration, to 150% of the working concentration (0.2, 0.4, 0.6, 0.8 and 1.2 μg/mL). For EP impurity D, method linearity was determined from six standard solutions prepared at concentration levels ranging from the reporting threshold (0.05%) corresponding to 5% of the working concentration, to 150% of the working concentration (0.2, 1, 2, 3, 4 and 6 μg/mL for imp. D). The obtained results from linear regression analysis (Table IV) confirmed the method's linearity in the defined range.

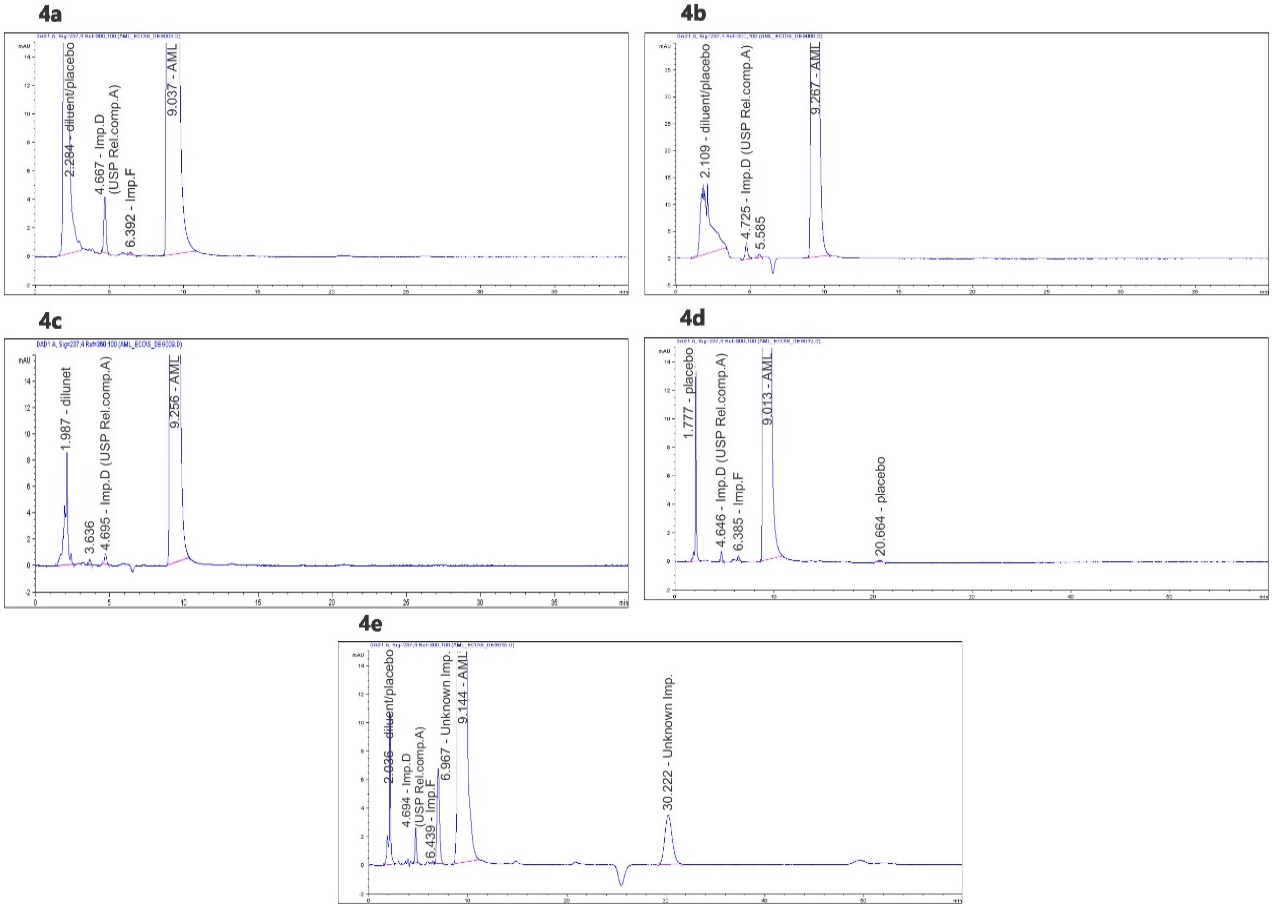


Figure 4. Chromatograms obtained after degradation: a) oxidative; b) acidic, c) basic, d) photolytic, and e) thermal

Slika 4. Hromatogrami dobijeni nakon degradacije: a) oksidativne; b) kisele, c) bazne, d) fotolitičke, e) termalne

Table IV Results from the determination of linearity, DL and QL

Tabela IV Rezultati određivanja linearnosti, DL i QL

Compound	Regression equation (y)				Correlation coefficient	DL / QL (μg mL^{-1})	
	Slope (b)	Intercept (a)	Intercept in % (bias)	Standard error		From calibration data	Experimentally obtained
AML imp. D	22861.8	-0.9558	-1.02	0.495	0.9998	0.09 / 0.28	0.09 / 0.21
AML imp. F	47827.3	-0.5831	-1.55	0.359	0.9997	0.02 / 0.07	0.05 / 0.10
AML	48338.5	-1.2278	-3.24	0.134	0.9999	0.01 / 0.03	0.05 / 0.10

Accuracy: The results obtained for the analytical recovery at concentration levels: reporting threshold, 50%, 100% and 150% of the working concentration of AML, imp. D and imp. F in spiked placebo (Table V) confirmed the accuracy of the method.

Precision: The repeatability of the method was evaluated by analyzing 6 independent test solutions prepared from AML tablets from manufacturer 1 and spiked with imp. D and imp. F at the reporting threshold concentration (Table V). The obtained RSD values for both impurities were below 10.0%; therefore, the method precision was confirmed. The intermediate precision was assessed by analyzing another set of 6 independent test solutions prepared in the same manner by a second analyst. The F-test showed that there was no statistically significant difference between the two sets of results (Table V).

Table V Accuracy and precision data for the proposed green HPLC method

Tabela V Podaci o tačnosti i preciznosti za predloženu „zelenu“ HPLC metodu

Accuracy of the proposed method			
Concentration level (%)	AML imp. D	AML imp. F	AML
% Recovery ± % confidence interval (95% level of confidence)			
Reporting threshold*	100.60 ± 2.06	98.69 ± 1.72	102.23 ± 1.32
50 %	102.27 ± 1.34	101.58 ± 0.57	97.56 ± 1.14
100 %	100.49 ± 0.60	100.65 ± 1.93	99.32 ± 2.03
150 %	101.20 ± 0.24	99.75 ± 1.35	98.76 ± 0.96

Method repeatability and intermediate precision data of the proposed method		
	Analyst 1	Analyst 2
RSD (n=6) AML imp. D	3.01%	4.01%
RSD (n=6) AML imp. F	7.94%	6.30%
F-test (F critical = 6.39)	F = 2.85	F = 1.18

*Reporting threshold for imp. D: 5% concentration level, reporting threshold for imp. F and AML: 25% concentration level.

Sensitivity: The sensitivity of the method was estimated from the obtained values for detection limit and quantification limit (DL & QL) for AML, EP imp. D and EP imp. F. Both DL and QL were calculated using two different approaches: SD of the linear response and the slope of the curve, and the signal-to-noise approach (Table IV). The DL and QL values which were calculated from the calibration data for EP imp. D corresponded to the experimentally obtained values. For AML and EP imp. F, the experimentally confirmed values were higher than those theoretically calculated from the calibration data.

The proposed ethanol-based method in this study has better sensitivity (QL value for AML of 0.10 μg/mL) compared with the previously reported green method (QL for AML 0.17 μg/mL) (14). In addition, the method proposed in this study was fully validated by quantitative determination of imp. D and imp. F.

Robustness: Eleven experiments defined using the CCF Centered design were performed for evaluation of the robustness of the method, by injection of Amlodipine for peak identification CRS (containing AML and EP impurities D, F and E). Even though impurity E is a process-related impurity and is not found in the finished product, it was included in robustness testing to potentially enhance the method's applicability for the impurity profile of the active substance. Two critical experimental factors were evaluated: ethanol content (EtOH, *% v/v*) in the mobile phase (40 ± 2% *v/v*) and pH value of the buffer (4.0 ± 0.2); while the considered responses were resolution between the critical pair of peaks (*Rs* imp. D/imp. F; *Rs* imp. F /AML; *Rs* AML/imp. E), as well as the *k'* of imp. D (Table VI). From Figure 4 it can be seen that satisfactory values for the evaluated response factors were obtained in the whole tested range of chromatographic factors. The method was proven to be robust over the entire tested experimental variation of the pH value of the buffer and the percentage of EtOH.

Table VI Critical factors and chromatographic responses for robustness testing of the green RP-HPLC method for related substances quantification of AML in tablets using full factorial CCF DoE

Tabela VI Ključni faktori i hromatografski odgovori za testiranje robusnosti „zelene" RP-HPLC metode za kvantifikaciju srodnih supstanci amlodipina u tabletama korišćenjem punog faktorskog dizajna – CCF DoE

Experimental factors			Responses			
Exp.	EtOH content (*%, v/v*)	pH value of the buffer	*Rs* (imp. D/ imp. F)	*Rs* (imp. F/ AML)	*Rs* (AML / imp. E)	*k'* imp. D
N1	38	3.8	6.30	6.79	7.97	3.79
N2	42	3.8	4.63	5.34	6.3	2.61
N3	38	4.2	6.28	6.97	8.2	3.80
N4	42	4.2	4.82	5.41	6.27	2.63
N5	38	4.0	6.31	6.89	8.54	3.89
N6	42	4.0	4.87	5.41	6.2	2.63
N7	40	3.8	5.61	6.74	7.58	3.12
N8	40	4.2	5.57	6.14	6.99	3.13
N9	40	4.0	5.66	6.18	7.24	3.16
N10	40	4.0	4.574	6.295	9.04	3.16
N11	40	4.0	4.564	6.263	8.958	3.15

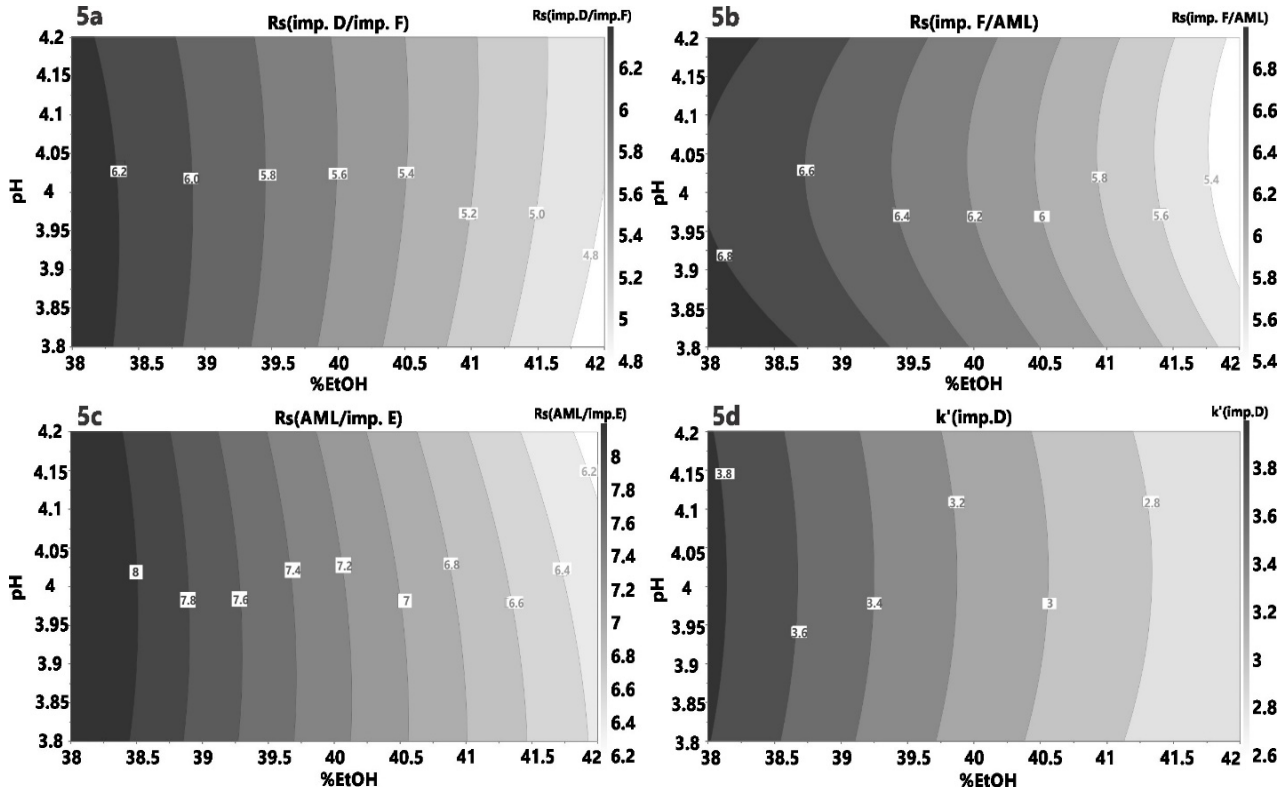


Figure 5. **Response contour diagram obtained during robustness testing for: a) Resolution between imp. D/imp. F, b) Resolution between imp. F/AML, c) Resolution between AML/imp. E, d) *k'* imp. D**

Slika 5. **Dijagram kontura odgovora dobijenih tokom testiranja robusnosti za: a) Rezoluciju između nečistoće D/nečistoće F, b) Rezoluciju između nečistoće F/AML, c) Rezoluciju između AML/ nečistoće E, d) *k'* nečistoće D**

Applicability of the method

The validated method was applied for the determination of AML-related substances in film-coated tablets (10 mg AML) obtained from three different manufacturers. Each detected related substance was calculated using the external standard method. For calculation of EP imp. D and EP imp. F, the external standard of the impurities at their working concentrations was used (4 μg/mL and 0.8 μg/mL, accordingly). Unknown impurities were calculated using AML besylate standard solution at its working concentration (0.8 μg/mL). The obtained results (Table VII) demonstrate the suitability of the proposed method for its intended purpose.

Table VII Results for related substances quantification in AML 10 mg tablets from three different manufacturers available on the Macedonian market

Tabela VII Rezultati kvantifikacije srodnih supstanci u tabletama amlodipina od 10 mg od tri različita proizvođača koji su dostupni na tržištu Makedonije

Compound	Manufacturer 1			Manufacturer 2			Manufacturer 3		
	Rt/min	RRT	% imp.	Rt	RRT	% imp.	Rt	RRT	% imp.
Amlodipine	9.032	/	/	9.114	/	/	9.027	/	/
Impurity F	6.402	0.71	< LOQ	6.438	0.71	< LOQ	6.398	0.71	< LOQ
Impurity D	4.671	0.52	< LOQ	4.684	0.51	0.06	4.669	0.52	0.13
Unknown impurity 1	6.881	0.76	< LOQ	2.926	0.32	< LOQ	3.826	0.42	< LOQ
Unknown impurity 2	7.970	0.87	< LOQ	/	/	/	13.074	1.45	< LOQ
Total	NA			0.06 %			0.13 %		

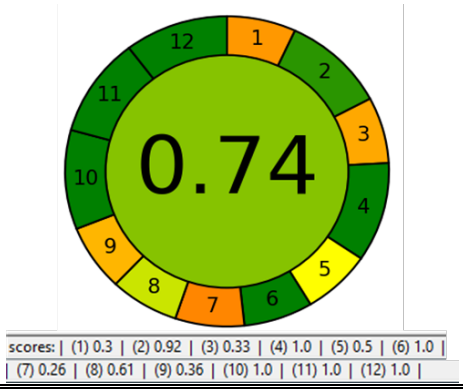
Greenness assessment of the proposed RP-HPLC method for impurity profile of amlodipine

Two quantitative tools for greenness assessment, the AGREE software (18) and the analytical Eco-scale index (19) were used to evaluate the environmental suitability of the proposed RP-HPLC method for the impurity profile of AML. The obtained overall value for the AGREE score of 0.74 confirmed the "greenness" of the proposed method (Table VIII). The evaluation of individual criteria showed that the proposed method is in line with seven out of twelve GAC principles (assigned with green colour). The 1st, 3rd, 5th, 7th and 9th principles are assigned with orange/yellow colour, meaning that these principles do not completely follow the GAC principles. Generally, in cases where HPLC is used as a technique, the stated principles have a lower "green" score. For instance, RP-HPLC methods are usually: procedures with external sample preparation (1st principle), offline methods with no degree of automation and energy consumption of 1 kW per hour (3rd, 5th and 9th principles). The lowest individual score was obtained for the 7th principle – the amount of waste, which is understandable in cases where HPLC is used as a technique. However, the generated waste is non-toxic (only ethanol and aqueous solution were used). Thus, it does not pose a risk to the analysts and the environment.

The greenness of the proposed method was also confirmed with the analytical Eco-scale index. The total Eco-scale score of 90 indicates that the method has excellent greenness (Table VIII). The obtained high value of the Eco-scale index is a result of the use of ethanol and aqueous buffer solvent for sample preparation and instrumental analysis, as well as the reduction of the solvent volume used for the sample preparation process. The EtOH-based method developed in this study has better AGREE and Eco-scale scores compared with the previously published green ACN/MeOH method (15).

Table VIII Greenness assessment using the AGREE software and analytical eco-scale index

Tabela VIII Procena ekološke prihvatljivosti korišćenjem AGREE softvera i metode *analytical eco-scale index*

AGREE score	
Analytical eco-scale	Penalty points
Sample preparation	
Ethanol	6
NaH_2PO_4 aqueous solution	0
Analysis	
HPLC	1
Occupational hazard	0
Chemical waste	3
Total points	13
ESA total score	100-10 = 90

Conclusion

A green, sustainable and robust ethanol-based RP-HPLC method for the determination of the related substances of AML in film-coated tablets was developed. The optimized conditions enabled the separation of two specified impurities (imp. D and imp. F) and showed satisfactory selectivity in the samples from forced degradation studies. The validation parameters confirmed that the method is selective, precise, accurate, and sensitive, with a quantification limit of 0.10 and 0.21 μg/mL for specified and unspecified impurities, respectively. The robustness of the optimized method was evaluated using the DoE approach, which follows the "green" analytical chemistry principles. In addition, the obtained results of 0.74 and 90 for AGREE metrics and Eco-scale, respectively, demonstrated that the method conforms to the GAC principles.

The proposed ethanol-based RP-HPLC method was applied for the determination of related substances in AML 10 mg tablets obtained from three different manufacturers available on the market in the Republic of North Macedonia. The ecological and

analytical advantages of the method provide a basis for its applicability in routine analysis for quality control of medicinal products in the pharmaceutical industry, as well as in market surveillance as a generic method.

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N/A.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Olga Gigopulu: Investigation; Writing - original draft. **Hrisanta Godzo:** Investigation; Writing - original draft. **Biljana Atanasovska:** Formal analysis; Investigation; Validation. **Marija Zafirova Gjorgievska:** Data curation. **Ana Poceva Panovska:** Writing – review & editing. **Jasmina Tonich-Ribarska:** Writing – review & editing. **Jelena Acevska:** Visualization; Methodology; Software. **Katerina Brezovska:** Visualization; Writing – review & editing. **Natalija Nakov:** Conceptualization; Project administration; Supervision.

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„Zelena“ RP-HPLC metoda za određivanje profila nečistoća amlodipina u tabletama

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Kratak sadržaj

Porast nivoa svesti o važnosti očuvanja prirode podstakao je uvođenje koncepta zelene analitičke hemije (GAC), koji se odnosi na nekoliko važnih aspekata, uključujući održivi razvoj, uticaj na životnu sredinu i minimizaciju otpada. Cilj ovog istraživanja bio je da doprinese implementaciji ovog pristupa u farmaceutskoj industriji, zadržavajući pritom ključne aspekte i stroge zahteve kontrole kvaliteta lekova. Stoga je razvijena i optimizovana ekološki prihvatljiva i robusna metoda tečne hromatografije visokih performansi (HPLC) za određivanje srodnih supstanci amlodipina (AML) u filmom obloženim tabletama, uz upotrebu dizajna eksperimenata (DoE). Hromatografska separacija izvršena je na RP-select B koloni (250 x 4,0 mm, 5 μm), pri čemu je korišćena mešavina 0,04 M natrijum dihidrogen fosfat monohidrata (pH 4,0) i etanola (60:40 % v/v) kao mobilna faza. Optimizovani uslovi omogućili su separaciju dve određene nečistoće (nečistoća D i nečistoća F). Selektivnost metode potvrđena je upotrebom studija prinudne degradacije. Pristup analitičke ekološke skale i metrika AGREE potvrdili su da metoda odgovara principima GAC. Validirana metoda uspešno je primenjena za određivanje srodnih supstanci u tri uzorka sa tržišta, što pokazuje primenljivost ove metode u rutinskoj analizi.

Ključne reči: amlodipin, profil nečistoća, „zelena“ metoda, HPLC, procena ekološke prihvatljivosti