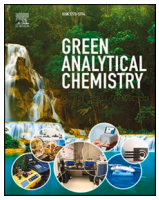
Green Analytical Chemistry 8 (2024) 100103

Contents lists available at ScienceDirect

Green Analytical Chemistry

journal homepage: www.elsevier.com/locate/greeac



Sustainable and white HPLC method for simultaneous determination of amlodipine and atorvastatin in film-coated tablet

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ARTICLE INFO

Keywords:
Amlodipine
Atorvastatin
Sustainable method
HPLC
Whiteness assessment
Greenness evaluation

ABSTRACT

This study presents a collaboration between academia and the pharmaceutical industry with one goal, to implement the transformation of the conventional into sustainable HPLC methods and to use these methods in the routine quality control of medicines. Therefore, a sustainable, fast, and robust high-performance liquid chromatography (HPLC) method for the determination of amlodipine (AML) and atorvastatin (ATV) in film-coated tablets was developed. The chromatographic separation was performed on stable bond C8 column (150 × 4.6 mm, 5 μm), using a mixture of ethanol and 0.02 M sodium dihydrogen phosphate monohydrate, pH 3.0 (63:37%, *v/v*) as a mobile phase. The optimized conditions enabled the determination of AML and ATV within 5 min, providing satisfactory results for system suitability parameters. The method was validated in accordance with the ICH guideline in terms of specificity, linearity, accuracy, and precision (repeatability and intermediate precision). The robustness of the method was confirmed using the Plackett-Burman experimental design. The greenness features of the method were assessed using the Eco-scale index, the AMGS calculator, and the AGREE tool. The high value of the whiteness score (93.5) confirmed that the method meets the requirements for the three main pillars (analytical, ecological, and economical) for the sustainable method development. The method was applied for the determination of both analytes in pharmaceutical dosage form (film-coated tablets that contains 10 mg AML and 10 mg ATV).

Introduction

Cardiovascular disease, as one of the leading causes of death in developing countries, is mostly caused by a combination of arterial hypertension and dyslipidemia as major risk factors [1]. In 2004, a fixed-dose tablet containing amlodipine (AML) besylate as a calcium channel blocker and atorvastatin (ATV) calcium as an HMG-CoA reductase inhibitor, was introduced for the treatment of hypertension, angina pectoris and for prevention of cardiovascular disease [2]. In the last ten years, several generic versions with different dosage strengths of AML and ATV, were approved. Considering the huge market demand, a large number of production batches of tablets containing this fixed combination of active pharmaceutical ingredients (API) are controlled on a daily basis, whether by the quality control (QC) laboratories of the pharmaceutic industry or by the network of the Official Control Medicine Laboratories (OMCL), networked under auspice of the European Directorate for Quality of Medicines and Health Care (EDQM), Council of Europe.

The global challenges related to environmental protection, energy consumption, and public health are constantly increasing. Therefore, the use of sustainable methods, as an integral part of quality control of medicines, would have a positive environmental and economic impact. The QC laboratories worldwide aim towards replacement of the conventional (not eco-friendly) methods with green ones. Considering that the liquid chromatographic (LC) methods are the most commonly used methods for quality control of medicines, there is a growing trend in the pharmaceutical industry for the introduction of eco-friendly LC methods as a part of module 3 of the Common Technical Documentation (CTD) for marketing authorization.

Although the use of green LC methods for QC of medicines is inevitable, the majority of the methods for simultaneous determination of AML and ATV in film-coated tablets don't comply with green analytical

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https://doi.org/10.1016/j.greeac.2024.100103
Received 18 December 2023; Received in revised form 9 February 2024; Accepted 13 February 2024
Available online 17 February 2024

chemistry (GAC) principles [3]. Namely, the compendial high-performance liquid chromatography (HPLC) method described in USP monograph for the assay of AML and ATV in tablets [4], as well as the LC methods described in the literature, use toxic solvents (such as acetonitrile or methanol) for the sample preparation process, as well as eluents in the mobile phase [5–14]. The literature survey revealed that there are only two eco-friendly LC methods for the simultaneous determination of AML and ATV in tablets [15,16]. Hemdan et al. [15] proposed an ethanol-based HPLC method for the determination of AML and ATV, but this method doesn't fulfill the required system suitability criteria in terms of peak symmetry and the number of theoretical plates. Habib et al. [16] developed a micellar liquid chromatography (MLC) method with fluorescent detection using sodium dodecyl sulfate and 10 % butanol as a mobile phase. Surfactants are nontoxic and biodegradable [17], so their use as eluents in the RP-HPLC mobile phase is considered an effective strategy for greening the HPLC methods [18]. However, the obstacles related to the wider use of MLC methods in QC labs are seen in the special considerations that need to be taken for the prevention of column clogging and system blockage, as well as the long time needed for column equilibration which makes MLC methods more time-consuming [19]. Recently, the micellar electrokinetic chromatography (MEKC) method based on the GAC principles was published for determination of valsartan, ezetimibe, simvastatin, AML and ATV [20]. Besides the advantages of this type of electrophoretic technique [21], the MEKC instrumentation is still not widely assessable in the QC laboratories for routine quality control of medicines.

The aim of this research was to develop a sustainable, fast, and robust HPLC method for simultaneous determination of AML and ATV in film-coated tablets, which at the same time will fulfill all the required chromatographic criteria. Robustness testing, as a critical parameter for method transfer among different QC laboratories, was evaluated using the Design of experiments (DoE) approach. Analytical Eco-scale index [22], AMGC calculator [23], and the AGREE metric [24] were used for the assessment of the green features of the method. In addition, the capability of these assessment tools for distinguishing the characteristics of methods with similar green features was evaluated. Besides the method's greenness, for the QC laboratories, it is equally important to use methods that also meet the analytical and efficiency requirements. Therefore, the whiteness assessment algorithm [25] was used for the overall assessment of the proposed ethanol-based method.

These research results present the successful implementation of scientific findings for fulfilling the industry effort of introducing sustainable analytical methods for QC practice.

Experimental materials and method

Reagents and chemicals

AML besylate CRS and ATV calcium CRS were purchased from EDQM, Council of Europe. Ethanol (HPLC gradient grade), sodium dihydrogen phosphate monohydrate, and orto-phosphoric acid (99,0%) were purchased from Merck (Darmstadt, Germany). Water was purified using the Werner water purification system, obtained in-house at Alkaloid, AD, Skopje. Regenerated cellulose membrane syringe filters (RC), pore size 0.2 μm, were purchased from Phenomenex (Torrance, CA. USA). Film-coated tablets containing 10 mg AML and 10 mg ATV were used to test the applicability of the method.

Instrumentation

The chromatographic analysis was performed on Agilent Technologies 1260 Liquid Chromatography system (Agilent technologies, USA) equipped with a binary pump, a column compartment, autosampler and a photo-diode array detector. Instrument control, data acquisition and processing were performed by Chromeleon Chromatography Data System (version 7.2 SR5.).

Chromatographic conditions

The separation was achieved on Zorbax SB-C8 (150 × 4.6 mm, 5 μm) chromatographic column (Agilent, USA) using a mixture of ethanol and 0.02 M sodium dihydrogen phosphate monohydrate (adjusted with orto-phosphoric acid to pH 3.0) (63:37%, *v/v*) as a mobile phase. The flow rate was 0.8 mL/min and the injection volume was 10 μL. The column temperature was set to 40 °C. The analytes were monitored at a wavelength of 254 nm. The chromatographic run time was 5 min.

Preparation of standard solution and sample solution

The standard solution (0.1 mg/mL for both analytes) was prepared by dissolving AML besylate CRS (accurate weight, equivalent to 5 mg of AML) and ATV calcium CRS (accurate weight, equivalent to 5 mg of ATV) in a 50 mL amber glass volumetric flask and dissolved with approximately 25 mL of a mobile phase. The solution was treated for 15 min in an ultrasonic bath and afterward filled up to the mark with the same solvent. For preparation of the sample solution (0.1 mg/mL for both analytes), half of one average tablet mass (corresponding to 5 mg AML and 5 mg ATV) was weighted and transferred into a 50 mL amber glass volumetric flask. 5 mL of deionized water was added and the volumetric flask was vigorously mixed until the tablets were dissolved, followed by the addition of 30 mL mobile phase, treatment of the solution in an ultrasonic bath for 20 min and dilution to volume with the same solvent.

Before the injection, all solutions were filtered through a 0.20 μm regenerated cellulose (RC) membrane filter.

Method validation

Validation of the proposed method was performed in accordance with ICH guideline [26] and included the testing of the method specificity, linearity, accuracy, precision, and robustness.

System suitability test

The system suitability was assessed from six replicate injections of a standard solution containing AML and ATV, both in the concentration of 0.1 mg/mL. Following parameters were considered for the system suitability: relative standard deviation (RSD) of the retention times (*Rt*) of AML and *Rt* of ATV (acceptance criteria RSD $\leq$ 2.0 %), RSD of the peak areas of AML and ATV (acceptance criteria RSD $\leq$ 2.0 %), peak symmetry (*As)* for both analytes (acceptance criteria 0.8 $\geq$ *As* $\leq$ 1.8), number of theoretical plates (*N*) per column (acceptance criteria *N* $\geq$ 2000) and resolution (*Rs)* between AML/ATV (acceptance criteria *Rs* $\geq$ 2).

Specificity

The specificity of the method was evaluated by injection of solvent, placebo solution containing film used for coating, standard solution, and sample solution, prepared according to the specified analytical procedure.

Linearity

Linearity of the method was evaluated from five standard solutions prepared in the concentration range from 0.05 to 0.15 mg/mL (50 - 150 % of the working concentration). The regression analysis was performed on the relationship between the responses (peak areas) of AML and ATV and the corresponding concentrations.

Accuracy

The accuracy of the method was evaluated at three concentration levels (50 %, 100 % and 150 % of the working concentration) for both

analytes using spiked placebo. The analytical recovery, as well as the repeatability of three individual determinations at each concentration level, was calculated.

Precision

The system repeatability was evaluated from six replicate injections of standard solution (100 % of the working concentration), while the method repeatability was assessed by preparing six independent sample solutions. The intermediate precision of the method was evaluated by calculation of the mean, SD, and RSD, as well as the overall RSD of the content of AML and ATV in twelve independent sample solutions analyzed on two consecutive days by two different analysts and on two different HPLC systems.

Robustness testing using design of experiments (DoE) approach

The robustness of the method was evaluated by 11 experiments using the Plackett-Burman DoE with MODDE Go software (Umetrics). Critical experimental factors evaluated were: ethanol content (EtOH,*% v/v*) in the mobile phase (62 ± 1 *% v/v*), flow (0.8 ± 0.2 mL/min), and column temperature (40 ± 5 °C). The evaluated critical parameters were: the resolution between AML and ATV (*Rs* AML/ATV), *Rt* of AML and *Rt* of ATV, as well as the *N* and the *As* for both analytes. The acceptance criteria used for the critical parameters were the same as the ones used for the system suitability: *Rt* of the analytes within the range of 1.5 to 6 min, *As* factor from 0.8 to 1.8, $N \geq 2000$ and *Rs* AML/ATV ≥ 2.

Result and discussion

Green method development

Greening the LC methods can be achieved by using green mobile phases and/or reducing the solvent consumption through optimization of the column-related parameters (dimension, particles size, etc.) such as development of an Ultra-high LC method [27]. Our experience in development of green LC methods [28] showed that it is more feasible and timesaving to replace the toxic eluents from the mobile phase of an already established conventional LC method with green solvents (such as ethanol), instead of development of new UPLC method. Therefore, we decided to transform an already established conventional LC method for determination of AML and ATV in film-coated tablets into green one, by replacing the toxic eluents (acetonitrile and methanol) with ethanol, as the most preferred green solvent. In addition, the reduction of the solvent consumption was introduced with the use of smaller solvent quantities for the sample preparation process.

The starting chromatographic conditions were chosen taking into consideration the conditions described in the USP monograph for determination of AML and ATV in tablets [4], as well as the conventional method described by Chaudari and coworkers [5]. The USP method uses acetonitrile (ACN): methanol: acetate buffer at pH 5.0 (38:15:47% *v/v/v*) as a mobile phase, while the method proposed by Chaudari [5] uses ACN and phosphate buffer at pH 3.0 (60:40% *v/v*) as a mobile phase. The separation on the analytes in both methods was achieved using C18 stationary phase (250 × 4.0 mm, 5 μm). Considering the chromatographic conditions stated in the conventional methods, the LiChrospher C18 column (250 × 4.0 mm, 5 μm) and mobile phase containing EtOH and phosphate buffer at pH 3.0 (60:40% *v/v*), were chosen as a starting point for the green method development. The column temperature was set at 25 °C and the detection wavelength was set at 254 nm, as per the conventional methods [4,5]. The flow was set on 0.8 mL/min instead of 1 mL/min due to the higher viscosity of the mobile phase. Under these conditions the *Rt* of AML was 5.6 min, while the *Rt* of ATV was 9.4 min. However, these starting conditions didn't satisfy the system suitability criteria in terms of peak symmetry (*As* above 2) and the number of theoretical plates (*N* below 2000). In order to improve the peak symmetry, as well as to decrease the column pressure, the column temperature was further increased to 40 °C (in 5 °C increment). As expected, the higher column temperature provided acceptable column pressure (around 200 bar), but the peak symmetry was not improved. Although, the increase of ethanol content in the mobile phase led to acceptable column efficiency (N above 2000) for both analytes and excellent peak symmetry for ATV (*As* 1.0), the AML peak symmetry didn't comply with the required system suitability criteria (*As* 1.9). Considering the high symmetry value of the AML peak, it was decided to proceed with the method development on Zorbax SB C8 chromatographic column. The choice of C8 as a stationary phase was primarily based on the theoretical knowledge that the shorter hydrocarbon chain (C8 compared to C18) provide less interaction, which would improve the symmetry of the peak and in the same time will reduce the retention time of the analytes. In addition, this stationary phase is designed to reduce or to eliminate strong adsorption of basic compounds [29], such as AML, thus improving the peak symmetry. Considering that it is an assay method, the dimension of the column was reduced (from 250 mm to 150 mm). The optimized chromatographic conditions on the used C18 chromatographic column were used for the separation of the analytes on this C8 stationary phase. Best chromatographic responses were obtained using EtOH and phosphate buffer at pH 3.0 in ratio 63:37 (*% v/v*) as a mobile phase, column temperature of 40 °C and a flow rate of 0.8 mL/min.

Under the defined chromatographic conditions, an ideal separation of the analytes within 5 min was achieved ($Rs > 5$), with suitable peak symmetry values (*As* 1.0–1.2) and high column efficiency ($N > 4000$) (Fig. 1). In addition, the viscosity of the mobile phase was not an issue, because the operating pressure of the column was approximately 130 bar.

Considering that one of the goals was to develop a method that could find its applicability in the QC labs in pharma industry, it was important to evaluate the common system suitability (SS) parameters (*Rt, As, N, Rs* AML/ATV). In addition, a comparison between the critical SS parameters between the proposed method, the conventional method [5] and previously published ethanol-based method [15] was performed (Table 1).

The comparison of the SS parameters showed that the proposed method, compared to the conventional [5] and previously published EtOH-based method [15], enables a reduction of the analysis time, as well as an improved symmetry of the two peaks. The shorter analysis time had no influence on the resolution between the peaks (Rs 5.6) and provided significantly higher column efficiency.

An interesting observation was that there was a change in the elution order of the AML and ATR peaks compared to the method described by Hemdan and coworkers [15]. The mobile phase described by Hedman et al. [15] consists of EtOH and a buffer solution at pH 7.0. Under these conditions, ATV was first eluted analyte (*Rt* 3.3), while AML was the second (*Rt* 6.8). The mobile phase of the method proposed in our study consists of ethanol and buffer solution at pH 3.0. ATV has a pKa value of 4.46 and a partition coefficient (logP) value of 6.36 [30], while the pKa value for AML is 9.26 and logP value is 3.0 [31]. At pH 7.0, both analytes are in their ionized forms and the elution order depends on the analyte's affinity to stationary phase. The ionized form of ATV molecule elutes before the ionized form of AML. In the case where mobile phase with pH value of 3.0 is used, ATV is in its non-ionized form, which has higher affinity to the non-polar stationary phase and elutes after the ionized form of AML. This research showed that acidic conditions are more suitable for separation of AML and ATV, because the retention time is shorter and the peak symmetry is improved. The MLC method described by Habib et al. [16] wasn't included in the comparison of the SS parameters because the data were not available. The MLC method, compared with the method presented in this study, has longer run time (8 min). Additionally, the MLC methods are not suitable for routine analysis in the QC labs in the pharmaceutical industry because of the drawbacks arising from the use of the surfactants in the mobile phase

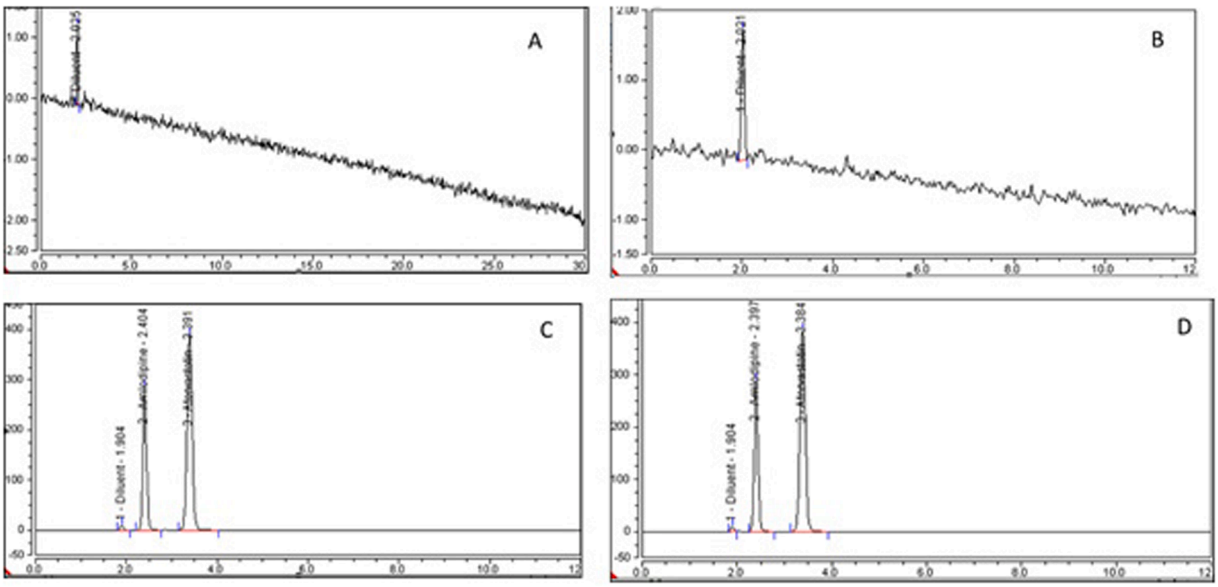


Fig. 1. Chromatograms obtained from specificity investigation: A) Solvent; B) Placebo containing film coating excipients, C) Standard solution and D) Sample solution.

Table 1
Comparison of the system suitability parameters for the proposed eco-friendly method, the conventional method and previously published ethanol-based method.

Chromatographic parameter	Acceptance criteria	Proposed method		Conventional method by Chaudhari et al. [5]		EtOH-based method by Hemdan et al. [15]	
		AML	ATV	AML	ATV	AML	ATV
Rt (min)	2 to 7	2.5	3.5	2,8	5,0	6.8	3.3
As	0.8 −1.2	1.1	1.0	1,2	1,3	1.4	1.4
N	≥ 2000	4094	4116	1677	727	1044	1438
RS	≥ 2	5.6		> 2		3.5	

[19].

Method validation

The validation of the method was performed according to the ICH guideline for validation of analytical methods (ICH Gudeline Q2(R1): "Validation of Analytical procedures: Text and Methodology", 2021) [26]. The following parameters were tested: specificity, linearity, accuracy, precision and robustness.

Selectivity/specificity - The representative chromatograms of solvent, placebo solution that contains the film coating excipients, standard solution and sample solution confirm that there are no peaks originating from the solvent or the placebo that interfere with the elution of AML and ATV (Fig. 1).

Linearity - The linearity of the method was confirmed at five concentration levels from 50 % to 150 % of the working concentration (0.05–0.15 mg/mL). The obtained value for the correlation coefficient for both analytes was found to be 0.9999, confirming the linearity of the proposed method (Table 2).

Accuracy – The recovery values obtained at the investigated levels (50 %, 100 % and 150 % from the working concentration) were in the acceptable limits form 100 ± 2 %, confirming the accuracy of the method (Table 2).

System and method repeatability - The RSD values of the AUC for both analytes obtained from six consecutive injections of the standard solution at 100 % working concentration was found to be less than 2%, thus the repeatability of the system was confirmed (Table 3). The RSD values of the content of AML and ATV in film-coated obtained from six independent preparations of the sample solution was less than 2 %, indicating the repeatability of the method (Table 2).

Intermediate precision – The overall RSD of the content of AML and ATV in film-coated tablets obtained by two different analysts on two consecutive days was found to be less than 2.0 % (0.7 % and 0.6 %, respectively), thus confirming the intermediate precision of the method (Table 2).

Table 2
Summary of results obtained from the validation study of the eco-friendly HPLC method for AML and ATV in film-coated tablet.

Validation parameter	AML	ATV
Linearity		
Concentration range (mg/mL)	0.05 - 0.15	0.05 - 0.15
Correlation coefficient	0.9999	0.9999
Intercept	0.0896	−0.0996
Slope	267.03	268.28
Accuracy (Recovery,% ± confidence interval at 95 % level of confidence/ RSD, n = 3)		
50 %	100.30 % ± 0.7 % / 0.65 %	100.80 % ± 0.7 % / 0.65 %
100 %	100.43 % ± 0.6%/ 0.57 %	100.10 % ± 0.3 % / 0.30 %
150 %	99.87%± 0.4%/ 0.40 %	100.00 % ± 0.3 % / 0.30 %
Repeatability (n = 6)		
System repeatability (RSD of AUC of Standard) solution)	0.13 %	0.15 %
Method repeatability (average value / RSD)	100.9% / 0.7 %	100.7 / 0.4%
Intermediate precision (average value / RSD)		
Analyst 1 (n = 6)	100.9% / 0.7%	100.7% / 0.4%
Analyst 2 (n = 6)	100.4% / 0.6%	100.5% / 0.6%
Overall RSD	0.7%	0.5%

Robustness testing - The evaluated critical experimental parameters (*Rt, As, N* and *Rs* AML/ATV), under all deliberately varied chromatographic conditions using the Plackett-Burman experimental design,

Table 3
Critical factors and chromatographic responses for robustness testing of the eco-friendly method for AML and ATV using the Plackett-Burman design.

Exp. No	Flow (mL/min)	T (°C)	EtOH (% v/v)	Rt (min) AML	Rt (min) ATV	As (AML)	As (ATV)	N (AML)	N (ATV)	Rs AML/ATV
N1	0.6	35	62	3.26	4.80	1.16	1.06	4858	5011	6.72
N2	1.0	35	62	1.99	2.92	1.15	1.03	3550	3580	5.65
N3	0.6	35	64	3.15	4.33	1.19	1.10	4906	5002	5.54
N4	1.0	35	64	1.93	2.64	1.11	1.03	3601	3621	4.70
N5	0.6	45	62	3.21	4.64	1.14	1.05	5020	5239	6.57
N6	1.0	45	62	1.96	2.84	1.12	1.01	3583	3619	6.52
N7	0.6	45	64	3.10	4.21	1.16	1.06	5115	5273	5.48
N8	1.0	45	64	1.86	2.58	1.14	1.10	3599	4196	4.81
N9	0.8	40	63	2.47	3.59	1.17	1.08	3926	3786	5.73
N10	0.8	40	63	2.46	3.57	1.16	1.09	3923	3793	5.71
N11	0.8	40	63	2.46	3.59	1.17	1.11	3943	3787	5.70

were within the acceptance criteria (Table 3). The design space was modeled using a MODDE integrated sweet spot analysis tool. The sweet spot diagram was created by simultaneous change of the tested factors (percentage of EtOH in the mobile phase, flow rate and column temperature) (Fig. 2). The green area represents the part of the design space where all criteria for the evaluated critical responses were met. Considering that the obtained sweet spot diagram was green in the entire range of tested conditions, the robustness of the method was confirmed (Fig. 2). The validation of the method, using the analysis of variance (ANOVA), showed that the percent of variation of the response explained by the model (*R-squared,* R^2), as well as the prediction ability of the model (Q-squared, Q^2), were greater than 0.5 in all cases, indicating that the model reasonably fits the experimental data. The validation of the method demonstrated that the relationship between the factors and the responses was adequately presented by the model, with a substantial portion of the response variation explained by the model and a good ability to predict responses.

Applicability of the method

The validated method was applied for determination of the content of AML and ATV in film-coated tablets (10 mg AML / 10 mg ATV). In addition, the content of both analytes was determined using conventional method [5] as reference. The assay of both analytes in the medicinal product was calculated using the external standard method. The results obtained with both methods (Table 4) were statistically evaluated using the variance ratio F-test and the Student T-test. The obtained values for the F-test (2.28 for AML and 2.28 for ATV), and for the T-test (1.17 for AML and 1.63 for ATV) were below the critical value of 5.05 for the F-test and 2.57 for T-test, indicating that there was no statistically significant difference between the performance of the proposed sustainable method and the conventional method (Table 4). The assay of AML and ATV (expressed as a percentage of the declared content for both active pharmaceutical substances) in film-coated tablet was found to be 100.9% and 100.5 %, respectively (Table 4), thus the applicability of the method was confirmed.

Table 4
Determination of the content of AML and ATV in film-coated tablet (10 mg AML / 10 mg ATV) obtained with the proposed sustainable method and the conventional method [5].

	AML content (%) in film-coated tablet		ATV content (%) in film-coated tablet	
Number of tablets	Proposed sustainable HPLC method	Conventional HPLC method [5]	Proposed sustainable HPLC method	Conventional HPLC method [5]
1	101.4	100.2	100.2	100.9
2	99.9	100.7	100.8	99.7
3	100.4	101.0	100.1	99.5
4	101.7	100.8	100.8	100.5
5	100.7	99.8	100.7	100.1
6	101.2	100.3	100.3	99.8
Mean (n = 6)	100.9	100.5	100.5	100.1
SD	0.67	0.45	0.32	0.53
RSD	0.67	0.44	0.32	0.53
F critical	5.05		5.05	
F value	2.28		2.28	
T critical	2.57		2.57	
T value	1.17		1.63	

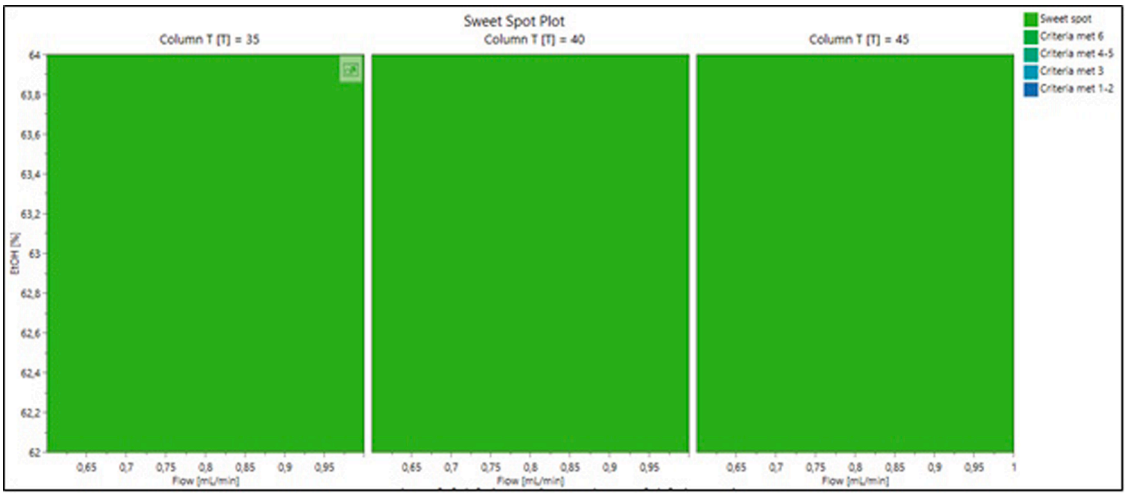


Fig. 2. Sweet spot diagram for robustness testing.

Evaluation of the greenness and whiteness features of the proposed method

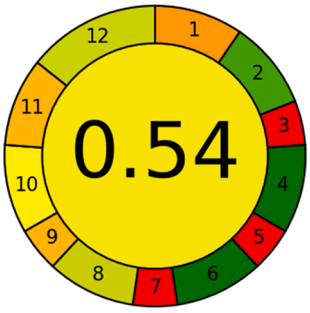
The green features of the method developed in this study were evaluated using three most commonly used quantitative tools: the analytical eco-scale index, the AMGS calculator and the AGREE software. The obtained greenness scores for the proposed sustainable method were compared with the scores obtained for the previously published EtOH-based method [15], as well as with the MLC method [16]. In addition, the capability of the employed greenness assessment tools for distinguishing the difference between green methods was discussed.

The eco-scale score was calculated based on the tool proposed by Galuszka et.al. [22], where the harmful effects of the solvents, energy consumption and amount of waste is presented by penalty points (PP). According to this tool, method with eco-scale score above 75 is assigned as a green method. The major impact on the PP for the evaluated methods arose from the reagents used (Table 5). The PP for each reagent were calculated based on the amount used, the number of pictograms and the severity of the pictogram's signal word. For example, the amount PP for each reagent were assigned with 3, indicating that the evaluated methods require more than 100 mL reagent. Regarding the reagents hazard PP, ethanol has two pictograms: one is more severe hazard (2 PP) and one is less severe hazard (1 PP); thus the subtotal hazard PP is 3. Combining the amount PP and hazard PP, the total reagent PP for ethanol was 9. Butanol has 3 pictograms, one more severe hazard and two less severe hazard, so the total PP were 12 (3 × 4). The SDS aqueous solution has no hazard points, thus the total reagent PP was zero. For energy consumption, HPLC as a technique corresponds to 1 PP. For the calculation of waste PP, the run time and the flow rate of the methods were taken into consideration. The eco-scale score value of the method proposed in this study was 87, which is higher (Table 5) than the previously published green methods for AML and ATV determination [15,16]. The improvement of the Eco-scale score of the proposed method is due to the shorter run-time and the reduction of the quantity of the solvent needed for the sample preparation process. The MLC method [16] has slightly lower eco-scale score of 82 which is a result of the addition of n-butanol in the mobile phase.

The second tool that was used for the evaluation of the greenness features of the methods was the AMGS calculator, available at the American Chemical Society (ACS) webpage [23]. Data relating to the flow rate, analysis run time, mobile phase composition, the type and amount of solvents used for the sample preparation for each method were entered into the AMGS calculator. The obtained AMGS score for the proposed method was found to be 171.44, compared to the previously published EtOH-based method [15] that has an AMGS score of 251.99 (Table 5). The MLC method [16] was not evaluated with this calculation tool, because the software does not cover the surfactants as an option for the calculation of the AMGS greenness score. Considering that a lower AMGS score indicates that the method is more environmentally friendly, the proposed sustainable method has around 1.4 times better AMGS index in relation to the existing EtOH-based method [15]. In addition, the evaluation showed that the proposed method offers a significant improvement in terms of "Instrument energy score" and "Solvent EHS score" compared to the existing one, which is a result of the shorter run time (Table 5). All this implies that the newly developed method is more eco-friendly compared to the green method available in the literature.

The results obtained for the AGREE scores [24] for the evaluated methods were in line with the scores obtained with the Eco-scale index and the AMGS calculator. The method developed in this study has the highest AGREE score of 0.73, while the scores for the previously published EtOH-based method [15] and MLC method [16] were found to be

Table 5
Assessment of the green features of the proposed sustainable method, EtOH-based green method [15] and MLC method [16] for determination of AML and ATV in tablets.

	Sustainable EtOH-based HPLC method proposed in our study	Previously published EtOH-based HPLC method [15]	MLC-based HPLC method [16]
Eco-scale index			
Reagent Hazard related PP	Total reagents PP = Amount PP * Hazard PP [22]		
EtOH	9 (3 * 3)	9 (3 * 3)	/
n-butanol	/	/	12 (3 * 4)
SDS aqueous solution	/	/	0 (3 * 0)
Phosphate buffer solution	0	0	/
Energy consumption PP (≤ 1.5 KW per sample)	1	1	1
Occupational hazard PP	0	0	0
Chemical waste PP	3	5	5
Total PP	12	15	18
Eco-scale score	**87**	**85**	**82**
AMGS calculation tool			
Instrument energy score	55.81 (32.55 %)	100.46 (39.87 %)	/
Solvent energy score	11.00 (6.42 %)	16.44 (6.53 %)	/
Solvent EHS score	104.63 (61.03 %)	135.09 (53.61 %)	/
AMGS Greenness score	**171.44**	**251.99**	**Not applicable**
AGREE pictogram			
AGREE score	 **scores: 1) 0.3, 2) 0.98, 3) 0.0, 4) 1.0 5) 0.0, 6) 1.0 7) 0.05, 8) 0.72, 9) 0.36, 10) 1.0, 11) 1.0, 12) 1.0**	 **scores: 1) 0.3, 2) 0.75, 3) 0.0, 4) 1.0 5) 0.0, 6) 1.0 7) 0.0, 8) 0.55, 9) 0.36, 10) 1.0, 11) 1.0, 12) 1.0**	 **scores: 1) 0.3, 2) 0.82, 3) 0.0, 4) 1.0 5) 0.0, 6) 1.0 7) 0.0, 8) 0.61, 9) 0.36, 10) 0.5, 11) 0.36, 12) 0.6**

0.69 and 0.54, respectively (Table 5). The scores for each of the twelve GAC principles, calculated with the AMGS calculator, for the evaluated methods are given in Table 5. The evaluated methods have same scores for the GAC principle 1, 3, 4, 5, 6,7 and 9. The AGREE evaluation confirm that all evaluated green methods are colored red (not in accordance with GAC principles) for the third principle (Off-line methods), the fifth principle (Degree of automation - manual) and the seventh principle (Analytical waste). In cases where HPLC is used as a technique for quantitative determination, these three principles of GAC are commonly colored red. The proposed method exhibits a slightly higher score of 0.05 for the 7th principle, as a result of waste volume reduction, in comparison to 0.0 for EtOH-based method [15] and MLC method [16]. However, HPLC is a technique that generates large volume of waste, so for this principle the evaluated methods are colored red. Due to its shorter run-time, the proposed method achieves a higher score of 0.72 for the 8th principle (Sample throughput) compared to the EtOH-based method [15] and MLC method [16], which scored 0.55 and 0.61 respectively. For principles 10, 11 and 12, the proposed method and the EtOH-based method [15] achieve maximum scores of 1.0. However, the MLC method [16] obtains a lower score for these principles due to the utilization of methanol and n-butanol.

It could be summarized that the best AGREE score obtained for the proposed method is a result of the double reduction of the solvent employed for the preparation of the standard solution and sample solution, as well as the runtime of only 5 min. Although the surfactants are defined as green eluents for LC mobile phase, still the MLC method for determination of AML and ATV [16] had the lowest AGREE score of 0.54. Several factors of the MLC method have an influence on this score: low amount of methanol used for the sample preparation process; n-butanol included as an eluent in the mobile phase, the flow of 1.5 mL/min and the runtime of 9 min.

The evaluation of the greenness features, using three different assessment tools, gave consistent results. The proposed method was found to have better greenness features compared to the previously published green methods [15,16], which was confirmed with the Eco-scale index, the AMGS calculator and the AGREE tool. The eco-scale index as an assessment tool gives similar scores for different methods when same technique and similar reagents are used. This assessment tool doesn't directly include the run time of the method and the calculation of the penalty points (PP) for chemical waste is more general. The AMGS calculator is more powerful tool compared to the Eco-scale index in distinguishing the differences between methods that are in line with the GAC principles. The reason is probably because the AMGS calculator takes into account the injection volume, the flow rate, the run time, volume of solvent used for standard preparation, etc. However, the current limitation of the AMGS tool over the Eco-scale index is that this calculation software is not applicable in cases where more than three eluents in the mobile phase are used or the eluents are not included in the calculator's predefined list (such as surfactants). In our opinion, the AGREE tool has the best capability for distinguishing the differences between green methods because this tool doesn't have limitations for solvents that could be included; it takes into account the sample amount, the chromatographic runtime expressed by the sample throughput and it offers more comprehensive evaluation of the toxicity of the used solvents. The results for this study align with those reported in the comparative study of four greenness assessment tools conducted by Gamal et al. [32]. According to this study, the AGREE metric was identified as the most suitable option, while the eco-scale tool was ranked third in terms of accuracy and reliability. In another review article [33], it is suggested that the eco-scale index has the disadvantage of providing only general information without any qualitative insights for critical steps in the method. In contrast, the AGREE tool combines the positive aspects of the eco-scale score and the Green Analytical Procedure Index (GAPI).

The main demand of the pharmaceutical industry for use of an LC methods as a method for routine quality control of medicines is not just the greenness itself, but the analytical and the economical attributes of the method. Therefore, it was essential to evaluate the whiteness features of the proposed method using the RBG 12 algoritm [25] and to compare it with the existing methods [15,16]. The input data used for the assessment of the red principle (analytical performance) were: the scope of application (R1), system suitability (R2) evaluated by the value of the *As* and *N*, method's precision (R3) and accuracy (R4). The primary distinction observed between the evaluated methods was in terms of the R2, resulting in a higher red score for the proposed method (Fig. 3a). The green principle was assessed through the toxicity of reagents (G1), amount of reagents and waste (G2), consumption of reagents (G3) and occupation hazard and safety (G4). The higher overall green score of the proposed method was mostly based on the lower reagent consumption and waste generation (Fig. 3a). The blue principle (practical side) was assessed through the cost-efficiency (B1), time-efficiency (B2), requirements (B3) and operational simplicity (B4) (Fig. 3a). Due to the shorter run-time and reduced amount of sample and volume requirements for sample preparation, the proposed method demonstrates better cost-efficiency, resulting in a higher blue score.

The evaluation of the whiteness of the method showed that the proposed method has the highest white score of 93.5, while the whiteness score of the previously published green methods [15,16] for AML and ATV determination were found to be below 90 (Fig. 3b). The whiteness assessment confirmed that the method developed during this study provided several advantages such as: improved analytical attributes, lower negative impact on the environment and better cost-effectiveness, thus it could be considered as sustainable method for determination of AML and ATV in film-coated tablets.

Conclusion

A sustainable, white and robust HPLC method for determination of AML and ATV in film-coated tablet was develop and validated. The proposed method allows simultaneous determination of AML and ATV in 5 min, using reduced amount of green solvent for the sample preparation process, as well for the chromatographic separation. The method presented in this study fulfills all the criteria for the chromatographic system suitability parameters in terms of peak symmetry, number of theoretical plates and resolution. The validation results confirm that the method is specific, linear, accurate, precise and robust. The comparative study, based on statistical evaluation of the results obtained with the proposed sustainable and the conventional method for determination of AML and ATV in film-coated tablet, confirmed the applicability of the method.

The obtained values for the Eco-scale index (score 87), the AMGS calculator (score 171.4) and the AGREE tool (score 0.73) confirm that the proposed method has excellent green features. In addition to the compliance with the GAC principles, the whiteness score of 93.5 showed that the proposed method meets the performance criteria of the method and improves the quality of analytical results. The whiteness assessment confirmed that the method offers a synergy between the analytical, ecological and economical aspects, thus it could be considered as a sustainable method and could be used for quality control of this medicinal product.

The approach used in this research for the selection of the starting conditions during the method development could be used for the transformation of the other existing conventional LC methods for quality control of medicines into green ones.

CRediT authorship contribution statement

Marija Tomikj: Writing – original draft, Investigation, Formal analysis. **Marijana Božinovska:** Validation. **Natasha Anevska-Stojanovska:** Resources. **Jelena Lazova:** Resources. **Jelena Acevska:** Visualization. **Katerina Brezovska:** Writing – review & editing. **Jasmina Tonich-Ribarska:** Writing – review & editing. **Natalija Nakov:**

a)

RED PRINCIPLES (analytical performance)			R1: Scope of application	R2: System suitability parameters			R3: Precision			R4: Accuracy		
	Method number	Method name	0-100	As	N	0-100	RSD% (repeatability)	RSD% (reproducibility)	0-100	Relative error (%)	Recovery (%)	0-100
	1	Sustainable	100	1.1	4100	100	0.7	0.15	100	0.6	100.43	100
	2	EtOH-based	100	1.4	1438	80	0.9	1.17	97	0.88	100.51	100
	3	MCL method	100	1.4	3000	90	1.3	1.3	95	0.74	100.68	100

GREEN PRINCIPLES (green chemistry)			G1: Toxicity of reagents (impact and biodegradation)		G2: Amount of reagents and waste		G3: Concumption of energy and other media	G4: Direct impacts (safety, use of animals and GMOs)			
	Method number	Method name	Total number of pictograms	0-100	Reagent consumption	Waste production	0-100	1-100	Occupational hazards	Safety of users (0-100)	Use of animals (0 if no, 1 if yes)	Use of GMO (0 if no, 1 if yes)
	1	Sustainable proposed method	2	100	110	110	100	90	95	95	0	0
	2	EtOH-based method	2	100	220	220	85	90	95	95	0	0
	3	MCL method	3	80	250	250	80	90	97	97	0	0

BLUE PRINCIPLES (practical side)			B1: Cost-efficiency		B2: Time-efficiency		B3: Requirements			B4: Operational simplicity		
	Method number	Method name	Total cost	0-100	Run time	0-100	Sample consumption (mg)	Sample consumption (0-100)	Other needs: advanced instruments, skills, facilities (0-100)	Miniaturization (0-100)	Integration and automation (0-100)	Portability (0-100)
	1	Sustainable proposed method	low	100	5	100	200	100	100	100	0	0
	2	EtOH-based method	medium	95	9	95	300	95	100	95	0	0
	3	MCL method	medium to high	90	8	90	300	95	90	90	0	0

b)

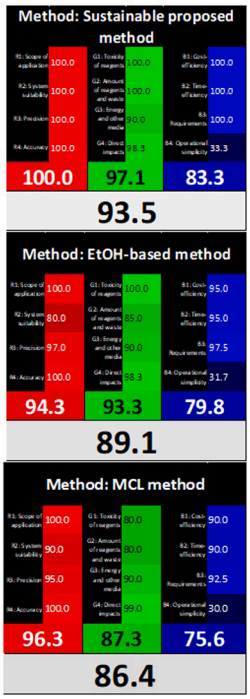


Fig. 3. a) Input data used for assessment of the red, green and blue principle for the evaluated methods, b) Whiteness assessment of the proposed sustainable method, EtOH-based green method [15] and MLC method [16] for determination of AML and ATV in tablets.

Supervision, Conceptualization.

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.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by the Ss. Cyril and Methodius University in Skopje, Republic of North Macedonia (project code NIP.UKIM.22-23.19) and the pharmaceutical company ALKALOID AD Skopje, R. North Macedonia.

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