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ORIGINAL RESEARCH PAPER

Green RP-HPLC methods for assay and related substances in rivaroxaban tablets

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ABSTRACT

In this study, two different ethanol-based RP-HPLC methods for assay and quantification of rivaroxaban related substances in tablets were developed, based on green analytical chemistry (GAC) principles, using the design of experiments approach. The chromatographic separation was performed on X-Bridge C18 column (250 × 4.6 mm, 5 µm particle size), using isocratic elution with ethanol : water (35:65, % *v/v*) for the assay and gradient elution with ethanol/water mobile phase, for related substances, with a flow rate of 1.0 mL min^{-1}. The gradient method was optimized for the separation of three specified impurities (impurity G, impurity H, and impurity 14) and the selectivity was further confirmed using forced degradation studies. Both methods were validated in accordance with ICH guidelines. The robustness of the methods was confirmed with the Central Composite Face Design of Experiments. Analytical Eco-scale approach and AGREE metrics confirmed that both methods are in accordance with the GAC principles. The proposed ethanol-based RP-HPLC methods were applied for assay and determination of related substances in rivaroxaban 10 mg tablets obtained from three different manufacturers available on the Macedonian market.

KEYWORDS

rivaroxaban, green analytical chemistry, ethanol-based HPLC method, greenness evaluation, pharmaceutical analysis

1. INTRODUCTION

Modern pharmaceutical analysis, as a fundamental part of the pharmaceutical industry, is continuously implementing the Green analytical chemistry (GAC) principles aiming to reduce environmental hazards and to improve the health of the analysts by usage of environmentally benign chemicals, optimization of energy consumption, as well as reduction of toxic waste [1–3]. The implementation of GAC principles in pharmaceutical analysis is highly required since the one of the most commonly used separation techniques for drug quality control, high-performance liquid chromatography (HPLC), generates huge amounts of organic toxic waste [3–5]. Although the number of published papers elaborating green chromatography approaches is constantly increasing, the use of eco-friendly HPLC methods in the pharmaceutical industry is still not widely implemented. The reasons for this may include lack of confidence that the green methods will provide the same or better analytical results [3].

Rivaroxaban (RIV) is a unique anti-thrombotic medicine that has been approved by the European Commission and United States FDA (FDA) as an oral anticoagulant for prevention

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of venous thromboembolism in adult patients, after total hip replacement or total knee replacement surgery, as well as a variety of other thrombotic vascular events [6–8]. Additional indication for administration of RIV was invcluded during the SARS-COVID-19 pandemic, making RIV one of the most used oral anticoagulants in the treatment of COVID-19 complications [9, 10].

Pharmaceutical companies throughout the world should provide safe and efficient medicines to patients. As recommended by International Conference on Harmonization (ICH) guidelines, the content of the active pharmaceutical ingredient (API), as well the content of related substances in medicines, as key parameters in finished product specification, should be determined using suitable stability-indicating HPLC methods [11]. The development of reverse-phase (RP) HPLC methods for simultaneous analysis of API and its related substances for quality control purposes is often a challenging task because of their structure's similarity and the big difference in quantity between the API and the impurities [1]. Design of Experiments (DoE) is usually used as a tool for the rational development of methods by performing the optimal number of experiments [12].

The methods for assay and determination of related substances in RIV tablets are described in the European Pharmacopoeia monograph for Rivaroxaban tablets (01/2022:3021) [13] and USP monograph for Rivaroxaban tablets [14]. A thorough review of the analytical methods for the assay and determination of RIV related substances in bulk, finished products, as well as in biological samples such as plasma, is presented in the manuscript written by Reçber and coworkers [15]. The reported methods include HPLC-UV as most frequently applied technique, followed by UV-spectrophotometry, LC-MS/MS, Q-TOF LC-MS, MEKC, HPTLC, and square wave voltammetry. The proposed HPLC methods for assay and related substances determination of RIV in finished medicinal products are based on mobile phases containing hazardous solvents such as acetonitrile (ACN) or methanol (MeOH) [4, 13–18].

According to the author's knowledge, no green ethanol-based RP-HPLC methods for assay and the determination of related substances of RIV in tablets have been reported in the literature, except the one green RP-HPLC method for simultaneous determination of RIV, metformin and linagliptin content in tablets. However, this method uses toxic organic solvent (ACN) for the sample preparation and for the mobile phase [19].

Considering that the modern RP-HPLC stability indicating methods for the determination of impurities in medicines should implement the GAC principles, the aim of this study was to develop two robust ethanol-based RP-HPLC methods for assay and determination of related substances of RIV in tablets, using experimental design approach (DoE). The greenness profiles of the proposed methods were evaluated using Analytical Eco-Scale approach (ESA) and the Analytical GREEnness calculator (AGREE).

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Ethanol 96% (for analysis) (Emsure, Ph. Eur) and ethanol (gradient grade for LC) were provided by Merck, Germany. Water (highly purified) was obtained with a TKA-LAB Reinstwasser system (Niederelbert, Germany). NaOH (analytical reagent grade), as well as HCl (37%, analytical reagent grade), were also purchased from Fisher Scientific, UK. Rivaroxaban, RIV related compound G (((2-Oxo-3-(4-(3-oxomorpholino) phenyl)oxazolidin-5-yl)methyl)isoindoline-1,3-dione) and RIV related compound H (S)-4,5-dichloro-N-((2-oxo-3-(4-(3-oxomorpholino)phenyl)oxazolidin-5-yl)methyl)thiophene-2-carboxamide USP reference standards were used. RIV related compound 14 (S)-2-(2-((4-(5-((5-chlorothiophene-2-carboxamido)methyl)-2-oxooxazolidin-3-yl)phenyl)amino)ethoxy) acetic acid was supplied from TLC Pharmaceuticals Standards Ltd. For investigation of method applicability RIV 10 mg tablets, manufactured by three different manufacturers, were purchased from local pharmacies.

2.2. Chromatographic conditions

The chromatographic separation was performed on an Agilent HPLC 1100 series (Agilent Technologies, USA) equipped with PDA detector and Chem Station for LC3D software using X-Bridge C18 chromatographic column (250 × 4.6 mm, 5 µm) (Waters, Milford, MA, USA). Mobile phase consisted of a mixture of ethanol and water, with isocratic elution for the assay (35:65, % *v/v*) and gradient elution for related substances (expressed by the content of EtOH): 0–3 min 8% *v/v* EtOH; 3–6 min from 8% to 23% *v/v* EtOH; 6–14 min from 23% to 44% *v/v* EtOH; 14–17 min 44% *v/v* EtOH; 17–18 min from 44% to 8% *v/v* EtOH (returning to the initial conditions); 18–27 min 8% *v/v* EtOH (equilibration time). The flow rate was 1.0 mL min^{-1}, the column temperature was 45 °C and volume of injection was 5 µL for both methods. The analytes were monitored at a wavelength of 250 nm. All solutions were filtered before injection through 0.45 µm PTFE syringe filters.

2.3. Standard and test solutions

2.3.1. Standard and test solutions for assay. The standard solution of RIV (0.5 mg mL^{-1}) was prepared in a solvent containing mixture of EtOH and 0.1% acetic acid (50:50, % *v/v*), by dissolving the reference substance in US bath for 30 min. The resolution solution (Res. sol.) containing 0.2 mg mL^{-1} RIV, 0.4 µg mL^{-1} impurity (imp.) G and 0.4 µg mL^{-1} imp. H was prepared in EtOH/water mixture (40:60, % *v/v*). For the test solution preparation, twenty tablets containing 10 mg RIV were weighed and triturated in a clean dry mortar. Tablet mass corresponding to 10 mg RIV was transferred in 50 mL volumetric flask, dissolved in a mixture of EtOH and 0.1% acetic acid (50:50, % *v/v*) and treated on an ultrasonic bath for 15 min. 5.0 mL of this solution were diluted to a volume of 10.0 mL using EtOH/water mixture (40:60, % *v/v*). The placebo solutions

for each medicinal product were prepared in the same manner as the test solution, by using placebo mixtures. Standard and test solutions for related substances quantification.

2.3.2. Standard and test solutions for related substances. Standard stock solution of RIV (0.2 mg mL^{-1}) was prepared in a solvent containing a mixture of EtOH and 0.1% acetic acid (50:50, % *v/v*) in the same manner as the preparation of standard solution of RIV for assay determination. Stock solutions of imp. G, imp. 14 and imp. H (0.2 mg mL^{-1} each) were also prepared in solvent containing mixture of EtOH and 0.1% acetic acid (50:50, % *v/v*) and treated on an ultrasonic bath for 15 min. Impurity stock solutions were further diluted with EtOH/water mixture (40/60, % *v/v*) to obtain final concentration of 0.4 µg mL^{-1}, which is the working concentration of the method for each impurity (0.2% of working concentration, corresponding to the Ph. Eur. limits for unspecified impurities of RIV). The system suitability solution (Res. sol.) was the same as for the assay determination.

For the preparation of the test solutions, twenty tablets containing 10 mg RIV were weighed and triturated in a clean dry mortar. Tablet mass corresponding to 20 mg RIV was transferred in 50 mL volumetric flasks, solvent mixture of EtOH/0.1% acetic acid (50:50, % *v/v*) was added and the solution was treated on a magnetic stirrer for 15 min, and 30 min on an ultrasonic bath. The solution was filled up to volume with the same solvent. A part of this suspension was centrifuged for 15 min at 4,000 rpm. 5.0 mL of the supernatant were transferred in 10 mL volumetric flask and diluted up to volume with EtOH/water mixture (40/60, % *v/v*). The placebo solutions for each medicinal product were prepared in the same manner as the test solution, by using placebo mixtures.

2.4. Forced degradation studies

Standard solutions of RIV (1 mg mL^{-1}) were prepared in 1 M HCl and 1 M NaOH and exposed to stress degradation such as acid hydrolysis (1 M HCl, 1 h at 80 °C) and alkaline hydrolysis (1 M NaOH, 1 h at 80 °C). The degradation samples were first neutralized by diluting each solution with 1 M NaOH and 1 M HCl respectively, to obtain concentration of 0.5 mg mL^{-1} RIV. The neutralized degraded solutions were diluted with EtOH/water mixture (40:60, % *v/v*) to final concentration of 0.1 mg mL^{-1}. Mixed solution was prepared from equal quantities of final acid and alkaline neutralized degradation solutions. Blank solutions were also prepared during analysis.

2.5. Design of experiments (DoE) for method optimization and robustness evaluation

DoE was applied for related substances method optimization and for robustness testing for both methods. MODDE 10.0 Software (Umetrics, Umea, Sweden) was used to build the design of experiments and to model the experimental responses.

2.5.1. DoE plan for related substances method optimization. During method optimization for related substances determination two sets of experiments were planed using DoE. The first set of experiments for screening the conditions was performed using Fractional Factorial DoE at two levels (11 experiments) with four experimental factors (results not shown): the percentage of EtOH in the isocratic part of the gradient (C_0 EtOH from 5 to 10% *v/v*), the length of the isocratic part of the gradient defined as gradient delayed time (t_D from 1 to 5 min), gradient time (t_G from 8 to 12 min) and final percentage of EtOH in the mobile phase (C_1 EtOH from 36 to 40%, *v/v*).

The second set of experiments (2^3 Central Composite Face Design of Experiments, CCF DoE) was performed for the final optimization of the gradient elution, that required 17 experiments (2^k+2k+n experiments, where k was the number of parameters studied and *n* was the number of central points included, $n = 3$). Three repetitions at the central points are required to determine the experimental error variance and test the predictive validity of the model [20]. Chromatographic behavior was evaluated through three factors: C_0 EtOH (8–12% *v/v*), t_G (8–12 min) and C_1 EtOH (40–50% *v/v*), as shown in Table 1.

2.5.2. DoE plan for robustness testing for assay and related substances method. The robustness of both methods (for assay and related substances) was evaluated using 2^3 Central Composite Face DoE, where small changes of values for the selected experimental factors, respectively critical for both methods, were made (Table 2).

Table 1. Critical factors and chromatographic responses for optimization of the green RP-HPLC method for related substances quantification of RIV in tablets using 2^3 Central Composite Face DoE

	Experimental factors			Responses		
Exp.	C_o EtOH content (%, v/v)	t_G (min)	C_1 EtOH content (%, v/v)	*Rs* (RIV/imp. H)	Rt RIV (min)	k' imp.1
N1	8	8	40	1.53	15.276	0.98
N2	12	8	40	1.54	14.199	0.65
N3	8	12	40	1.82	17.291	0.98
N4	12	12	40	1.79	16.618	0.51
N5	8	8	50	1.26	13.141	0.92
N6	12	8	50	1.38	12.768	0.45
N7	8	12	50	1.56	15.38	0.92
N8	12	12	50	1.62	14.754	0.56
N9	8	10	45	1.53	15.032	0.78
N10	12	10	45	1.61	14.528	0.39
N11	10	8	45	1.34	13.594	0.73
N12	10	12	45	1.62	15.918	0.58
N13	10	10	40	1.61	15.738	0.54
N14	10	10	50	1.52	14.058	0.6
N15	10	10	45	1.53	14.792	0.62
N16	10	10	45	1.54	14.792	0.62
N17	10	10	45	1.54	14.793	0.62

Table 2. Results obtained from robustness testing of the green RP-HPLC methods for assay and related substances quantification of RIV in tablets using 2^3 Central Composite Face DoE

Assay method							Related substances method					
Experimental factors				Responses			Experimental factors			Responses		
Exp.	EtOH content (%, v/v)	Flow rate (mL min^{-1})	Column temp. (° C)	Rt RIV (min)	*Rs* (RIV/imp. H)	*Rs* (imp. G/RIV)	t_D	t_G	Flow rate (mL min^{-1})	Rt RIV (min)	*Rs* (RIV/imp. H)	*Rs* (imp. G/RIV)
N1	33	0.8	43	8.33	2.38	14.7	2	7	0.9	14.249	1.95	18.47
N2	37	0.8	43	6.414	2.04	11.7	4	7	0.9	16.139	1.93	19
N3	33	1.2	43	5.506	2.07	12.3	2	9	0.9	14.946	1.84	19.86
N4	37	1.2	43	4.25	1.48	9.6	4	9	0.9	16.84	2	20.87
N5	33	0.8	47	7.87	2.31	13.9	2	7	1.1	13.004	1.72	18.3
N6	37	0.8	47	7.859	2.36	13.8	4	7	1.1	14.882	1.75	19.27
N7	33	1.2	47	5.174	1.86	11.7	2	9	1.1	13.605	1.98	19.67
N8	37	1.2	47	4.081	1.46	9.4	4	9	1.1	15.446	2.02	20.62
N9	33	1	45	6.457	2.21	13.0	2	8	1	13.934	1.77	19.15
N10	37	1	45	5.025	1.82	10.6	4	8	1	15.804	1.75	19.8
N11	35	0.8	45	7.04	2.16	12.0	3	7	1	14.527	1.82	19.12
N12	35	1.2	45	4.658	1.87	10.6	3	9	1	15.176	2.01	20.37
N13	35	1	43	5.802	1.97	11.7	3	8	0.9	15.558	1.86	19.8
N14	35	1	47	5.528	1.69	11.0	3	8	1.1	14.251	1.86	19.02
N15	35	1	45	5.654	1.94	11.3	3	8	1	14.866	1.9	19.4
N16	35	1	45	5.656	2	11.5	3	8	1	14.869	1.91	19.68
N17	35	1	45	5.652	2.01	11.6	3	8	1	14.872	1.75	20.02

Critical experimental factors for the assay method were: C_1 EtOH (35 ± 2%, v/v), flow rate (0.8–1.2 mL min^{-1}) and column temperature (45 ± 2 °C). The evaluated critical factor for related substances methods were: t_D (3 min ±1 min), t_G (8 min ±1 min) and flow rate (0.9–1.1 mL min^{-1}), as presented in Table 2.

2.6. Assessment of method greenness

The greenness of the developed methods in this study was evaluated using two quantitative tools: the Analytical Eco-scale approach (ESA) [21] and the Analytical GREEnness calculator (AGREE) [22].

3. RESULTS AND DISCUSSION

3.1. Green RP-HPLC method for assay of RIV in tablets

3.1.1. Method development. Green analytical chemistry (GAC) principles which initiated a shift of attitudes and behavior in the chemical and pharmaceutical industry, can be seen as a critical tool for achieving sustainability [23]. As a multistep approach, it starts from the selection or modification of the appropriate analytical method that should meet the specified performance criteria, followed by the use of less solvents and/or less toxic solvents in the sample preparation and measurements. According to the physico-chemical properties, especially solubility tests, several solvents were evaluated in order to dissolve RIV [24]. RIV is practically insoluble in anhydrous EtOH, while it is freely soluble in DMSO [13]. However, during the chromatographic analysis of DMSO solutions, several peaks appeared that interfered with the peaks from RIV impurities. The RIV standard was found to be freely soluble in solvent containing mixture of EtOH and 0.1% acetic acid (50:50, % *v/v*). The selection of mixture of EtOH and acetic acid resembles the mobile phase composition and is much eco-friendlier than the solvents for sample preparation described in literature [13–18].

Considering the GAC principles, the starting point of the method development was the replacement of the acetonitrile in the mobile phase that is mostly used in the methods described in the literature with EtOH, as a more eco friendlier organic solvents [16–19, 23]. Similar retention time of RIV was observed with EtOH-based mobile phase (Rt 4.3 min), compared to ACN-based mobile phase (Rt 3.37 min) [23]. Although these chromatographic conditions satisfy the common system suitability requirements (retention time, symmetry factor, number of theoretical plates), still the method didn't have the required specificity regarding the imp. H as its closest eluting impurity. The resolution between the RIV and imp. H (*Rs* RIV/imp. H), using mobile phase of EtOH :H_2O (40:60%, *v/v*) was lower than 1.5. Therefore, the percentage of EtOH was decreased to 35% (*v/v*), which resulted in acceptable system suitability (*Rs* RIV/imp. H > 1.5), as well as improved greenness of the method. The *Rs* between imp. G and RIV (*Rs* imp. G/RIV) for assay determination is not critical, because the imp. G is well separated from the RIV peak (relative Rt imp. G is 0.6). Representative chromatograms for assay determination obtained under final conditions are presented in Fig. 1.

3.1.2. Method validation. Validation of the analytical methods included the determination of method specificity, linearity, accuracy, precision, detection limit, and quantification limit according to ICH guideline [11].

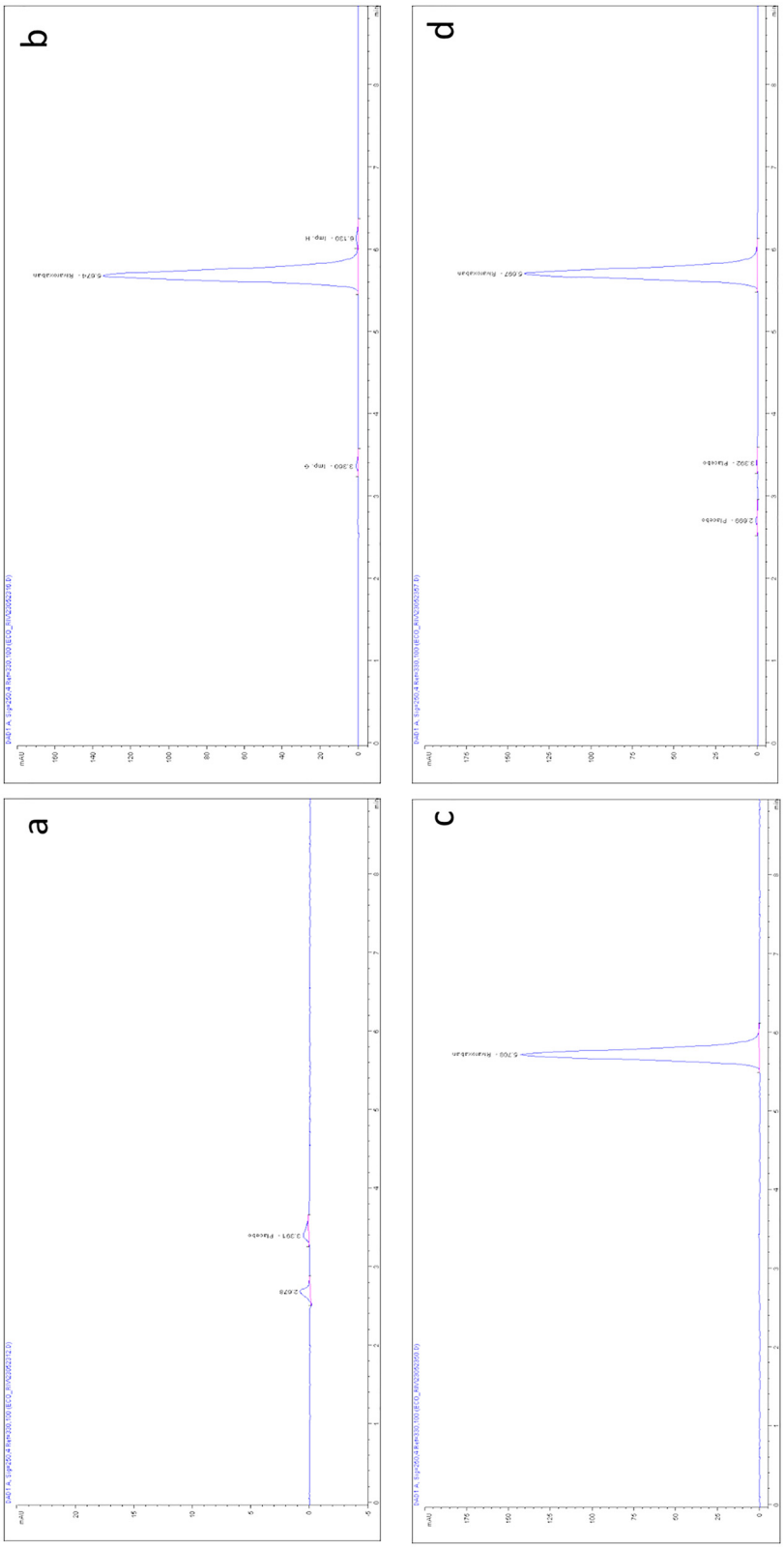

Fig. 1. Chromatogram for assay determination under final conditions: a) Placebo solution b) Res. sol. c) Standard sol. and d) Test sol

Specificity/selectivity and system suitability: The system suitability was assessed by the following parameters: retention time of RIV (Rt RIV), peak symmetry (*As* factor 0.8–1.8), number of theoretical plates per column ($N > 2000$) and *Rs* RIV/imp. H $\geq$ 1.5. The obtained values for Rt RIV (5.67 min), *N* (7,928), *As* (1.2) and the resolution between the critical pair of peaks of 2.1, confirm the suitability of the proposed method. The specificity of the method was evaluated by injection of blank (solvent), placebo, resolution solution, standard solution of RIV, test solution, as well as separate standard solutions of its impurities (imp. G and imp. H). No interfering peaks were observed with the retention time of RIV: none of the examined impurities eluted at the retention time of RIV and there were no interfering peaks from the placebo (Fig. 1).

Linearity, accuracy and precision: Linearity of the method was evaluated from the five standard solutions prepared in the concentration range from 0.05 mg mL^{-1} to 0.15 mg mL^{-1} (50 – 150% of the working concentration). The obtained value of the correlation coefficient R^2 (0.9999) confirm the linearity of the method in the evaluated range. The RSD of the peak areas of the lowest and the highest concentration of the calibration curve obtained from six determinations for the studied range was 0.25% and 0.27%, respectively. The accuracy of the method was evaluated on spiked solutions of RIV at three concentration levels (70%, 100% and 130% of the working concentration). The confirmed accuracy of the method (Table 3) in the wider accuracy range (70%–130% instead of 80%–120%) confirms that the proposed method could be used for content uniformity determination as well. System precision and method precision were confirmed from six replicate injections of RIV standard at 100% working concentration (RSD = 0.34%) and six test solutions (RSD = 0.34%).

Robustness testing: The critical experimental responses (Rt RIV, *Rs* imp. G/RIV and *Rs* RIV/imp. H) from the robustness testing using the 2^3 CCF DoE are presented in Table 2. The response surface plot from the robustness investigation is given in Fig. 2a. In all of the cases of the deliberately varied chromatographic conditions, the monitored system suitability parameters were within the acceptance criteria, confirming the robustness of the method.

3.2. Green RP-HPLC method for related substances determination

3.2.1. Method development. Green, ethanol-based RP-HPLC method for determination of related substances of RIV in tablets was developed. According to official methods for determination of related substances in rivaroxaban tablets described in Ph. Eur [13] and the USP pharmacopeia [14], impurity G and impurity H are crucial for demonstrating the system suitability. Considering the structural characteristics of imp. 14, which provide different polarity of this impurity compared to imp. H, in addition to specified impurities, imp. 14 was also included in this study. This approach provides more comprehensive evaluation of the chromatographic behavior of impurities with diverse polarities during the optimization of the method. Forced degradation studies were performed for the optimization of the method for determination related substances. The mixed solution was used to enable identification of all possible degradation products that could be produced in addition to imp. G, imp. H and imp. 14., thus improving the resolving capacity of the method. Taking into account the inherent stability of the molecule under influence of neutral hydrolysis, photolysis, thermolysis and oxidative forced degradation, these conditions were not considered for chromatographic optimization [16]. Four degradation products were obtained from force degradation under acidic conditions, with domination of imp. 14 and imp. H. Under alkaline conditions, a total of five degradation products were observed, including four unknown impurities (with one dominant early eluting impurity, assign as imp. 1) and the imp. H.

The initial mobile phase composition was selected based on comprehensive literature search and replacing most commonly used acetonitrile and water (or buffer) with EtOH and water respectively, as mobile phase eluents, aiming towards development of a green method. In order to obtain optimal separation between RIV and its related substances with a minimal number of experiments, the gradient elution conditions were evaluated using two sets of screening experiments: Fractional Factorial DoE (first set) and Central Composite Faced DoE (second set). The first DoE set was used to assess the effects of different

Table 3. Accuracy data for the proposed green HPLC methods

Accuracy of the assay method				
Concentration level (%)		Recovery (%)		RSD (n = 3)
70%		98.9 ± 0.008		0.003
100%		98.2 ± 0.008		0.001
130%		98.3 ± 0.009		0.001
Accuracy of the related substances method				
Concentration level (%)	RIV	RIV imp. G	RIV imp. H	RIV imp. 14
% Recovery				
LOQ	93.15 ± 0.91	93.44 ± 1.09	92.31 ± 1.52	94.63 ± 1.25
50%	98.92 ± 0.35	98.72 ± 0.43	98.61 ± 0.45	98.97 ± 0.37
100%	99.15 ± 0.15	99.50 ± 0.35	98.41 ± 0.38	99.01 ± 0.22
150%	98.86 ± 0.24	98.96 ± 0.39	98.74 ± 0.54	99.26 ± 0.28

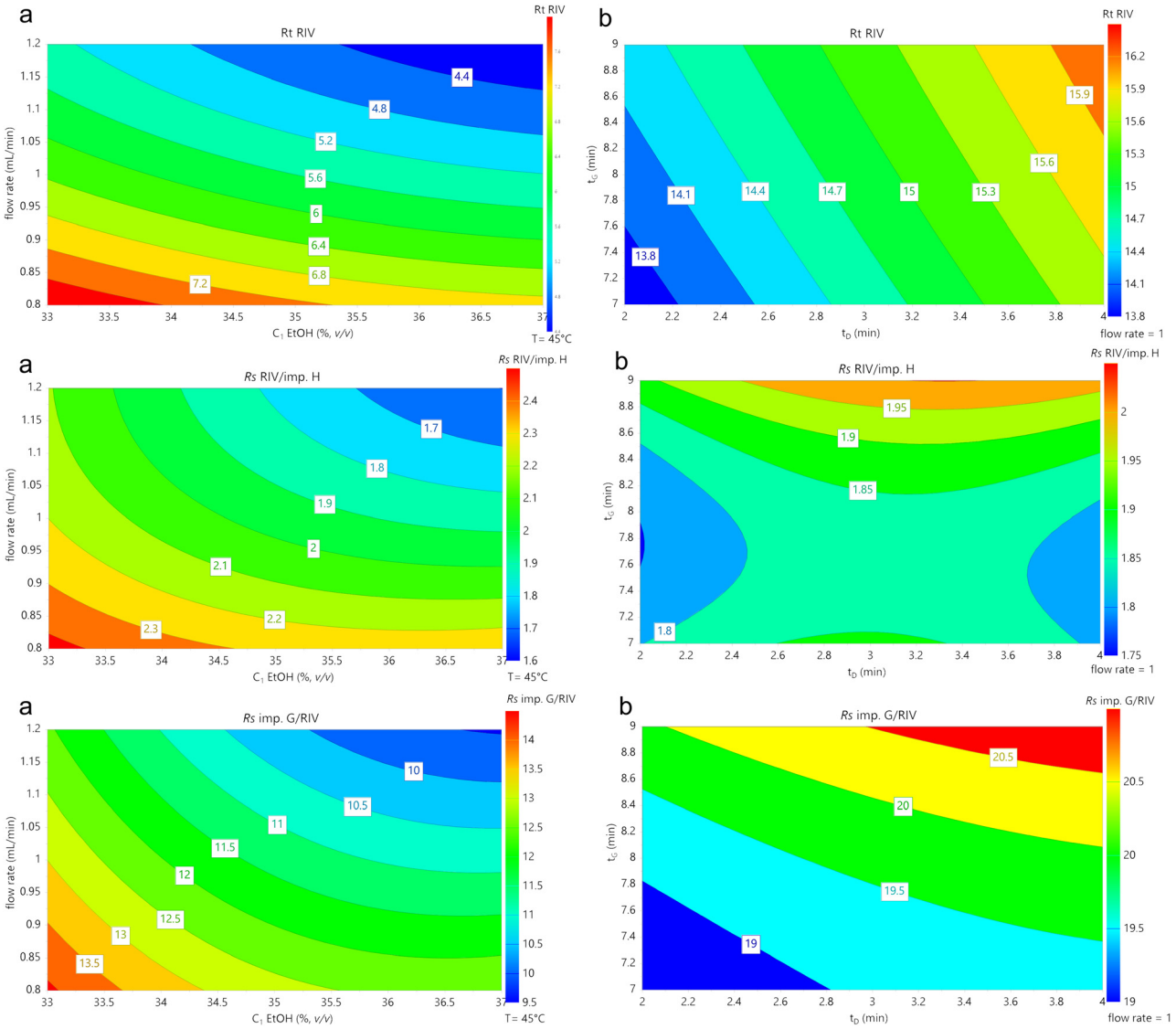

Fig. 2. Response contour plot of robustness testing for: a) assay method, b) related substances method

chromatographic conditions (percentage of EtOH in the isocratic part of the gradient, C_0 EtOH; the length of the isocratic part of the gradient defined as gradient delayed time, t_D; gradient time, t_G; and final percentage of EtOH in the mobile phase, C_1 EtOH) on desired chromatographic responses: resolution between RIV and imp. H (*Rs* RIV/imp.H) and the capacity factor of the early eluting peak (*k'* imp. 1). However, under investigated conditions the *Rs* RIV/imp.H was below 1.5 and the *k'* imp. 1 was below 0.5. In all experiments, the resolution between imp. G and RIV was more than 5, as required for system suitability according to Ph. Eur, thus the response of this parameter was not considered as critical. In addition, there was no interference between the imp. 14 and other degradation peaks obtained with forced degradation. The evaluation of the critical responses obtained from the first DoE set, revealed that the delayed gradient time had no significant influence on the responses, therefore in the second DoE this experimental factor was excluded. The value of delayed gradient time was selected to be 3 min in the gradient program of the method, in order to eliminate the influence of the different dwell volumes in different HPLC instruments, enabling easy gradient method transfer between the different laboratories.

Central Composite Faced DoE was used for further optimization of the factors that have the highest effect on the chromatographic response (C_0 EtOH, t_G and C_1 EtOH). Seventeen experiments were performed to reveal the optimal chromatographic conditions for desired method performance: Rt RIV lower than 15 min, *Rs* RIV/imp. H higher than 1.5 and suitable retention of the early eluting peak (*k'* imp. 1 more than 0.5). The assessment of the critical chromatographic responses (Rt, *Rs* RIV/imp. H and the *k'* imp. 1) (Table 1) showed that satisfactory chromatographic performance related to the Rt and *Rs* could be easily achieved in a wide range of combinations of investigated experimental factors, while satisfying the criteria for *k'* imp. 1 could be obtained in a very narrow area of experimental factors (Fig. 3). Rt RIV lower than 15 min and *Rs* RIV/imp.

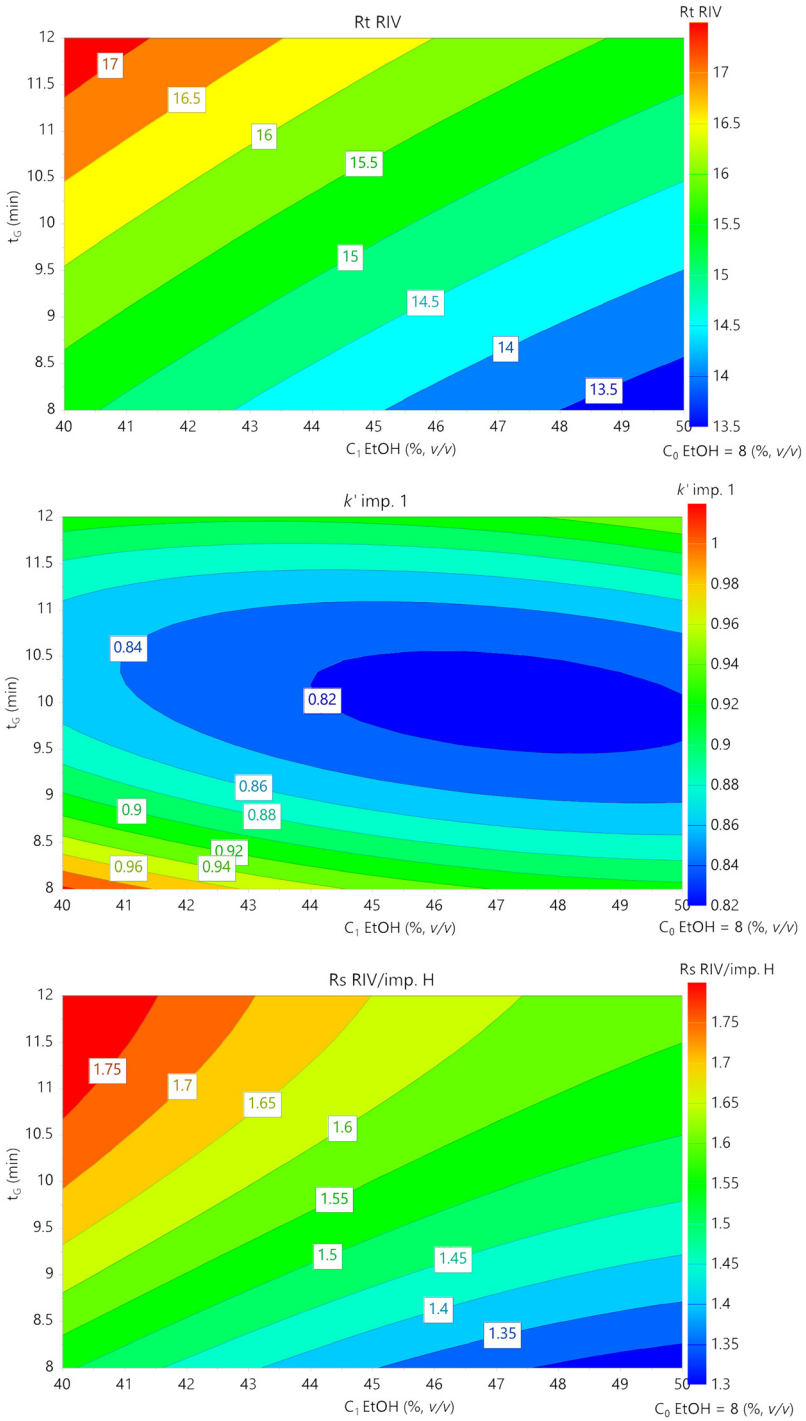

Fig. 3. Response contour plot obtained with the 2^3 Central Composite Faced DoE for the related substances method optimization

H higher than 1.5 could be achieved with lower percentage of EtOH in the mobile phase (C_1 ~ 41%) and shorter gradient time (t_G ~ 8 min) or with higher percentage of EtOH and longer gradient time. Suitable retention of early eluting peak (*k'* imp. 1–1) is observed only if C_1 is between 40 and 41% and the t_G ranges between 8 and 9 min (Fig. 3). The C_0 EtOH did not influence critical chromatographic responses and it was set to 8%. After careful evaluation of the obtained results, the final gradient elution program was established.

3.2.2. Method validation. *Specificity/selectivity and system suitability*: The system suitability was evaluated by injection of the resolution solution. The method was found to be suitable for use because the *Rs* imp. G/RIV was 17.6 (≥5.0), the *Rs* RIV/imp.H was 2.1 (≥1.5) and the Rt for RIV was 14.9 min. The system suitability results imply that the green method developed in this study fulfills the system suitability criteria. Forced degradation studies were performed to assess the specificity/selectivity of the developed method for related substances determination. Representative chromatograms of

diluent, placebo, each impurity solution at the specification limit (0.2%) and neutralized solutions obtained after acidic and alkaline hydrolysis are presented in Fig. 4. The peaks from all impurities are separated from each other and from RIV, without interfering peaks from the diluent or placebo, therefore the method was found specific and selective for the quantification of RIV related substances in tablets. In addition, peak purity analysis was conducted for all obtained peaks from chromatograms of the forced degradation solutions (Fig. 4a and 4b) in the wavelength range from 210 to 400 nm, using ChemStation for LC3D software. The peaks were considered to be spectrally pure if the peak purity factor value was above the set purity threshold of minimum 990. The absorbance threshold was set on 1 mAU for all relevant peaks and 0.5 mAU for the imp. 2 peak in the acidic degradation and imp. 3 peak in the alkaline condition. The obtained peak purity factor values for RIV (999.6), imp.H (999.8), imp. 14 (999.7) and for the unspecified impurities (the lower value was 993.7 for imp.1 in acidic condition) showed that the peaks are spectrally pure.

Linearity, accuracy and precision: Five solutions (prepared from the standard substances) at concentration levels ranging from quantification limit (QL) to 150% of the working concentration (0.05, 0.1, 0.15, 0.2, 0.4 and 0.6 μg mL^{-1} for RIV and for each impurity) were used for evaluation of linearity of the method. The results from the linear regression analysis (Table 4) confirm the linearity of the method in the specified range. The obtained results for the analytical recovery in the entire linearity range (concentration levels: LOQ, 50%, 100% and 150% of the working

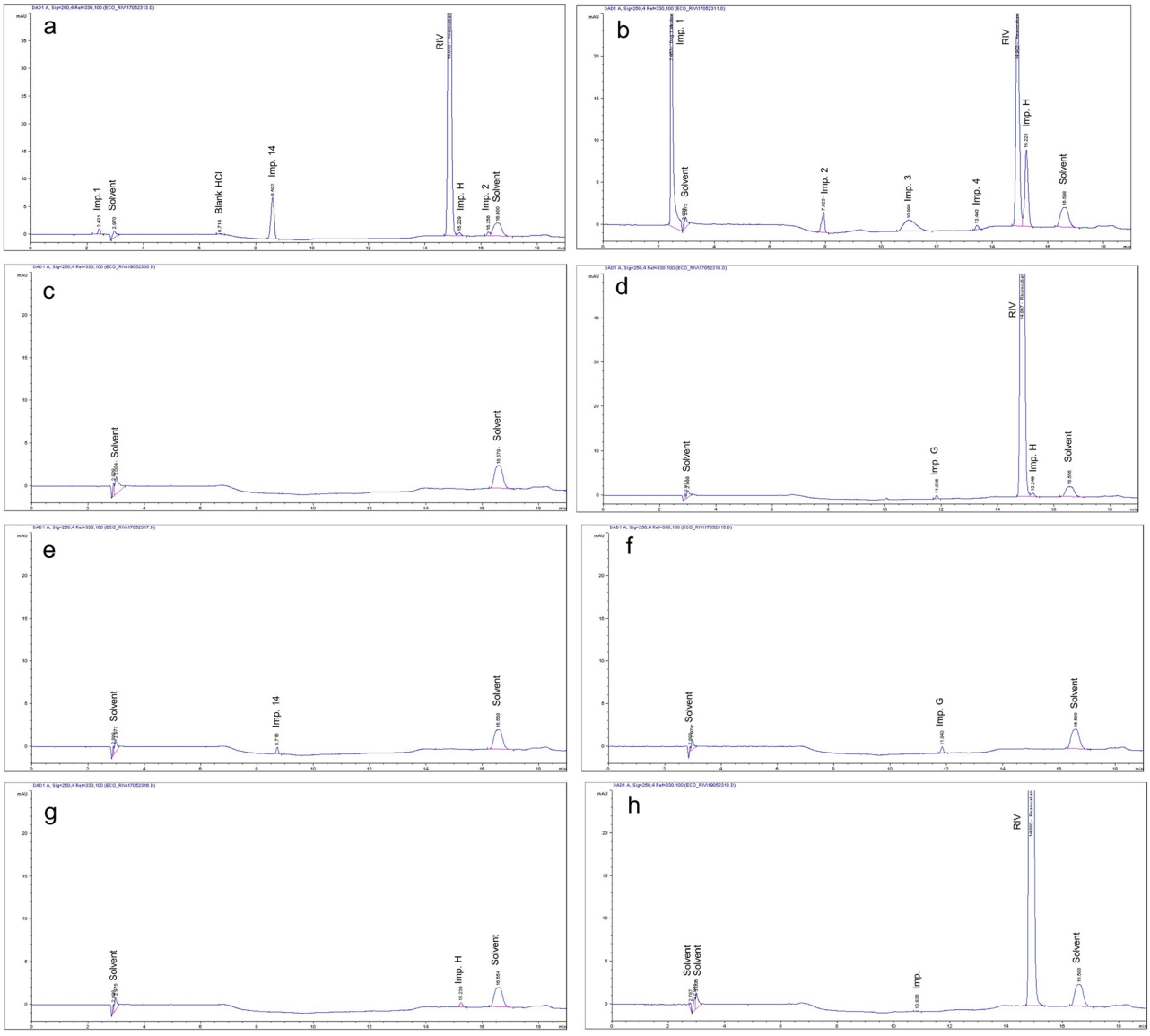

Fig. 4. Representative chromatograms for the related substances method under final conditions: a) St. solution of RIV degraded under acidic condition, b) St. solution of RIV degraded under alkaline condition, c) Placebo, d) Resolution solution, e) St. sol. imp. 14, f) St. sol. imp. G, g) St. sol. imp. H, and h) Test solution

Table 4. Results for linearity, DL and QL obtained during validation of the method for related substances quantification

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
RIV	0.1046	13.204	1.95	0.129	0.9973	0.03/0.10	0.05/0.15
Imp. 14	0.0827	10.579	2.01	0.091	0.9977	0.03/0.09	0.05/0.10
Imp. G	−0.0731	11.002	−1.53	0.101	0.9980	0.03/0.09	0.05/0.10
Imp. H	−0.258	10.676	−4.91	0.098	0.9975	0.03/0.09	0.05/0.15

Table 5. Results for assay and related substances quantification in Rivaroxaban 10 mg tablets from three different manufacturers available on the Macedonian market

Compound	Manufacturer 1	Manufacturer 2	Manufacturer 3
RIV assay (%)	97.67	100.10	97.69
Imp. G (%)	ND*	ND*	0.02
Imp. 14 (%)	ND*	ND*	0.03
Imp. H (%)	ND*	ND*	0.01
Unspecified impurities (%)	ND*	0.02	0.03
Total (%)	/	0.03	0.05

*ND – not detected.

concentration) of RIV, and the imp. G, imp. 14 and imp. H in spiked placebo (Table 3) confirmed the accuracy of the method. The repeatability of the system was confirmed by the obtained values for RSD (≤5%) of the peak area for six replicate injections of standard solutions containing RIV and impurities (imp. G, imp. H and imp. 14). RSD values for each detected and quantified related substance from analysis of 6 different test solutions spiked with the investigated impurities at a concentration level of 0.2% was below 5%, confirming the method repeatability.

Sensitivity: The detection and quantification limits (DL & QL) for RIV and RIV imp. G, imp. H and imp. 14 were calculated using the two different approaches: SD of the

Table 6. Analytical eco-scale and AGREE score for the proposed ethanol-based HPLC methods

Proposed method for assay		**Proposed method for related substances quantification**	
AGREE		**AGREE**	
ESA	Penalty points	**ESA**	Penalty points
Sample preparation		Sample preparation	
Ethanol	4	Ethanol	4
Acetonitrile	/	Acetonitrile	/
Phosphoric acid	/	Phosphoric acid	/
Acetic acid	/	Acetic acid	2
Analysis		Analysis	
HPLC	1	HPLC	1
Reagents	4	Reagents	4
Waste	5	Waste	4
Total points	14	Total points	15
ESA total score	100-14 =**86**	AES total score	100-15=**85**

linear response and the slope of the curve, and the signal-to-noise approach (Table 4). The method shows satisfactory sensitivity for the intended use of the method, allowing quantification of the investigated impurities in the concentration range of 0.10 μg mL^{-1} to 0.15 μg mL^{-1}.

Robustness testing: A rational assessment of the method robustness was achieved using the 2^3 CCF DoE. The deliberate changes in the tested experimental factors didn't influence the critical resolution between RIV/imp. H (Table 2). The obtained response contour plots for the related substances method (Fig. 2b) show that deliberate alterations didn't affect method performance, confirming the robustness of the method.

3.3.3. Application of the proposed green RP-HPLC methods. The proposed ethanol-based HPLC methods were applied for assay and determination of related substances in rivaroxaban 10 mg tablets obtained from three different manufacturers. The content of RIV in tablets was calculated using the external standard method, whereas the percentage of the detected related substances was calculated using the peak area of RIV in diluted test solution (0.4 μg mL^{-1}) [13]. The obtained results (Table 5) confirm that the proposed methods are suitable for their intended purpose.

3.3.4. Greenness assessment of the proposed green RP-HPLC methods. The objective evaluation of greenness of the developed ethanol-based methods for determination of content and related substances of RIV in tablets was performed using the two quantitative tools ESA and AGREE. The penalty points for ESA score for both methods were calculated taking into account the amount and the toxicity of the solvents, the used technique, as well as the amount of waste generated from the whole procedure (sample preparation and instrumental analysis). The obtained ESA score for the assay method and for the related substances method were found to be 86 and 85, respectively (Table 6), showing excellent greenness of the proposed methods.

The AGREE metric was applied to assess the compliance of each segment of the proposed methods with the twelve GAC principles. The results confirmed excellent agreement with the GAC p. 2, p. 4, p. 6, p. 10, p. 11 and p. 12 (green color in the pictograms) of both methods. Deviation from the GAC p. 1, p. 7 and p. 9 (expressed as orange colour in the pictograms) was observed due to the use of external sample pretreatment (p. 1); the amount of the analytical waste between 25 and 100 mL (p. 7); and the level of energy consumption, because LC is technique demanding high energy (p. 9). Considering that both methods are off-line, red color in the AGREE pictogram (non-compliance of the methods) is obtained for principle 3, as expected. The overall color of AGREE pictograms for both methods is pale green color (AGREE score of 0.66 for the assay method and 0.65 for related substances method) (Table 6). The results from assessment indicate that the proposed methods for determination of content and related substances of RIV in tablets, can be considered as eco-friendly.

4. CONCLUSION

Development, applicability and greenness assessment of two ethanol-based HPLC methods for quantitative determination of rivaroxaban and its related substances in tablets is presented in this study. Rational optimization of the developed methods, in accordance with the GAC principles was achieved using the DoE approach. The first method enables determination of RIV in the presence of its impurities within 7 min, while the second method provides determination of rivaroxaban impurities (imp. G, imp. H and imp. 14 and other unspecified impurities) with a simple gradient, based on EtOH-water mobile phase, within 27 min. The validation parameters confirm that both methods are selective, precise and accurate, and that the method for determination of related substances is sensitive, with a quantification limit of 0.1 ppm. Therefore, the method is suitable for routine analysis and stability studies of tablets containing rivaroxaban. The robustness of both methods, confirmed through the use of DoE approach, is suitable for easy method transfer between different laboratories for quality control of medicines.

The analytical Eco-scale score for the proposed methods (above 80) and the AGREE score (0.66 for the assay method and 0.65 for the related substances method) confirmed the eco-friendliness of both methods.

This study demonstrated that the ethanol-water based HPLC methods can deliver the required selectivity, accuracy, precision and robustness needed for quality control methods used in the pharmaceutical industry. Additionally, it was confirmed that the ecological aspects of the method can be improved, while preserving the required analytical method attributes.

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REFERENCES

1. Yabré, M.; Ferey, L.; Touridomon Somé, I.; Sivadier, G.; Gaudin, K. *J. Pharm. Biomed. Anal.* **2020**, *190*, 113507.
2. Kokilambigai, K. S.; Lakshmi, K. S. *J. Chrom. Open* **2022**, *2*, 100052.
3. Nakov, N.; Acevska, J.; Brezovska, K.; Kavrakovski, Z.; Dimitrovska, A. In *High Performance Liquid Chromatography – Recent Advances and Applications*; Núñez, O., Sentellas, S., Granados, M., Saurina, J., Eds. IntechOpen: London, **2023**; pp 119–40.
4. Rao, W.; Li, L.; Zhang, C.; Zheng, J.; Fan, J.; Luan, B.; Sun, J.; Qui, M.; Wu, S.; Li, Y.; Wang, Z. *J. Pharm. Biomed. Anal.* **2023**, *228*, 115325.
5. Stojanovic, J.; Krmar, J.; Otasevic, B.; Protic, A. *Arh. Farm.* **2023**, *73*, 146–71.
6. Bonde, A. N.; Marinussen, T.; Lee, C. J.; Lip, G. Y. H.; Staerk, L.; Bang, C.; Bhattacharya, J.; Gislason, G.; Torp-Pedersen, C.;

Olesen, J. B.; Hlatky, M. A. *Circ. Cardiovasc. Qual. Outcomes* **2020**, *13*, e006058.
7. Lim, G. B. *Nat. Rev. Cardiol.* **2020**, *17*, 320.
8. Malik, A. H.; Yandrapalli, S.; Shetty, S.; Frishman, W. H.; Aronow, W. S. Am *J. Ther.* **2022**, *29*(3), e373–6.
9. Berger, J. S.; Connors, J. M. *The Lancet* **2021**, *397*(10291), 2226–8.
10. Kumar, D.; Kaimaparambil, V.; Chandralekha, S.; Lalchandani. *J. Assoc. Physicians India* **2022**, *70*(2), 11–2.
11. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology Q2(R1). ICH, **2005**. https://www.ema.europa.eu/documents/scientific-guideline/ich-q-2-r1-validation-analytical-procedures-text-methodology-step-5_en.pdf.
12. Deidda, R.; Orlandini, S.; Hubert, P.; Hubert, C. *J. Pharm. Biomed. Anal.* **2018**, *161*, 110–21.
13. European Pharmacopoeia. General monographs: rivaroxaban tablets. 11th Ed. **2022–2023.** Strasbourg France: European Directorate for the quality of medicines & Healthcare, 3904–6.
14. United States Pharmacopeia *USP Monographs, Rivaroxaban Tablets*. USP-NF; United States Pharmacopeia: Rockville, MD, **2023**.
15. Reçber, T.; Haznedaroğlu, C. I.; Çelebier, M. *Crit. Rev. Anal. Chem.* **2020**, 1–13.
16. Arous, B.; Al-Mardini, M. A.; Karabet, F.; Daghestani, M.; Al-Lahham, F.; Al-Askar, A. *Pharm. Chem. J.* **2018**, *52*, 483–90.
17. Ramisetti, R. N.; Kuntamukkala, R. *RSC Adv.* **2014**, *4*, 23155–67.
18. Rao, W.; Li, L.; Zhang, C.; Zheng, J.; Fan, X.; Luan, B.; Sun, J.; Qui, M.; Wu, S.; Li, Y.; Wang, Z. *J. Pharm. Biomed. Anal* **2023**, *228*, 115325.
19. Alam, I.; Ali, N. S.; Qayoom, A.; Bibi, R.; Aqeel, M.; Muhammad, H. *Microchem. J.* **2023**, *193*, 109025.
20. European Pharmacopoeia draft chater 5.33 design of experiments (PA/PH/Exp. SDA/T (22) 4 ANP), Pharmeuropa 34.4 | October 2022, 7-15, Strasbourg France: European Directorate for the quality of medicines & Healthcare.
21. Gałuszka, A.; Konieczka, P.; Migaszevski, Z. M.; Namieśnik, J. *TrAC* **2012**, *37*, 61–72.
22. Pena-Pereira, F.; Wojnowski, W.; Tobiszevski, M. *Anal. Chem.* **2020**, *92*, 10076–82.
23. Turner, C. *Pure Appl. Chem.* **2013**, *85*, 2217–29.
24. Çelebier, M.; Reçber, T.; Koçak, E.; Altınöz, S. *Braz. J. Pharm. Sci.* **2013**, *49*, 359–66, 2013.