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Concept of ecologically acceptable chromatographic methods: Case study on the separation of dronedarone hydrochloride and its degradation products

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Abstract

Recently, concern about the environmental impact of drug analysis methods has increased significantly. Reversed-phase high-performance liquid chromatography (RP-HPLC), the predominant technique in drug analysis, relies heavily on organic solvents such as acetonitrile, which is known for its chromatographic efficiency, but also for its toxicity and flammability. To address these concerns, it is essential to minimize the use of toxic organic solvents. The aim of this study is to explore greener RP-HPLC modifications and evaluate their applicability in the pharmaceutical industry. Methods were developed for the separation of dronedarone hydrochloride and its degradation products based on experimental design, including micellar liquid chromatography (MLC), β-cyclodextrin (CD) modified RP-HPLC and ultra-high performance liquid chromatography (UHPLC). The eco-friendliness of these methods was assessed using the analytical eco-scale score, green analytical procedure index (GAPI) and analytical greenness (AGREE). AGREE appears to be the most suitable, as it revealed the greatest differences between the compared methods, as well as insights into critical aspects of the methods. UHPLC and β-CD modified RP-HPLC have been shown to be superior to MLC, and both methods can be a good choice, depending on whether the ease of implementation or energy efficiency is considered to be a more important criterion.

Key words: RP-HPLC, MLC, UHPLC, cyclodextrin modified HPLC, greenness evaluation methods

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Introduction

Concerns about the negative impact of analytical methods applied in pharmaceutical analysis on human health and the environment are justified and have increased dramatically over the past few years (1). For this reason, the subject of greening the analytical methods that are frequently used in industry has gained attention. Green analytical chemistry (GAC) is mostly directed towards the minimization of the amount of produced waste, associated with either sample preparation or analysis (2). The quality control of bulk drugs and pharmaceutical formulations, as well as the determination of drugs and their metabolites in biological samples, are mostly performed with reversed-phase high performance liquid chromatography (RP-HPLC) (3, 4). Despite being the gold standard among the methods applied in pharmaceutical industry, the amount of waste produced by each liquid chromatograph per day should not be neglected, especially taking into account the number of liquid chromatographs simultaneously employed in large pharmaceutical companies. It is approximated that one liquid chromatograph equipped with a traditional column and with mobile phase flow rate set to 1 mL min^{-1} generates 1.5 L of waste daily (2). This amount of waste is substantial if extrapolated on the whole year and the number of instruments working in parallel worldwide. Apart from the harmful effect, waste disposal contributes to the overall costs of the analysis.

In RP-HPLC, organic solvents, as mobile phase components, play a vital role. Most of the solvents consumed are often highly volatile, flammable, and toxic. In order to reduce the harmful effects of these hazardous chemicals, the development of eco-friendly techniques and methodologies is being significantly promoted (5–7). Some of the typical organic solvents most widely used for mobile phases in RP-HPLC are acetonitrile and methanol. Although acetonitrile is less stable, more toxic and more expensive than methanol, it has a higher eluotropic strength, a lower UV cut-off (8) and forms low-viscosity mobile phases, which in turn leads to a reduction in analysis time. Acetonitrile or methanol could be substituted with ethanol, as an eco-friendly alternative. The benefits of ethanol are reflected in lower volatility, lower toxicity and lower disposal costs in comparison to acetonitrile or methanol (9). On the other hand, the high viscosity of ethanol limits its regular use with standard LC systems (400 bar), which prevents its widespread use in industry. Although acetone is more environmentally friendly compared to other solvents, its use is limited due to its incompatibility with UV detectors, mainly because of its high UV cut-off (329 nm) (7). Detectors compatible with acetone, such as the Corona charged aerosol detector (10, 11), are used to develop eco-friendly methods. However, CAD is rarely used in the industry, which is due to the limited presence of CAD in the pharmacopoeial methods. Hopefully, this could be changed in the future, especially if CAD was manufactured by multiple producers. Since UV detection is the most commonly used technique for monitoring HPLC elution in the pharmaceutical industry, only the "greening" modifications that are possible with this type of detector were considered in this study. However, plenty of other approaches to attach an eco-friendly character to the LC method are at our

disposal (12). Mobile phase modifications leading to the development of ecologically acceptable RP-HPLC methods, but without the need to upgrade the instruments, include the utilization of different organic additives, such as surfactants or cyclodextrins (CDs). Micellar liquid chromatography (MLC), as a HPLC eco-friendly alternative, uses aqueous micellar solution as a mobile phase; thus the concentration of organic modifier is usually low. Moreover, the biodegradable character of surfactants used in the analysis is another advantage of micellar mobiles phases in terms of ecological acceptability (2). Reduced organic solvent consumption is also achieved by adding CD to the mobile phase (1). In this way, the retention time of the analytes is shortened due to complexation with CD, which reduces the run time and solvent consumption and produces less waste (13). The pharmaceutical industry is urgently looking for technologies that enable significantly shorter run times without compromising separation performance (8). This could be accomplished by using fully porous stationary phase particles with a size of less than 2 μm, which offer a higher resolution compared to the particle size of stationary phases traditionally used in HPLC. Consequently, stationary phase modifications induce high column backpressure, which is not a problem if relying on ultra-high performance liquid chromatography (UHPLC) instruments. In that respect, UHPLC also follows the GAC principles, as it uses higher pressure in a shorter column with reduced sorbent particle size and provides shorter analysis time, further reducing the amount of produced waste in comparison to HPLC.

In this study, MLC and β-CD-modified RP-HPLC, as mobile phase modifications, and UHPLC, as an instrument modification, were developed to separate a complex mixture consisting of dronedarone hydrochloride and its degradation products. The ability to separate degradation products makes the developed methods stability-indicating. The applicability of the aforementioned approaches as strategies for the development of eco-friendly methods was tested on this particular model mixture, as it includes compounds with diverse physicochemical characteristics and thus adequately represents the complexity of separation problems in pharmaceutical quality control. Dronedarone hydrochloride (N-[2-butyl-3-[4-[3-(dibutylamino) propoxy] benzoyl]-1-benzofuran-5-yl] methanesulfonamide) is a derivative of benzofuran with lipophilic characteristics (log P = 7.35) which has been relatively recently approved for the treatment of atrial flatter and atrial fibrillation (14). Dronedarone hydrochloride is susceptible to degradation under stress conditions, especially in a basic environment (14). The polarity of degradation products is miscellaneous and different from dronedarone hydrochloride itself. Due to the pronounced differences in the polarity of its components, this mixture is considered quite challenging from an analytical point of view. As a lipophilic compound, dronedarone hydrochloride requires substantial amounts of organic solvent for its elution, which is harmful for the environment. For all these reasons, the need was recognized to develop a chromatographic method for the separation of dronedarone hydrochloride and its degradation products with a lower consumption of organic solvents and/or shorter analysis time.

The overall goal of the presented work is to develop an ecologically acceptable chromatographic method which could be readily available in industry. Therefore, different strategies for greening were applied and methods were developed with the aid of experimental design methodology, which is in line with the concept of sustainability. MLC and β-CD-modified RP-HPLC could be classified as IA variations, while UHPLC falls under IB variation. Type IA and IB are both considered minor variations. Type IA variations are implemented prior to submission, which should be made in a one-year period after the implementation date, while IB variations could be implemented after agency approval, which takes approximately up to three months. The greenness of proposed chromatographic methods for separation of dronedarone hydrochloride and its degradation products was evaluated with three different metrics, namely the Analytical Eco-Scale score and Green Analytical Procedure Index (GAPI) and Analytical Greenness (AGREE). Taking into account the assessed ecological acceptability along with the necessity to submit variations, recommendations for the industry will be given.

Theory

Experimental design methodology

The old-fashioned trial and error approach to developing analytical methods included the investigation of the influence of a certain experimental factor on the selected system, while others remain constant. Thus, the approach is referred to as One-Factor-At-a-Time (OFAT). OFAT is time-consuming, followed by high expenses and reagents' consumption, and it does not exclusively guarantee meeting optimal separation conditions. Accordingly, a need for a more efficient method development approach has emerged. Design of Experiments (DoE) is a mathematical-statistical tool that can simultaneously assess the impact of multiple factors on a chosen response. DoE proposes a planned execution of experiments, within predefined factors' levels, rationalizing the time and resource requirements. The collected data are more informative and susceptible to mathematical modelling. Furthermore, DoE provides an insight into the entire experimental space, and not just for the derived experimental points. Therefore, the behaviour of investigated analytes within the defined experimental space is predictable, which contributes to the method's sustainability (15).

Method optimization as a part of method development strives for an experimental space domain that would provide optimal method performance. The most relevant optimization designs include Central Composite Designs (CCD), Box-Behnken design (BBD) and Doehlert design. CCD consists of factorial design, full or fractional (2^{k-p}), combined with star design ($2k$) and a number of replicates in the central point (C_p), where k represents the number of factors and p the fraction size. The number of experiments is defined by the following formula (1):

$$N = 2^{k-p} + 2k + C_p \quad (1)$$

Depending on the distance of star design points from the C_p, circumscribed and face-centred CCD are distinguished. In circumscribed CCD, all experimental points are equidistant from the C_p and are located on a hypersphere surrounding the experimental space. To achieve such a configuration, the levels of the factors at the star points must differ from the levels of the factors at the factorial points. The factors are therefore examined at five levels. The main advantage of circumscribed CCD is its rotatability, which means that the accuracy of the predictions is the same regardless of the direction of the point in the experimental space. However, if axial points are very distant from the C_p or examination of such extreme points is not feasible, face-centered CCD is the design of choice. Star design points of face-centered CCD lie on the sides of the cube encompassing the experimental space, which implies the examination of factors at three levels, and bypassing the extreme factor levels proposed in circumscribed CCD (16).

Tools for the evaluation of an analytical method's eco-friendly character

With the emergence of the green analytical chemistry concept, aspects of HPLC's environmental impact needed to be evaluated. In that respect, significant consumption of organic solvents and a large amount of generated waste could be highlighted as critical points (17). Although method greening is desirable, the reliability of the method must be maintained and should never be compromised by eco-advancement. The reduction of hazards and the development of an environmentally safe analytical method could be achieved by following the principles of green analytical chemistry from the earliest stages of method development (18).

Different tools for the evaluation of ecological acceptability of the analytical method are available. One of the firstly developed was the National Environmental Methods Index (NEMI). The method's greenness is assessed according to NEMI through a pictogram divided into four segments intended for evaluation (18–20). The segments in the pictogram represent PBT (persistent, bioaccumulative and toxic), hazardous, corrosive and waste, respectively. In case the utilized substances are not defined as PBT by the Environment Protection Agency's Toxic Release Inventory, the corresponding segment would be coloured green. In the same manner, the remaining segments in the pictogram would be coloured green if the used substances are not considered hazardous, the medium's pH is in the range of 2–12, and the amount of the produced waste is less than 50 g (17). Even though NEMI is easy to read, it is more of a qualitative than a quantitative tool, and requires time to individually evaluate each substance (19). As a result of the aforementioned shortcomings, more informative tools for the assessment of ecological acceptability have been developed, such as the Analytical Eco-Scale score, GAPI and AGREE.

Analytical eco-scale score

The analytical eco-scale score evaluates the physical, environmental, and health impact of an analytical method. Scoring is based on the Globally Harmonized System

of Classification and Labelling of Chemicals (GHS) due to its most comprehensive, state-of-the-art chemical classification (18).

The scoring system is organized in such a way that an ideally green analytical method has a score of 100. The analytical eco-scale score is obtained by subtracting the number of calculated penalty points from the ideal score of 100. The number of penalty points depends on the used reagents, hazards, energy, and produced waste (17). Penalty points are assigned to reagents according to their number of pictograms and signal words, proposed by the GHS. GHS characterizes substances as "warning" (1 penalty point) or "danger" (2 penalty points). The total penalty points assigned to a certain chemical are obtained by multiplying the number of pictograms with the number of penalty points given according to the signal word. When scoring, the amount of used energy, the occupational hazard and the amount of produced waste are taken into account as well (18). According to the achieved eco-scale score, the methods are classified as "excellent green" when scoring above 75, "acceptable green" when the score is greater than 50, and "inadequately green" methods, with a score below 50 (17, 18).

The analytical eco-scale score is a semi-quantitative tool suitable for use in laboratory practice and educational purposes. Although it has showed adequate performance in terms of well-defined evaluation criteria and ability to be applied to new methodologies, it does not provide information on the structure of the hazards. Moreover, it does not provide any information on the analytical procedure and its impact on the environment, waste generation, and exposure to professional hazards (19).

Green analytical procedure index (GAPI)

GAPI has emerged as a tool that allows not merely a general, but also a detailed qualitative assessment of the green profile of the entire analytical process, from sampling to the final analyses (19). Every step in analytical procedure, as well as the number of steps, plays a significant role in eco-assessment. Namely, the more steps there are, the higher is the risk of hazard's use, waste generation, and energy consumption.

Like with the NEMI pictograms, the evaluation with GAPI pictograms also offers a quick, easy-to-understand assessment (19). On the other hand, it also provides a semi-quantitative assessment, similar to the analytical eco-scale, which is achieved through colour scaling. In this regard, five colour-scaled pentagrams visually present a method's eco-friendly profile. Each pentagram evaluates the greenness of one aspect of an analytical procedure, while within each aspect several steps are taken into account, as shown in Figure 1. Colour scaling is carried out by three-color levels, namely green, yellow, and red, ranging from environmentally friendly to dangerous (17, 19).

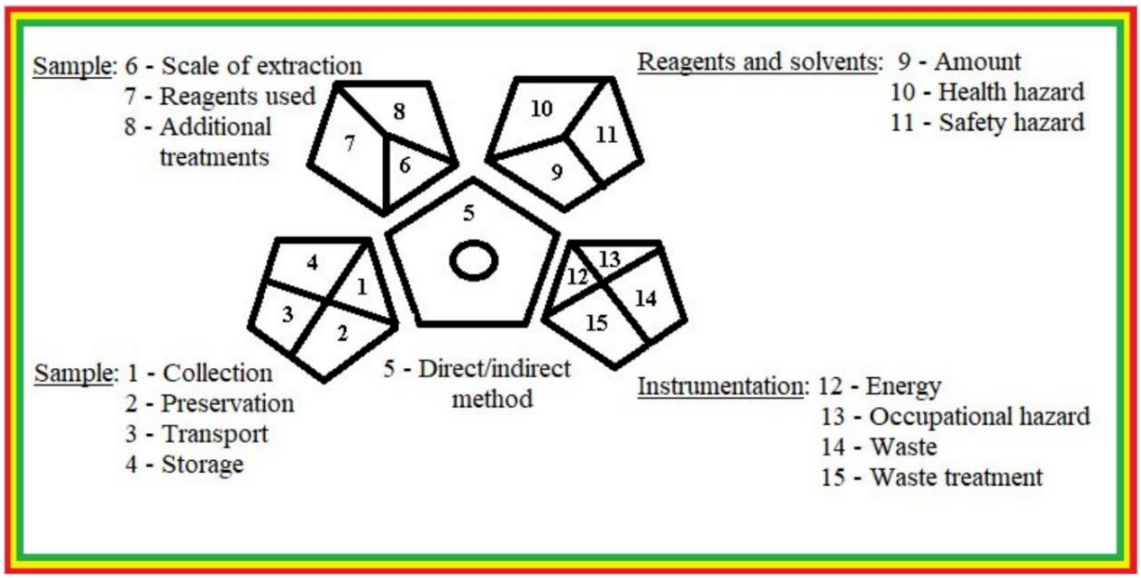

Figure 1. Criteria for the assessment of environmental friendliness according to the green analytical procedure index (GAPI)

Slika 1. Kriterijumi za procenu ekološke prihvatljivosti prema indeksu zelene analitičke procedure (GAPI)

GAPI's detailed visual representation of the eco-friendly characteristics of the analytical procedure allows analysts to self-assess ecological criteria, which positions this tool as invaluable for comparing the environmental friendliness of methods. In addition, GAPI clearly indicates the step with the least green character. In this respect, GAPI can be an extremely valuable tool for the development of analytical methods, as it evidently shows which aspects of the method can be further modified to improve its greenness (19).

Analytical Greenness (AGREE) Approach

In recent years, Analytical Greenness (AGREE) has emerged as a promising alternative to the above-mentioned metrics for assessing the environmental friendliness of analytical methods. The assessment by this tool considers all 12 principles of GAC as inputs and allows the weighting of the inputs according to their importance in the respective case (21). AGREE is therefore a comprehensive approach that can be applied to a wide range of analytical techniques. The result of the evaluation performed by AGREE is a clock-like pictogram consisting of the centre and the segmented border. Each segment of the border represents a GAC principle, while its colour indicates the extent to which the analytical method meets the respective criterion. Details of the principles can be found in the reference (21). Similar to the GAPI, such a representation makes it possible to identify critical aspects for the overall greenness of the method. On the other hand, the middle of the pictograms contains an overall score, expressed on a scale from 0 to 1, which together with the middle background colour indicates the overall environmental friendliness of the method and is suitable for comparing different methods. It could therefore be said that AGREE combines the advantages of the analytical eco-

scale score and GAPI. The availability of freely accessible software facilitates the implementation of the AGREE assessment.

Experimental

Chemical substances and reagents

Dronedarone hydrochloride (DH), N-debutyl-dronedarone (DBD) and dronedarone N-oxide (DNO) reference standard substances were procured from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). HPLC grade acetonitrile, Brij 35 and β-CD were also purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). Hydrochloric acid and sodium hydroxide pro analysis grade, used for adjusting the pH of the aqueous phase and preparation of stress samples, were purchased from Centrohem (Stara Pazova, Serbia). Deionized HPLC water was obtained from Simplicity 185 system (Milipore, USA). Ammonium acetate manufactured by Sigma Aldrich Chemie GmbH (Taufkirchen, Germany) was used as a mobile phase buffer, while ammonium hydroxide solution (Sigma Aldrich Chemie GmbH (Taufkirchen, Germany)) was used to adjust the pH of the aqueous phase when developing the UHPLC gradient method. Moreover, formic acid (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) was used to adjust the pH when developing β-CD modified RP-HPLC.

Preparation of the stock solution

The concentration of the DH stock solution was 1 mg mL^{-1}. It was prepared by weighing 10 mg of DH reference standard substance and adding 7 mL of acetonitrile:water mixture (50:50, v/v). After the dissolution in an ultrasonic bath (Fungilab, Spain), an appropriate volume of acetonitrile:water mixture (50:50, v/v) was added to supplement the 10 mL volumetric flask to the mark. The prepared stock solution was further used to obtain stressed solution.

Preparation of stressed samples

Previously prepared stock solution of DH was used to prepare the stressed samples. Firstly, 0.5 mL of stock solution of DH was transferred to a 5 mL volumetric flask and mixed with 0.5 mL of 1M solution of sodium hydroxide. The solution was exposed to heat (70°C) for 1h. Afterwards, it was neutralized with 0.5 mL of 1M HCl and the volumetric flask was supplemented to the mark with different mixtures, depending on the method. Depending on the method to be developed, a mixture of acetonitrile and an appropriate aqueous phase (50:50, v/v) was used as a diluent: 40 mM Brij 35 solution for MLC, 10 mM β-CD solution for β-CD modified RP-HPLC and 10 mM ammonium acetate solution.

In the basic environment, the following degradation products were formed: the first degradation product denoted as DP I, the second degradation product denoted as DP II, DBD and DNO.

Identification of degradation products

DBD and DNO stock solutions were prepared following the same procedure of preparation as in the case of DH stock solution (3.2) to obtain final concentrations of 1 mg mL^{-1}. Working solutions of DBD and DNO were prepared by diluting the stock solution with the mobile phase to obtain concentration of 100 μg mL^{-1}. Retention time of DBD reference standard and its UV spectra were compared to the retention time and UV spectra of the corresponding peak in the stressed sample. The same procedure was performed with the DNO sample. Structures of DP I and DP II were not elucidated.

Instruments and experimental conditions for method development

Experiments related to the development of MLC and β-CD modified RP-HPLC were conducted on Dionex Ultimate 3000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), while those related to UHPLC method development were carried out on UHPLC Accela (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The separation of analytes was achieved on a monolithic RP column, RP-18e Chromolith performance (100 mm x 4.6 mm, macropore size 2 μm, mesopore size 13 nm) (Merck, Germany) in the case of MLC and β-CD modified RP-HPLC, while in the development of UHPLC a Poroshell 120 EC-C18 column (2.5 mm x 50 mm, particle size 1.9 μm) (Agilent Infinity Lab, Santa Clara, USA) was utilized. In all systems, the detection was performed with the aid of a photodiode array (PDA) detector, at 270 nm. pH-meter with a combined electrode (PHM 210 Radiometer, Danish) was used during pH adjustment. Prior to use, the mobile phases were filtered through a membrane filter with 0.45 μm pore size (Agilent Technologies, Germany).

When developing MLC, mobile phases consisted of the mixture of acetonitrile and aqueous solution of Brij 35. pH of the Brij 35 solution was set to either 2, 3 or 4, with an addition of an appropriate amount of HCl or NaOH. Mobile phase flow rate was set to 2 mL min^{-1}, while the injection volume was 20 μL. Mobile phase composition varied in accordance with constructed experimental plan. Prior to defining the experimental space, preliminary investigation was undertaken to reveal the factor influential towards the retention behaviour of examined analytes in MLC. Consequently, pH value of Brij 35 solution (2–4), the content of acetonitrile in the mobile phase (18–22%, v/v) and column temperature (20–40°C) were used to create the plan of experiments. Experimental plan was constructed via CCD in Design-Expert® (Stat–Ease Inc., Minneapolis, MN, USA), and it is shown in Table I.

In β-CD modified RP-HPLC, the mobile phase consists of a mixture of acetonitrile and aqueous solution of β-CD (concentration range: 5–15 mM). The aqueous phase pH was set to 4, with an addition of formic acid. The mobile phase flow rate was set to 1 mL min^{-1}. Gradient elution was applied and mobile phases were mixed according to the gradient presented in Table II. The column temperature was set to 45°C, while the injection volume was 5 μL.

Table I CCD-aided experimental plan for MLC method development

Tabela I Plan eksperimenata za razvoj MLC metode dobijen primenom CCD

Experiment No.	Content of acetonitrile in the mobile phase (%, v/v)	pH of the aqueous phase	Column temperature (°C)
1	18	2.00	20
2	22	2.00	20
3	18	4.00	20
4	22	4.00	20
5	18	2.00	40
6	22	2.00	40
7	18	4.00	40
8	22	4.00	40
9	18	3.00	30
10	22	3.00	30
11	20	2.00	30
12	20	4.00	30
13	20	3.00	20
14	20	3.00	40
15	20	3.00	30
16	20	3.00	30
17	20	3.00	30
18	20	3.00	30

Table II Gradient elution programs for β-CD modified RP-HPLC and UHPLC method

Tabela II Programi gradijentnog eluiranja za β-CD modifikovanu RP-HPLC i UHPLC metodu

β-CD modified RP-HPLC				**UHPLC**			
t (min)	Mobile phase flow rate (mL min^{-1})	Acetonitrile content in the mobile phase (%, v/v)	Content of β-CD solution in the mobile phase (%, v/v)	t (min)	Mobile phase flow rate (mL min^{-1})	Acetonitrile content in the mobile phase (%, v/v)	Content of buffer solution in the mobile phase (%, v/v)
0	1	10	90	0	0.5	6	94
5	1	55	45	5	0.5	50	50
10	1	55	45	10	0.5	50	50
10.1	1	10	90	10.1	0.5	6	94
13	1	10	90	13	0.5	6	94

Preliminary experiments indicated which factor to vary to adequately define the experimental space. Furthermore, the factors included the initial (5–10%, v/v) and final (35–55%, v/v) content of acetonitrile in the mobile phase, the duration of the linear gradient (3–5 min), and the concentration of β-CD in the aqueous phase (5–15 mM). The experimental plan was constructed with the aid of CCD, and the corresponding plan of experiments is presented in Table III.

Table III CCD-aided experimental plan for β-CD modified RP-HPLC method development

Tabela III Plan eksperimenata za razvoj β-CD modifikovane RP-HPLC metode dobijen primenom CCD

Experiment No.	Initial content of acetonitrile (%, v/v)	Final content of acetonitrile (%, v/v)	β-CD concentration (mM)	Gradient time (min)
1	5	35	5	3
2	10	35	5	3
3	5	55	5	3
4	10	55	5	3
5	5	35	15	3
6	10	35	15	3
7	5	55	15	3
8	10	55	15	3
9	5	35	5	5
10	10	35	5	5
11	5	55	5	5
12	10	55	5	5
13	5	35	15	5
14	10	35	15	5
15	5	55	15	5
16	10	55	15	5
17	5	45	10	4
18	10	45	10	4
19	7.5	35	10	4
20	7.5	55	10	4
21	7.5	45	5	4
22	7.5	45	15	4
23	7.5	45	10	3
24	7.5	45	10	5
25	7.5	45	10	4
26	7.5	45	10	4
27	7.5	45	10	4
28	7.5	45	10	4
29	7.5	45	10	4
30	7.5	45	10	4

When developing the UHPLC method, the mobile phase consisted of acetonitrile and buffer solution of ammonium acetate pH = 7. pH of the buffer solution was adjusted with an addition of ammonium hydroxide. Mobile phases were mixed according to the

gradient elution programme shown in Table II. The flow rate of the mobile phase was 0.5 mL min^{-1}, while the column temperature and injection volume were the same as in the development of the β-CD modified RP-HPLC.

Preliminary experiments indicated the factors to be included in experimental design. These factors are the initial (5–10%, v/v) and final (40–55%, v/v) content of acetonitrile in the mobile phase, the duration of the linear gradient (4–10 min), and the concentration of buffer in the aqueous phase (5–15 mM). The obtained experimental plan is presented in Table IV.

Table IV CCD-aided experimental plan for UHPLC method development
Tabela IV Plan eksperimenata za razvoj UHPLC metode dobijen primenom CCD

Experiment No.	Initial content of acetonitrile (%, v/v)	Final content of acetonitrile (%, v/v)	Concentration of ammonium acetate buffer solution (mM)	Gradient time (min)
1	5	40	5	4
2	10	40	5	4
3	5	55	5	4
4	10	55	5	4
5	5	40	5	10
6	10	40	5	10
7	5	55	5	10
8	10	55	5	10
9	5	40	15	4
10	10	40	15	4
11	5	55	15	4
12	10	55	15	4
13	5	40	15	10
14	10	40	15	10
15	5	55	15	10
16	10	55	15	10
17	5	47.5	10	7
18	10	47.5	10	7
19	7.5	47.5	10	7
20	7.5	47.5	10	7
21	7.5	47.5	10	4
22	7.5	47.5	10	10
23	7.5	47.5	5	7
24	7.5	47.5	15	7
25	7.5	47.5	10	7
26	7.5	47.5	10	7
27	7.5	47.5	10	7
28	7.5	47.5	10	7

The construction of the plan of experiments and corresponding data analyses were performed in Design-Expert® 7.0.0, while indirect modelling and grid point search were processed in MATLAB® R2016a (9.0.0.341360) (Mathworks, Natick, MA, USA).

Results and discussion

Development of MLC method for separation of dronedarone hydrochloride and its degradation products

The preliminary experiments were conducted with the goal of choosing the adequate stationary phase and defining the ranges of significant experimental parameters towards the retention of examined analytes. Firstly, the chromatographic column compliant with the characteristics of the examined substances was selected. DH pH dependent lipophilicity required RP C18 stationary phase. Due to a high mobile phase flow rate and the ability to handle substantially viscous mobile phases, a monolithic column appeared to be adequate for separating DH and its degradation products in MLC.

Among the available organic solvents, acetonitrile was selected as the best option, taking into account the solubility, peak symmetry and chromatographic efficiency. The corresponding range was from 18% (v/v) to 22% (v/v). The range was chosen according to preliminary investigation and in order to preserve the integrity of micelles. The pH of the aqueous phase was kept below 6, to prevent the distribution of DH into different ionization forms and corresponding peak broadening. The column temperature was varied from 20°C to 40°C, while the detection was performed at 270 nm, according to the literature data. Further, the concentration of non-ionic surfactant Brij 35 in the mobile phase was investigated. Brij 35 concentration in the aqueous phase was varied in the range of 40–50 mM. The preliminary experiments show that increasing the Brij 35 concentration above 40 mM did not have any significant effect on the system. For that reason, it was decided to keep the Brij 35 concentration constant at 40 mM, which is far above CMC and enables good elution strength of the mobile phase.

After the preliminary experiments, the factors with significant influence on the examined system were included in the experimental plan. These factors were, namely, the pH of the aqueous phase (2–4), acetonitrile content in the mobile phase (18–22%, v/v), and column temperature (20–40°C). The column temperature interval was determined to include the Krafft point. Krafft point is a temperature at which the surfactant's solubility is the same as its CMC. It is advisable to perform MLC experiments above this temperature in order to prevent the precipitation of the surfactants (22). The selected factors were varied according to the experimental plan obtained by CCD and presented in Table I. Retention factors of DP I, DP II, DBD, DH and DNO were followed, and the responses acquired through the experimental plan are shown in Table V.

Table V Responses obtained in the MLC method

Tabela V Odgovori dobijeni za MLC metodu

Experiment No.	DP I *k*	DP II *k*	DBD *k*	DH *k*	DNO *k*
1	0.880	1.237	1.903	2.703	2.597
2	0.837	1.103	1.633	2.380	2.287
3	1.073	1.457	2.137	4.017	4.873
4	0.983	1.233	1.743	3.227	4.127
5	0.860	1.157	1.733	2.427	2.333
6	0.823	1.043	1.508	2.153	2.097
7	1.020	1.297	1.913	3.510	4.290
8	0.943	1.127	1.593	2.843	3.663
9	0.887	1.230	1.990	2.773	2.413
10	0.843	1.100	1.687	2.430	2.140
11	0.847	1.130	1.630	2.403	2.297
12	1.007	1.267	1.850	3.500	4.237
13	0.877	1.210	1.930	2.767	2.413
14	0.857	1.127	1.757	2.473	2.177
15	0.867	1.170	1.857	2.627	2.300
16	0.860	1.140	1.850	2.600	2.300
17	0.850	1.110	1.800	2.580	2.230
18	0.844	1.250	1.740	2.510	2.390

k – retention factor, DBD – N-debutyl-dronedarone; DH – dronedarone hydrochloride; DNO – dronedarone N-oxide; DP I – degradation product one; DP II – degradation product two.

The optimization goal was to find chromatographic conditions leading to satisfying separation of DH and its degradation products, as well as the adequate peak symmetry within the shortest possible analysis time. Processing the obtained data in Design Expert 7.0.0, mathematical models were obtained, which enabled interpreting the dependence of the selected responses on the examined factors in their corresponding ranges. The retention behaviour of DP I, DBD and DH was best described with the quadratic model (2, 3, 4), whereas the linear model was proposed for the retention behaviour of DP II (5). The retention behaviour of DNO was also described with the quadratic model, but the response required logarithmic transformation (6). The obtained mathematical models are as follows:

$$k_{DPI} = +1.41618 - 0.018824 * x_1 - 0.17344 * x_2 - 0.00385393 * x_3 - 0.00543750 * x_1 * x_2 + 0.000118750 * x_1 * x_3 - 0.000737500 * x_2 * x_3 + 0.000425595 * x_1^2 + 0.063702 * x_2^2 + 0.0000370238 * x_3^2 \quad (2)$$

$$k_{DBD} = +2.98806 - 0.11625 * x_1 + 0.77733 * x_2 - 0.26897 * x_3 - 0.013687 * x_1 * x_2 + 0.000743750 * x_1 * x_3 - 0.000987500 * x_2 * x_3 + 0.00148512 * x_1^2 - 0.066060 * x_2^2 + 0.000109405 * x_3^2 \quad (3)$$

$$k_{DH} = +2.10933 + 0.19427 * x_1 - 0.26533 * x_2 - 0.023823 * x_3 - 0.053750 * x_1 * x_2 + 0.00107500 * x_1 * x_3 - 0.00485000 * x_2 * x_3 - 0.00462798 * x_1^2 + 0.33149 * x_2^2 - 0.000000119048 * x_3^2 \quad (4)$$

$$k_{DPII} = +1.89362 - 0.038600 * x_1 + 0.071100 * x_2 - 0.000489 * x_3 \quad (5)$$

$$\log(k_{DNO}) = +2.76383 + 0.032453 * x_1 - 1.43582 * x_2 - 0.00000889152 * x_3 - 0.00565000 * x_1 * x_2 + 0.000178897 * x_1 * x_3 - 0.000659452 * x_2 * x_3 - 0.00136923 * x_1^2 + 0.31141 * x_2^2 + 0.0000309407 * x_3^2 \quad (6)$$

where x_1 represents acetonitrile content in the mobile phase (%, v/v), x_2 the pH of the aqueous phase, and x_3 column temperature (°C).

According to p values lower than 0.05, it can be concluded that all three examined factors contributed to the obtained models significantly. The models' coefficients indicate the influence of each of the examined factors on the selected response. ANOVA test for all models showed that there is no significant discrepancy between experimentally obtained and predicted values of the selected responses. Coefficient of determination (R^2) adjusted and predicted R^2 values were 0.9927, 0.9844 and 0.9700, respectively, for the mathematical model obtained for DP I (2), while in the case of DP II (5) these values were as follows: 0.8513, 0.8195 and 0.7608. Further, R^2, adjusted and predicted R^2 values for mathematical models obtained for DBD (3) and DH (4) were 0.9742, 0.9452 and 0.9488 for DBD, and 0.9924, 0.9838 and 0.9531 for DH. When discussing the mathematical model obtained to explain the retention behaviour of DNO (6), R^2, adjusted and predicted R^2 values were 0.9979, 0.9942 and 0.9952, respectively. Coefficient of determination (R^2), adjusted and predicted R^2 values are relatively close to 1, which confirms the validity of the models. In addition, no model showed a significant lack-of-fit ($p > 0.05$). Thus, the models are considered reliable in predicting the retention behaviour of analytes within the tested experimental space.

Mathematical models show that the retention factor of DP I is reduced with an increase in pH, acetonitrile content and column temperature, whereas the pH values have an inverse influence on the retention factor of DP II and DBD. Retention factors of DH and DNO are increased with higher acetonitrile percentages, whereas their values are lower with an increase in the pH of the aqueous phase and column temperature.

The graphical analysis was conducted by constructing the 3D response surface plots of a global desirability function. As in β-CD modified RP-HPLC, multicriteria approach, namely Derringer's desirability function, was used to search for optimal conditions. Firstly, the goals for each of the selected responses were set. The first goal was to achieve a retention factor of DP I higher than 1 to prevent its elution with the mobile phase peak. Furthermore, the duration of the chromatographic run was supposed to be as short as possible. The separation of peaks was not taken into account, because it was noticed that an adequate separation among the examined analytes was achieved across the investigated experimental space. Therefore, the critical aspects were the non-retention behaviour of DP I and the duration of the chromatographic analysis. For all experimental conditions under which the predefined goals are achieved, the Desirability function has a value equal to 1. Figure 2 shows 3D response surface plots of desirability function against the pH of the aqueous phase and acetonitrile content in the mobile phase at column

temperature of 20 °C (Figure 2a), 30 °C (Figure 2b) and 40 °C (Figure 2c). In-depth assessment of the obtained 3D plots spawned the conditions providing reduced analysis time. The optimal chromatographic conditions included 18% (v/v) acetonitrile in the mobile phase, the pH of the aqueous phase equal to 4 and column temperature of 40 °C. Under these conditions, total analysis time was less than 8 minutes. Moreover, the conditions chosen with respect to overall analysis time provided satisfying peak symmetries and resolution of all peaks. The representative chromatograms obtained under optimal chromatographic conditions are illustrated in Figure 3.

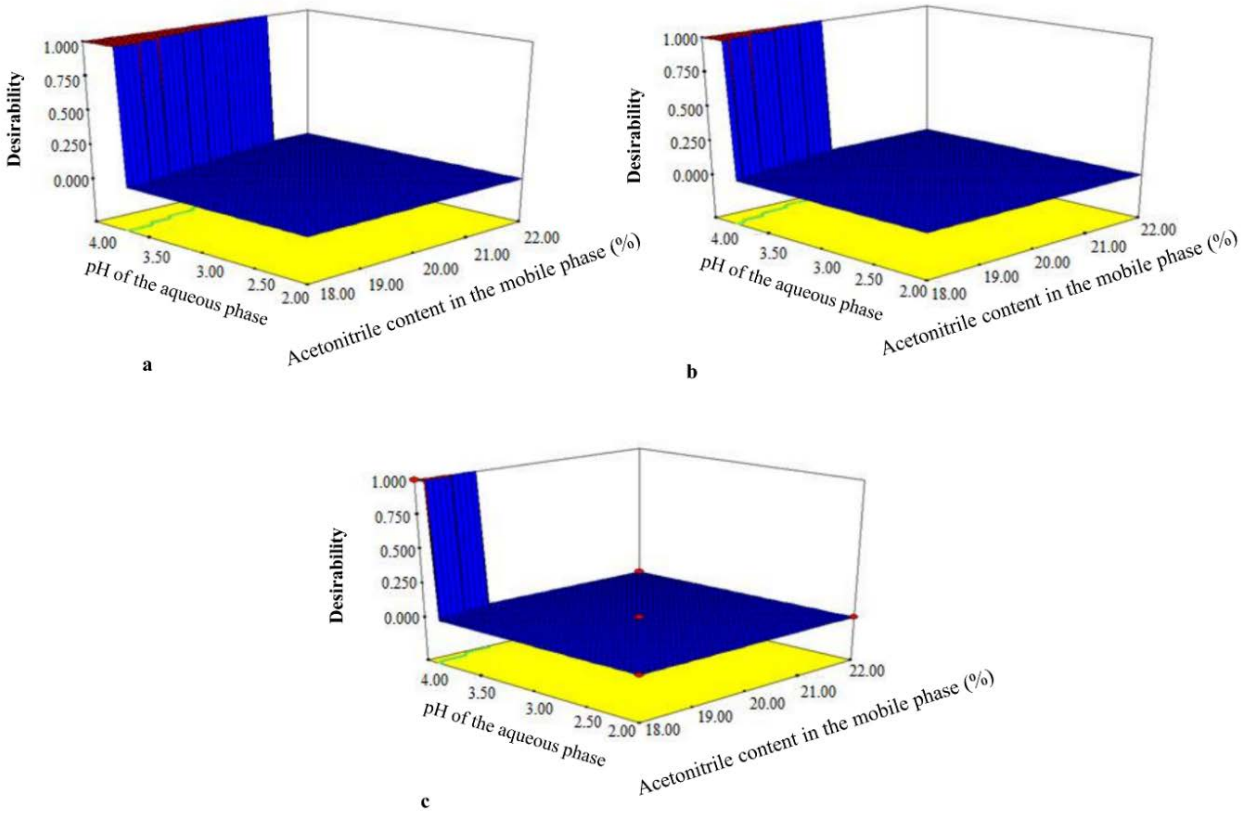

Figure 2. **3D response surface plots of the desirability function against the pH value of the aqueous phase and the acetonitrile content in the mobile phase at a column temperature of: a) 20 °C; b) 30 °C; and c) 40 °C for the MLC method**

Slika 2. **3D površina odgovora koja prikazuje zavisnost funkcije poželjnih odgovora od pH vrednosti vodenog dela mobilne faze i sadržaja acetonitrila u mobilnoj fazi pri temperaturi kolone: a) 20 °C; b) 30 °C i c) 40 °C za MLC metodu**

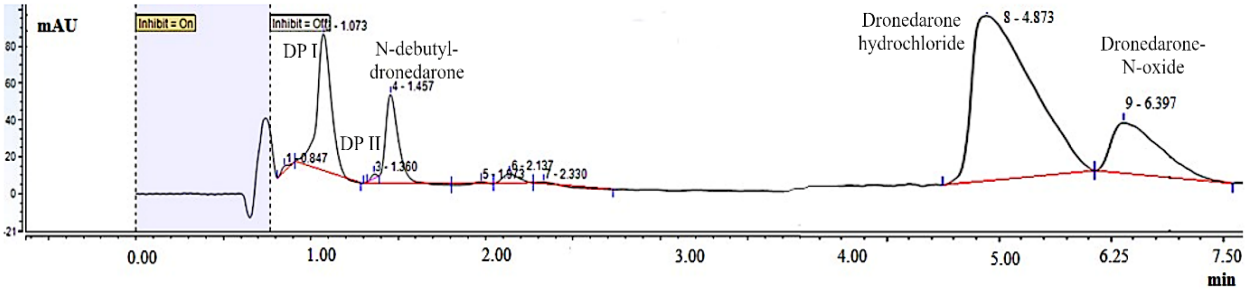

Figure 3. **Representative chromatogram for the separation of dronedarone hydrochloride and its degradation products obtained under optimal conditions by the MLC method**

Slika 3. **Reprezentativni hromatogram dobijen razdvajanjem dronedaron hidrohlorida i njegovih degradacionih proizvoda dobijen pri optimalnim uslovima za MLC metodu**

Development of β-CD modified RP-HPLC method for separation of dronedarone hydrochloride and its degradation products

Preliminary experiments were conducted to identify factors with significant influence on the retention of DH and its degradation products. Among the available chromatographic columns, an RP-18 monolithic column was used. The stationary phase of monolithic columns is composed of highly porous continuous silica network, with macro- and mesopores. Macropores are 2 μm in size and they are responsible for low resistance towards mobile phase flow. Therefore, monolithic columns are compatible with a high mobile phase flow rate, even up to 9 mL min^{-1}, accompanied with low pressure in the system. On the other hand, mesopores are smaller in comparison to macropores and they account for a huge active surface, approximately 300 m^2 g^{-1}, which enables efficient chromatographic separation (23). Allowing faster mobile phase flow rates and thus shortening the duration of chromatographic analyses, together with its compatibility with highly viscous mobile phases, such as CD-modified mobile phases, made the monolithic column the ideal choice for the separation of DH and its related compounds in β-CD modified RP-HPLC. Different polarity of DH and its degradation products dictated the conditions of gradient elution mode. Mobile phase composition in terms of initial and final acetonitrile content in gradient elution, gradient time and β-CD concentration in the mobile phase were investigated and appeared to be significantly related to retention behaviour of DH and its degradation products. To adequately describe the experimental space, experiments were conducted in accordance with the CCD plan of experiments, which is presented in Table III, while Table VI shows the obtained responses, namely the time of the end of the first peak (t_{end}) and the time of the beginning of the second eluting peak (t_{start}) of a critical peak pair.

Table VI Responses obtained in the β-CD modified RP-HPLC method

Tabela VI Odgovori dobijeni za β-CD modifikovanu RP-HPLC metodu

Experiment No.	DP I t_{end}	DP II t_{start}	DP II t_{end}	DBD t_{start}	DBD t_{end}	DH t_{start}
1	5.200	6.00	6.500	10.000	13.300	25.000
2	5.900	6.00	6.700	9.000	12.200	18.500
3	4.300	4.800	5.125	5.500	6.200	7.000
4	4.750	4.875	5.500	5.500	6.000	6.000
5	5.500	5.300	5.850	5.500	6.500	7.200
6	5.000	5.200	5.750	5.900	6.300	15.000
7	4.400	5.000	5.250	5.600	6.000	6.300
8	4.400	5.100	5.500	5.500	6.100	6.100
9	7.000	8.000	8.700	11.000	12.000	20.000
10	6.725	8.000	8.500	9.000	10.000	15.000
11	5.500	6.250	6.500	7.250	7.200	7.900
12	5.200	5.900	6.200	6.900	7.100	7.200
13	6.000	6.000	6.500	6.750	7.500	8.500
14	5.800	5.600	5.500	6.500	7.700	15.700
15	4.725	6.000	6.200	6.800	7.200	7.700
16	5.000	5.900	6.000	6.725	7.500	7.600
17	5.500	5.725	5.900	8.725	10.000	11.725
18	5.725	5.725	6.400	8.000	9.000	11.000
19	5.900	6.100	6.400	6.900	7.200	10.000
20	5.000	5.500	5.900	6.500	6.725	6.725
21	5.200	6.000	6.300	7.500	8.200	11.000
22	5.200	5.900	6.000	6.800	7.800	10.900
23	5.000	5.100	6.500	7.500	9.200	12.000
24	5.600	6.700	7.000	8.000	8.800	10.700
25	5.100	6.000	6.500	7.200	7.900	9.600
26	5.200	5.800	6.000	7.700	9.000	10.500
27	5.300	5.900	6.400	7.300	8.250	10.000
28	5.400	6.100	6.500	7.250	8.350	10.100
29	4.900	5.750	6.200	7.500	8.000	9.750
30	5.500	6.050	6.350	7.025	8.125	10.250

DBD – N-debutyl-dronedarone; DH – dronedarone hydrochloride; DP I – degradation product one; DP II – degradation product two.

The method aimed at efficient separation of all adjacent peaks, and therefore the authors decided to assess the separation criterion (*S*). *S* is considered convenient to evaluate resolution in gradient elution mode due to its simple calculation, accompanied with the fact that baseline separation is exclusively achieved if *S* is equal to or higher than 0. In this way, the problem of setting an appropriate resolution threshold is overcome (24). *S* is calculated by subtracting the time of the end of the first peak (t_{end}) from the time of the beginning of the second eluting peak (t_{start}).

There were three critical peak pairs, namely DP I and DP II, DP II and DBD, and DBD and DH. Therefore, to be able to assess S for each critical peak pair, mathematical models for t_{start} and t_{end} were firstly obtained in a direct mode.

The linear model for DP I t_{end}, two factor interaction models for DP II t_{start} and t_{end}, and quadratic models for DBD t_{start} and t_{end}, as well as for DH t_{start}, were selected. For each response, suitable transformations were selected on the basis of the Box-Cox statistic.

The obtained mathematical models are shown in Eq. (7) – (12).

$$1.0/(\text{DPI } t_{end}) = + 0.15060 - 0.000519416 * x_1 + 0.00188695 * x_2 + 0.00129465 * x_3 - 0.013682 * x_4 \quad (7)$$

$$1.0/(\text{DPII } t_{start}) = + 0.11357 - 0.00155529 * x_1 + 0.00375377 * x_2 + 0.00625312 * x_3 - 0.024385 * x_4 - 0.0000258588 * x_1 * x_2 + 0.0000407505 * x_1 * x_3 + 0.0000692985 * x_1 * x_4 - 0.0000192010 x_2 * x_3 - 0.0000155050 * x_2 * x_4 + 0.0000895428 * x_3 * x_4 \quad (8)$$

$$1.0/(\text{DPII } t_{end}) = + 0.10259 - 0.00403275 * x_1 + 0.00413744 * x_2 + 0.00420768 * x_3 - 0.025924 * x_4 - 9.54957e^{-5} * x_1 * x_2 + 1.77054e^{-4} * x_1 * x_3 + 0.00167696 * x_1 * x_4 - 1.70817e^{-4} * x_2 * x_3 - 1.65262e^{-4} * x_2 * x_4 + 9.84846e^{-4} * x_3 * x_4 \quad (9)$$

$$\text{DBD } t_{start} = + 14.41319 - 2.20458 * x_1 + 0.42623 * x_2 - 0.73937 * x_3 - 1.29308 * x_4 + 0.0058125 * x_1 * x_2 + 0.016625 * x_1 * x_3 - 0.049375 * x_1 * x_4 + 0.017281 * x_2 * x_3 + 0.017031 * x_2 * x_4 + 0.0015625 * x_3 * x_4 + 0.12554 * x_1^2 - 0.00877851 * x_2^2 - 0.017114 * x_3^2 + 0.17215 * x_4^2 \quad (10)$$

$$(\text{DBD } t_{end})^{-1.88} = + 0.018218 + 0.00969856 * x_1 - 0.00519026 * x_2 + 0.00452832 * x_3 + 0.023827 * x_4 - 1.50849e^{-5} * x_1 * x_2 - 5.02719e^{-5} * x_1 * x_3 - 6.44791e^{-5} * x_1 * x_4 - 8.42121e^{-5} * x_2 * x_3 - 1.68208e^{-4} * x_2 * x_4 - 3.26042e^{-4} * x_3 * x_4 - 5.37832e^{-4} * x_1^2 + 8.13191e^{-5} * x_2^2 + 8.39843e^{-5} * x_3^2 - 0.00189608 * x_4^2 \quad (11)$$

$$(\text{DH } t_{start} - 0.5)^{-1.82} = + 0.094804 + 8.35230e^{-4} * x_1 - 0.00846921 * x_2 + 0.00703797 * x_3 + 0.025591 * x_4 + 1.35653e^{-4} * x_1 * x_2 - 2.91688e^{-4} * x_1 * x_3 + 1.07352e^{-5} * x_1 * x_4 - 5.51540e^{-5} * x_2 * x_3 - 2.73700e^{-4} * x_2 * x_4 - 2.66378e^{-4} * x_3 * x_4 - 2.88731e^{-4} * x_1^2 + 1.13789e^{-4} * x_2^2 - 3.56216e^{-5} * x_3^2 - 0.0016937 * x_4^2 \quad (12)$$

In all presented equations, x_1 stands for initial acetonitrile percentage, x_2 for final acetonitrile percentage, x_3 for β-CD concentration in the mobile phase (mM) and x_4 for the gradient time.

Non-significant lack-of-fit tests ($p > 0.05$) and high values of R^2, adjusted and predicted R^2 values reflected the validity of all proposed models. For DP I t_{end}, R^2, adjusted and predicted R^2 values were 0.8504, 0.8264 and 0.7726. Further, for DP II t_{start} these values were 0.9510, 0.9252 and 0.8572, while for DP II t_{end} they were 0.8777, 0.8133 and 0.6335. For DBD t_{start} and t_{end}, R^2, adjusted and predicted R^2 values were 0.9423, 0.8884 and 0.7033 for the former, and 0.9312, 0.8669 and 0.6592 for the latter.

Finally, R^2, adjusted and predicted R^2 values for DH t_{start} were 0.9559, 0.9148 and 0.7219, respectively.

Analysing the obtained models by ANOVA leads to the conclusion that initial content of acetonitrile in the mobile phase is not significant towards the examined responses. Therefore, in the optimization of separation conditions phase, it was kept constant at 10% (v/v). A higher level was chosen because it is beneficial to shorten the chromatographic run and reduce the overall consumption of the organic solvent and electrical energy in this way. The responses followed in this phase are shown in the equations below (13–15).

$$Sa = \text{DPII } t_{start} - \text{DPI } t_{end} \quad (13)$$

$$Sb = \text{DBD } t_{start} - \text{DPII } t_{end} \quad (14)$$

$$Sc = \text{DH } t_{start} - \text{DBD } t_{end} \quad (15)$$

Satisfactory separation between DH and its degradation products is achieved if *Sa*, *Sb* and *Sc* are equal to or higher than 0. The optimization was performed via grid point search methodology, as a simple numerical optimization technique. Firstly, the discretization of the investigated factors was conducted and experimental space was divided into a grid. Experimental space was gridded by the discretization of final acetonitrile content [35:1:55], concentration of β-CD in the mobile phase [5:1:15], and gradient time [3:0.3:5]. 3D graph (Figure 4) shows the distribution of *Sa*, *Sb* and *Sc* for the given chromatographic conditions at 10% of the initial content of acetonitrile (v/v). Points in which the defined criteria are met are grid points coloured yellow. Therefore, optimal separation conditions were as follows: initial acetonitrile percentage 10% (v/v), final acetonitrile percentage 55% (v/v), gradient time 5 minutes, and 5 mM β-CD concentration. A detailed analysis of the 3D graph (Figure 4) indicated that the defined criteria are fulfilled if the final content of acetonitrile in the mobile phase is 50% (v/v). However, taking into account the total run time, the final content of acetonitrile of 55% (v/v) was favourable. As previously mentioned, reduced analysis time is beneficial in terms of the green analytical chemistry concept, because it implies lower organic solvents and energy consumption.

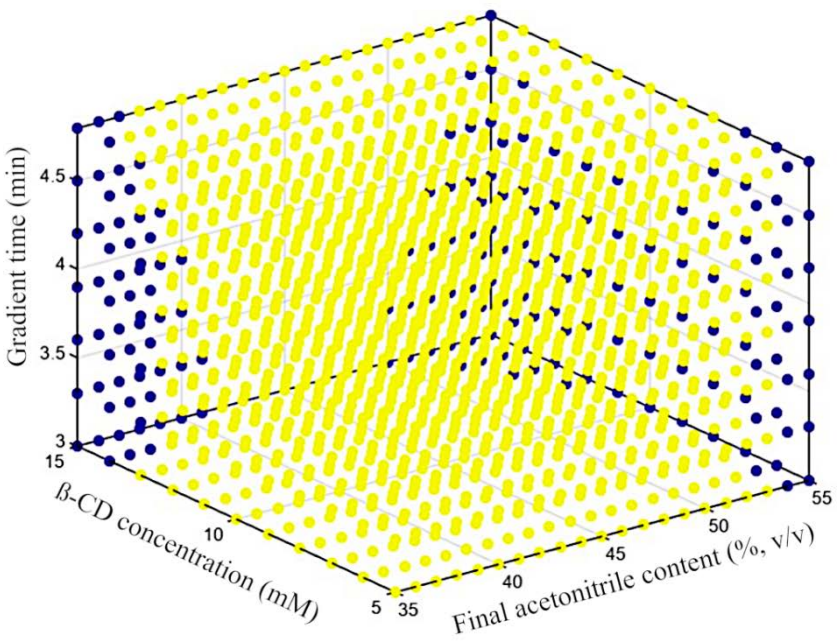

Figure 4. Three-dimensional representation of the grid points that meet the defined criteria for the β-CD-modified RP-HPLC method

Slika 4. Trodimenzionalni prikaz tačaka mreže pri kojima su ispunjeni definisani kriterijumi za β-CD-modifikovanu RP-HPLC metodu

The representative chromatogram obtained under selected optimal condition is presented in Figure 5.

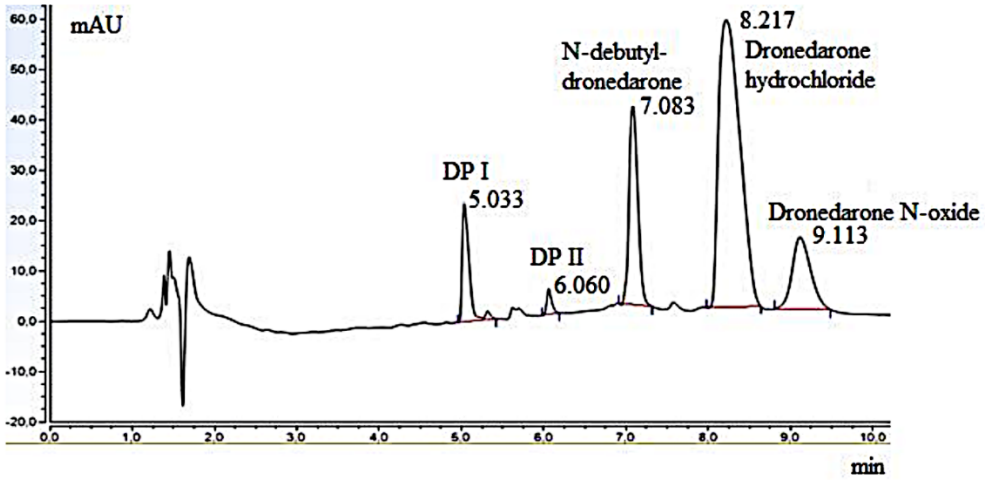

Figure 5. Representative chromatogram for the separation of dronedarone hydrochloride and its degradation products obtained under optimal conditions by the β-CD-modified RP-HPLC method

Slika 5. Reprezentativni hromatogram dobijen razdvajanjem dronedaron hidrohlorida i njegovih degradacionih proizvoda pri optimalnim uslovima za β-CD-modifikovanu RP-HPLC metodu

Development of UHPLC method for separation of dronedarone hydrochloride and its degradation products

Preliminary experiments enabled the selection of factors which showed the most profound influence towards the retention behaviour of DH and its degradation products. During the preliminary phase, different pH values of the buffer were investigated. It was shown that, under pH values lower than 7, DP I was eluted with the mobile phase peak, while pH values higher than 7 led to unnecessary prolongation of the chromatographic run. Therefore, the pH of the ammonium acetate buffer was set to 7 and it was kept constant during the experiments. Among other factors, the initial and final content of acetonitrile in the mobile phase (%, v/v), the gradient duration, and the molarity of the employed buffer showed the most significant effect on the retention behaviour of DH and its degradation products. Thus, these factors were varied in the following ranges: the initial content of acetonitrile from 5% (v/v) to 10% (v/v), the final content of acetonitrile from 40% (v/v) to 55% (v/v), the time of linear gradient from 4 min to 10 min, and the concentration of ammonium acetate in the aqueous phase from 5 mM to 15 mM. Experiments were conducted according to the experimental plan obtained by CCD and showed in Table IV. As with β-CD-modified RP-HPLC, the aim was to achieve satisfactory separation between critical peak pairs. Therefore, the same procedure as in the previous case was undertaken. Namely, mathematical models for t_{start} and t_{end} for each critical peak pair were obtained, followed by indirect modelling of *Sa*, *Sb* and *Sc*. *Sa* stands for the distance between the start of DBD peak and the end of DP II peak, *Sb* for the distance between the start of DNO peak and the end of DBD peak, while *Sc* shows the distance between the start of DH peak and the end of DNO peak.

The obtained t_{start} and t_{end} values for the identified critical peak pairs across the investigated experimental space are shown in Table VII.

DP II t_{end}, DBD t_{start} and DBD t_{end} were described with two-factor interaction mathematical models. For t_{start} and t_{end} of DNO, linear models were selected, while the quadratic model was the most appropriate for t_{start} of DH. Neither of the responses required transformation. The obtained mathematical models are shown below (16–21).

$$\text{DPII } t_{end} = +4.88720 - 0.10228 * x_1 - 0.063241 * x_2 + 1.28343 * x_3 - 0.077611 * x_4 + 0.00266667 * x_1 * x_2 - 0.00283333 * x_1 * x_3 - 0.0057 * x_1 * x_4 - 0.014167 * x_2 * x_3 + 0.00216667 * x_2 * x_4 + 0.00266667 * x_3 * x_4 \quad (16)$$

$$\text{DBD } t_{start} = +5.96794 - 0.15750 * x_1 - 0.084537 * x_2 + 1.41111 * x_3 - 0.10632 * x_4 + 0.00316667 * x_1 * x_2 + 0.00541667 * x_1 * x_3 - 0.00575 * x_1 * x_4 - 0.016806 * x_2 * x_3 + 0.00291667 * x_2 * x_4 + 0.00354167 * x_3 * x_4 \quad (17)$$

$$\text{DBD } t_{end} = +6.87994 - 0.035 * x_1 - 0.1 * x_2 + 1.34907 * x_3 - 0.14729 * x_4 + 0.0015 * x_1 * x_2 - 0.00125 * x_1 * x_3 - 0.00575 * x_1 * x_4 - 0.014028 * x_2 * x_3 + 0.00325 * x_2 * x_4 + 0.00395833 * x_3 * x_4 \quad (18)$$

$$\text{DNO } t_{start} = +12.51532 - 0.035111 * x_1 - 0.20319 * x_2 + 0.71907 * x_3 + 0.045222 * x_4 \quad (19)$$

$$\text{DNO } t_{end} = +14.23208 - 0.09 * x_1 - 0.22481 * x_2 + 0.76944 * x_3 + 0.019444 * x_4 \quad (20)$$

$$\text{DH } t_{start} = +71.60067 - 2.14418 * x_1 - 2.14749 * x_2 + 0.56419 * x_3 - 0.44274 * x_4 + 0.066533 * x_1 * x_2 + 0.070333 * x_1 * x_3 - 0.0623 * x_1 * x_4 + 0.00288889 * x_2 * x_3 - 0.00776667 * x_2 * x_4 + 0.00275 * x_3 * x_4 - 0.08487 * x_1^2 + 0.015459 * x_2^2 - 0.045048 * x_3^2 + 0.064783 * x_4^2 \quad (21)$$

Table VII Responses obtained in the UHPLC method

Tabela VII Odgovori dobijeni za UHPLC metodu

Experiment No.	DP II t_{end}	DBD t_{start}	DBD t_{end}	DNO t_{start}	DNO t_{end}	DH t_{start}
1	5.10	5.50	6.00	7.50	7.90	10.10
2	4.92	5.20	5.90	6.15	6.40	6.90
3	3.90	3.95	4.25	4.40	4.65	6.00
4	3.72	3.75	4.10	4.30	4.50	6.11
5	9.60	10.45	11.00	10.50	12.90	14.00
6	9.10	10.20	10.80	12.15	12.60	10.90
7	6.50	6.50	7.30	8.00	8.45	7.30
8	6.90	7.40	7.80	8.40	8.65	11.00
9	4.95	5.35	5.65	9.00	9.50	13.60
10	4.80	5.30	5.70	7.20	7.50	5.80
11	4.00	4.00	4.20	4.50	4.60	6.00
12	3.65	3.80	3.95	4.37	4.50	3.95
13	9.50	10.30	10.80	12.50	12.80	15.00
14	8.90	10.00	10.30	12.20	12.60	10.40
15	7.30	7.80	8.20	8.90	9.20	9.60
16	6.80	7.35	7.80	8.60	8.90	9.10
17	6.40	6.70	7.10	7.95	8.10	7.40
18	6.10	6.35	6.80	8.30	8.40	7.40
19	7.60	8.00	8.30	9.60	9.90	11.00
20	5.40	5.60	6.00	7.90	8.30	6.60
21	4.20	4.25	4.65	5.80	6.10	5.05
22	7.90	8.30	8.90	10.80	11.10	10.00
23	6.25	6.40	7.20	9.40	9.70	8.50
24	6.20	6.70	7.00	7.60	7.90	10.60
25	5.90	6.40	6.70	7.50	7.80	7.00
26	5.80	6.30	6.60	7.40	7.80	6.90
27	6.20	6.70	7.00	7.80	8.10	7.30
28	6.10	6.60	6.90	7.70	8.00	7.20

DBD – N-debutyl-dronedarone; DH – dronedarone hydrochloride; DNO – dronedarone N-oxide; DP II – degradation product two.

In the presented equations, x_1 stands for the initial content of acetonitrile in the mobile phase (%, v/v), x_2 for the final content of acetonitrile in the mobile phase (%, v/v), x_3 for gradient time (min), and x_4 for buffer molarity (mM).

The adequacy of all obtained mathematical models was confirmed with non-significant lack-of-fit tests ($p > 0.05$). Moreover, relatively high values of R^2, adjusted R^2 and predicted

R^2 also showed that the behaviour in well explained by the obtained model. The obtained R^2, adjusted and predicted R^2 values for DP II t_{end} were 0.9894, 0.9831 and 0.9663, for DBD t_{start} 0.9885, 0.9817 and 0.9464, while for DBD t_{end} these values were 0.9914, 0.9863 and 0.9656. For DNO t_{start} and t_{end}, R^2, adjusted and predicted R^2 were 0.9171, 0.9026 and 0.8775 for the former, and 0.9332, 0.9215 and 0.9037 for the latter. Finally, R^2, adjusted and predicted R^2 for DH t_{start} were 0.9507, 0.8977 and 0.7347, respectively.

Coefficients of the obtained models show that the buffer concentration is insignificant towards the examined retention behaviour. Thus, it was kept constant in further modelling. The separation of adjacent peaks was modelled indirectly, assessing the *S* values shown in the equations below (22–24).

$$Sa = \text{DBD } t_{start} - \text{DPII } t_{end} \quad (22)$$

$$Sb = \text{DNO } t_{start} - \text{DPII } t_{end} \quad (23)$$

$$Sc = \text{DH } t_{start} - \text{DNO } t_{end} \quad (24)$$

Sa, *Sb* and *Sc* should be equal to or higher than 0 to achieve an appropriate separation between DH and its degradation products. As in the case of β-CD-modified RP-HPLC, grid point search methodology was employed to optimize the separation conditions. Experimental space was gridded by the discretization of initial acetonitrile content [5:0.5:10], final acetonitrile content [40:1:55], and gradient time [4:1:10]. A 3D graph (Figure 6) shows the distribution of the examined responses for different

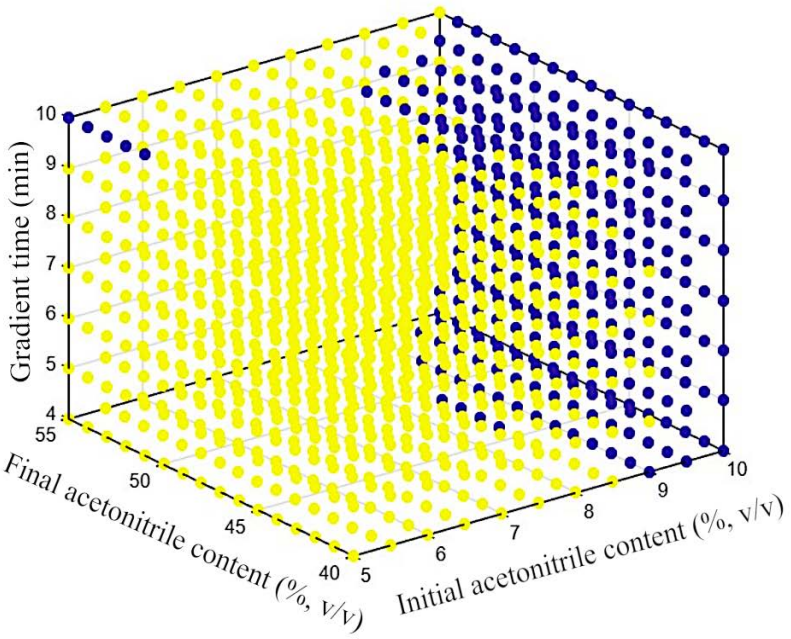

Figure 6. Three-dimensional representation of the grid points that meet the defined criteria for the UHPLC method

Slika 6. Trodimenzionalni prikaz tačaka mreže pri kojima su ispunjeni definisani kriterijumi za UHPLC metodu

combinations of chromatographic conditions if buffer concentration is kept constant at 15 mM (higher level). A higher level of buffer concentration was selected because it offers the possibility to choose optimal conditions from the middle of the region. In this way, the robustness of the method could also be assured. A detailed assessment of the 3D graph provides the following optimal separation conditions: the initial content of acetonitrile of 6% (v/v), the final content of acetonitrile in the mobile phase of 50% (v/v), the gradient duration of 5 min. The final acetonitrile content is set to 50% (v/v) to shorten the total analysis time, having in mind the eco-friendly character of the method. The representative chromatogram obtained under selected optimal conditions was shown in Figure 7.

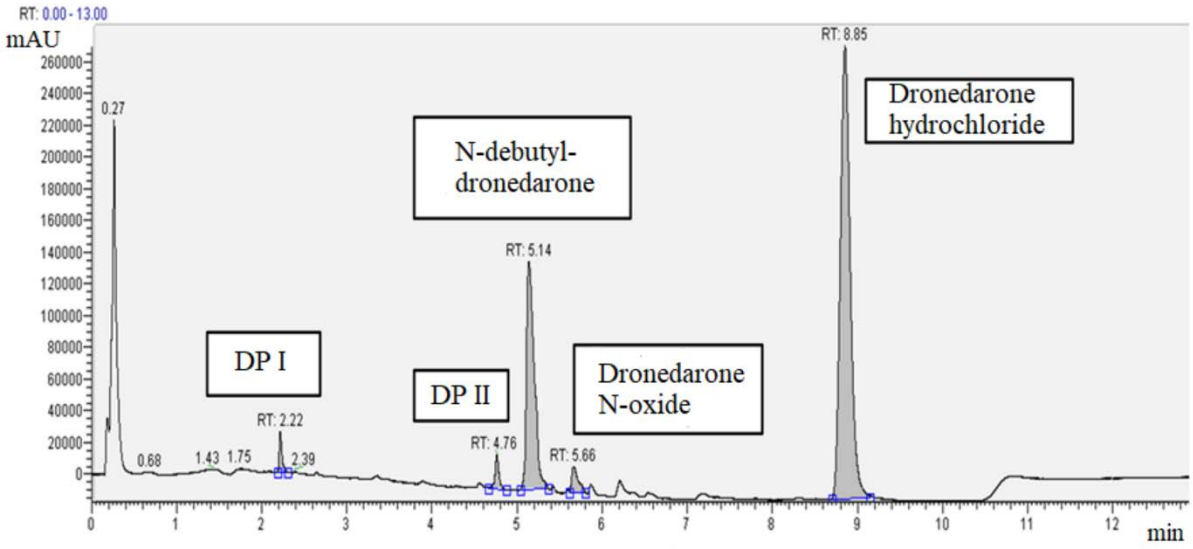

Figure 7. Representative chromatogram for the separation of dronedarone hydrochloride and its degradation products obtained under optimal conditions by the UHPLC method

Slika 7. Reprezentativni hromatogram dobijen razdvajanjem dronedaron hidrohlorida i njegovih degradacionih proizvoda pri optimalnim uslovima za UHPLC metodu

Comparison of methods' ecological acceptability according to their analytical eco-scale score and GAPI

The assignment of penalty points to every step of the analytical procedure in order to calculate analytical eco-scale scores was performed following the steps explained in the theory section. According to the GHS, Brij 35 is labelled with the signal word "warning" and has only one pictogram, while acetonitrile is labelled "danger" and has two pictograms. In contrast, ammonium acetate and β-cyclodextrin are considered safe as they do not meet the criteria for GHS classification. None of the used chemicals exceeded the amount of 10 mL per analysis. HPLC uses ≤ 1.5 kWh or > 0.5 kWh of electrical currency per sample, and therefore penalty points are assigned to this technique. On the other hand, UHPLC uses less than 0.5 kWh of electrical energy per sample and no penalty points are

assigned. All methods use a PDA detector, which spends less than 0.5 kWh of electrical energy per sample. In all three methods, the generated waste is collected and recycled, and therefore nothing is released in the environment. Moreover, the chromatographs used are hermetically closed systems. Therefore, penalty points accounting for produced waste are assigned only on the basis of its amount.

Furthermore, in order to get an insight into the critical segments of the proposed analytical methods, GAPI pentagrams for MLC, β-CD-modified RP-HPLC and UHPLC were obtained. GAPI offers a more extensive evaluation than the analytical eco-scale score, as it also takes into account sampling, preservation, transport, storage, the need for extraction and derivatization and the scale of extraction, in addition to the criteria mentioned above.

Another tool that provides a graphical and numerical representation of the estimated environmental friendliness of the method is the AGREE metric. The three methods developed were also evaluated using this tool. In the evaluation, the principles in which the three methods differed the most (principles 8–12) were weighted more heavily (a weighting value of 3) in order to achieve a greater discrepancy between the methods. The principles in relation to which all methods were similar (principles 1–6) were assigned a weighting value of 1. A weighting value of 1 was also applied to principle 7, which concerns the amount of waste, to take into account that waste is recycled. In addition to the ecological criteria evaluated by other tools, AGREE also takes into account the number of analytes determined in a single run, sample throughput, automation, and the use of chemicals from renewable sources.

Table VIII represents the calculated analytical eco-scale scores for all three developed methods. When using the analytical eco-scale score, all methods satisfied the criteria for ecological acceptability (> 75). β-CD-modified RP-HPLC (analytical eco-scale score: 92) and UHPLC (analytical eco-scale score: 93) had a slight advantage over MLC (analytical eco-scale score: 89). Although adding surfactants leads to a reduction in retention time, it should be noted that Brij 35 is considered irritant and hazardous towards the environment according to GHS, which is undesirable from the ecological perspective. Although the analytical eco-scale scores are almost the same for β-CD-modified RP-HPLC and UHPLC, they do not reflect the fact that β-CD-modified RP-HPLC uses approximately double the amount of acetonitrile in comparison to the latter and has significantly higher energy requirements. This example shows that an evaluation based solely on numerical values is not sufficient to judge or compare the eco-friendliness of the methods.

In contrast, GAPI pentagrams illustrate all segments of an analytical procedure, and critical aspects could be easily spotted and compared. GAPI pentagrams obtained in this study are represented in Figure 8. The critical aspects common to all three methods were off-line sampling and the use of non-green solvents. In the case of MLC, the amount of waste was identified as an additional critical element (Figure 8b). When discussing the eco-friendly character of β-CD-modified RP-HPLC and UHPLC having approximately equal eco-scale scores, GAPI pentagrams (Figure 8a and 8c) reveal the differences in

terms of the amount of expended energy. On that basis, an advantage could be given to the UHPLC method. Moreover, the GAPI pictograms show that the MLC method was undoubtedly inferior to the other two methods due to the significantly higher amount of waste. Although the analytical eco-scale score also takes into account the amount of waste produced, the penalization in relation to this criterion was obviously not sufficient to lead to a significant difference between MLC and other methods. On the contrary, the colour scaling applied in GAPI provided a clearer difference with respect to this criterion, with the corresponding field for the MLC method being red, while it was yellow for the other methods.

Table VIII Calculation of analytical eco-scale scores for MLC, UHPLC and β-CD modified RP-HPLC methods for separation of dronedarone hydrochloride and its degradation products

Tabela VIII Izračunavanje skora analitičke eko-skale za MLC, UHPLC i β-CD modifikovanu RP-HPLC metodu za razdvajanje dronedaron hidrohlorida i njegovih degradacionih proizvoda

MLC			**UHPLC**			**β-CD modified RP-HPLC**		
Reagents	**Amount**	**Penalty points**	**Reagents**	**Amount**	**Penalty points**	**Reagents**	**Amount**	**Penalty points**
Brij 35	9,84 mL	1	Ammonium acetate	2,65 mL	0	Acetonitrile	4,38 mL	4
Acetonitrile	2,16 mL	4	Acetonitrile	2,35 mL	4	β-CD		0
HPLC water		0	HPLC water		0	HPLC water		0
		∑ = 5			∑ = 4			∑ = 4
Instrument			**Instrument**			**Instrument**		
LC		1	UHPLC		0	LC		1
UV/vis detector		0	UV/vis detector		0	UV/vis detector		0
Waste	16 mL	5	**Waste**	5 mL	3	**Waste**	10 mL	3
Sum of penalty points		∑ = 11	**Sum of penalty points**		∑ = 7	**Sum of penalty points**		∑ = 8
Analytical eco-scale score		**100 – 11 = 89**	**Analytical eco-scale score**		**100 – 7 = 93**	**Analytical eco-scale score**		**100-8 = 92**

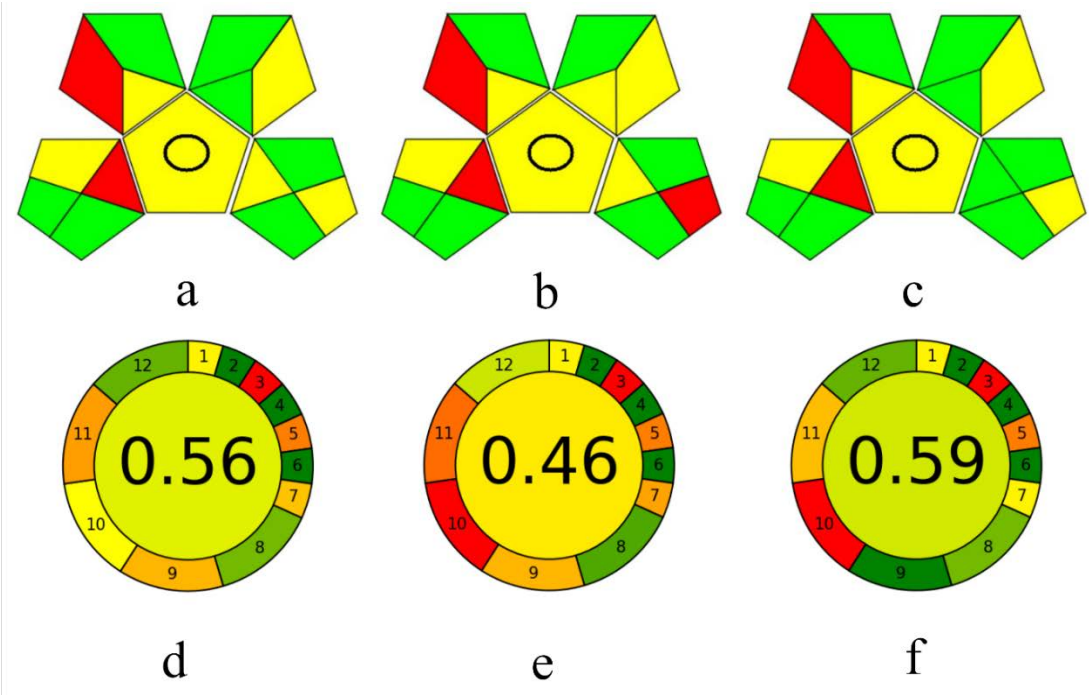

Figure 8. **GAPI pentagrams for: a) β-CD-modified RP-HPLC; b) MLC; C) UHPLC method, and AGREE pictograms for: d) β-CD-modified RP-HPLC; e) MLC; f) UHPLC method**

Slika 8. **GAPI pentagrami za: a) β-CD-modifikovanu RP-HPLC; b) MLC; C) UHPLC metodu i AGREE piktogrami za: d) β-CD-modifikovanu RP-HPLC; e) MLC; f) UHPLC metodu**

The result of the AGREE assessment was consistent with that of the previously used tools, as shown in Figure 8. From the colour in the middle of the AGREE pictograms, it can be seen that both β-CD-modified RP-HPLC and UHPLC (Figure 8d and 8f) can be considered environmentally friendly, while the MLC method (Figure 8e) is less suitable in this regard. A comparison can easily be made on the basis of the total scores achieved. In addition, the differences in the scores are more pronounced compared to the analytical eco-scale. On the other hand, as with GAPI, the edge of the pictogram indicates problematic aspects of the method. For example, the UHPLC method was problematic in relation to principles 3 and 10, which consider the positioning of the HPLC instrument in relation to the sampling location and whether the chemicals used are from renewable sources. Although the conclusions of the AGREE assessment are consistent with the conclusions of the analytical eco-scale-score and GAPI, the superiority of AGREE over other metrics was clearly demonstrated.

UHPLC is preferred over other methods due to the low amount of waste, low reagent consumption, high sample throughput and low energy requirements. However, if discussed from the perspective of industry, developing β-CD-modified RP-HPLC is more feasible since it does not require the adaptation of the instrument and it could be implemented without the need to inform regulatory authorities in advance.

Conclusion

The demand for the development of ecologically acceptable methods for the analysis of active pharmaceutical ingredients (APIs) and their related compounds arises from the mission of pharmacy to contribute to the maintenance and improvement of human health. For this reason, the effect of pharmaceuticals on human health is not the only thing that needs to be considered. The impact of all chemical substances and reagents used in the manufacture and quality control of pharmaceuticals on human health and the environment should not be neglected either. In this regard, experimental design methodology was applied in this study, which allowed the efficient optimization of MLC, β-CD-modified RP-HPLC and UHPLC for the separation of dronedarone hydrochloride and its degradation products. Different strategies for "greening" were tested and evaluated using the analytical eco-scale score, GAPI and AGREE. The applied metrics showed that the β-CD-modified RP-HPLC and UHPLC methods can both be considered ecologically acceptable, while GAPI pentagrams and AGREE slightly favour the UHPLC method due to energy savings. However, the modification of mobile phase with an addition of β-CD is more easily applied in industry, as it does not require the upgrade of the existing equipment, and also offers satisfying ecological acceptability of the method. In addition, among the tools used to assess greenness, AGREE proved to be the most appropriate, as it is sensitive to differences between the HPLC methods developed and provides comprehensive information on all environmental aspects of the methods.

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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

Nevena Djajić: Investigation, Formal analysis, Software, Methodology, Writing – original draft. **Jovana Krmar:** Investigation, Methodology. **Jevrem Stojanović:** Validation, Methodology, Writing – original draft. **Bojana Svrkota:** Software, Writing – review & editing, Visualization. **Biljana Otašević**: Writing – original draft, Formal analysis, Methodology. **Anđelija Malenović:** Supervision, Writing – review & editing. **Ana Protić:** Conceptualization, Supervision, Resources, Writing – review & editing.

References

1. Płotka J, Tobiszewski M, Sulej AM, Kupska M, Górecki T, Namieśnik J. Green chromatography. Journal of Chromatography A. 2013 Sep 13;1307:1–20.
2. Welch CJ, Wu N, Biba M, Hartman R, Brkovic T, Gong X, et al. Greening analytical chromatography. TrAC Trends Anal Chem. 2010 Jul 1;29(7):667–80.
3. Görög S. The changing face of pharmaceutical analysis. TrAC Trends Anal Chem. 2007 Jan 1;26(1):12–7.
4. Kazakevich YV, LoBrutto R. HPLC for Pharmaceutical Scientists [Internet]. Wiley; 2007 [cited 2024 Apr 5]. Available from: https://books.google.rs/books?id=csAk-MpeTocC.
5. Armenta S, Garrigues S, de la Guardia M. Green Analytical Chemistry. TrAC Trends Anal Chem. 2008 Jun 1;27(6):497–511.
6. Cielecka-Piontek J, Zalewski P, Jelińska A, Garbacki P. UHPLC: The Greening Face of Liquid Chromatography. Chromatographia. 2013 Nov 1;76(21):1429–37.
7. Yabré M, Ferey L, Somé IT, Gaudin K. Greening Reversed-Phase Liquid Chromatography Methods Using Alternative Solvents for Pharmaceutical Analysis. Molecules. 2018;23(5):1065.
8. Shaaban H. New insights into liquid chromatography for more eco-friendly analysis of pharmaceuticals. Anal Bioanal Chem. 2016 Oct 1;408(25):6929–44.
9. Tobiszewski M, Mechlińska A, Namieśnik J. Green analytical chemistry—theory and practice. Chem Soc Rev. 2010;39(8):2869–78.
10. Ligor M, Studzińska S, Horna A, Buszewski B. Corona-Charged Aerosol Detection: An Analytical Approach. Crit Rev Anal Chem. 2013 May 1;43(2):64–78.
11. Maljurić N, Otašević B, Golubović J, Krmar J, Zečević M, Protić A. A new strategy for development of eco-friendly RP-HPLC method using Corona Charged Aerosol Detector and its application for simultaneous analysis of risperidone and its related impurities. Microchem J. 2020 Mar 1;153:104394.
12. Koel M. Do we need Green Analytical Chemistry? Green Chem. 2016;18(4):923–31.
13. Maljurić N, Golubović J, Otašević B, Zečević M, Protić A. Quantitative structure – retention relationship modeling of selected antipsychotics and their impurities in green liquid chromatography using cyclodextrin mobile phases. Anal Bioanal Chem. 2018 Apr 1;410(10):2533–50.
14. Chadha R, Bali A, Bansal G. Identification and characterization of stress degradation products of dronedarone hydrochloride employing LC-UV/PDA, LC–MS/TOF and MSn studies. J Pharm Biomed Anal. 2016 Jan 25;118:139–48.
15. Sahu PK, Ramisetti NR, Cecchi T, Swain S, Patro CS, Panda J. An overview of experimental designs in HPLC method development and validation. J Pharm Biomed Anal. 2018 Jan 5;147:590–611.
16. Hibbert DB. Experimental design in chromatography: a tutorial review. J Chromatogr B Analyt Technol Biomed Life Sci. 2012 Dec 1;910:2–13.
17. Mohamed D, Fouad M. Application of NEMI, Analytical Eco-Scale and GAPI Tools for Greenness Assessment of Three Developed Chromatographic Methods for Quantification of Sulfadiazine and Trimethoprim in Bovine Meat and Chicken Muscles: Comparison to Greenness Profile of Reported HPLC Methods. Microchem J. 2020 Apr 1;157:104873.
18. Gałuszka A, Migaszewski ZM, Konieczka P, Namieśnik J. Analytical Eco-Scale for assessing the greenness of analytical procedures. TrAC Trends Anal Chem. 2012 Jul 1;37:61–72.

19. Płotka-Wasylka J. A new tool for the evaluation of the analytical procedure: Green Analytical Procedure Index. Talanta. 2018 May 1;181:204–9.
20. Tobiszewski M. Metrics for green analytical chemistry. Anal Methods. 2016;8(15):2993–9.
21. Pena-Pereira F, Wojnowski W, Tobiszewski M. AGREE—Analytical GREEnness Metric Approach and Software. Anal Chem. 2020 Jul 21;92(14):10076–82.
22. El-Shaheny RN, El-Maghrabey MH, Belal FF. Micellar Liquid Chromatography from Green Analysis Perspective. 2015;13(1). doi: 10.1515/chem-2015-0101.
23. Otašević B, Protić A, Golubović J, Zečević M. The application of ecologically acceptable concept in liquid chromatographic method development in drug analyses. Arhiv za farmaciju. 2015;65(3):178–90.
24. Sljivić J, Protić A, Malenović A, Otašević B, Zečević M. Simple and Efficient Solution for Robustness Testing in Gradient Elution Liquid Chromatographic Methods. Chromatographia. 2018;81(8):1135–45.

Koncept ekološki prihvatljivih hromatografskih metoda: Studija slučaja na primeru razdvajanja dronedaron hidrohlorida i njegovih degradacionih proizvoda

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Kratak sadržaj

U poslednje vreme, zabrinutost za negativan uticaj metoda koje se koriste u analitici lekova na životnu sredinu je u značajnom porastu. Reverzno-fazna tečna hromatografija visokih performansi (RP-HPLC) kao dominantno korišćena tehnika u velikoj meri se oslanja na primenu organskih rastvarača, poput acetonitrila, koji je poznat po hromatografskoj efikasnosti, ali i po toksičnosti i zapaljivosti. Kako bi se ovi problemi rešili i zaštitilo zdravlje ljudi i životna sredina, neophodno je upotrebu toksičnih organskih rastvarača svesti na minimum. Cilj ovog istraživanja bio je da preporuči „zelenije“ modifikacije RP-HPLC metoda. Primenom eksperimentalnog dizajna razvijene su metode za razdvajanje dronedaron-hidrohlorida i njegovih degradacionih proizvoda, uključujući micelarnu tečnu hromatografiju (MLC), RP-HPLC metodu modifikovanu β-ciklodekstrinom (CD) i tečnu hromatografiju ultra visokih performansi (UHPLC). Ekološka prihvatljivost ovih metoda je procenjena korišćenjem analitičke eko-skale, indeksa zelene analitičke procedure (GAPI) i pristupa analitičke zelenosti (AGREE). AGREE se izdvojio kao najpogodniji, jer je pokazao najveće razlike između navedenih metoda, kao i uvid u kritične aspekte metoda. UHPLC i β-CD modifikovana RP-HPLC metoda su se pokazale superiornim u odnosu na MLC. Koja metoda će biti metoda izbora zavisi od toga da li se lakoća implementacije ili energetska efikasnost smatraju važnijim kriterijumom.

Ključne reči: RP-HPLC, MLC, UHPLC, ciklodekstrinom modifikovana HPLC, procena ekološke prihvatljivosti