Macedonian Journal of Chemistry and Chemical Engineering, Vol. **42**, No. 2, pp. xx–xx (2023)

MJCCA9 –

ISSN 1857-5552
e-ISSN 1857-5625

Received: March 29, 2023
Accepted: June 26, 2023

DOI: 10.20450/mjcce.2023.2683
Original scientific paper

ECO-FRIENDLY RP-HPLC METHOD FOR DETERMINATION OF DIAZEPAM IN COATED TABLET

Marija Tomić, Marijana Božinovska[1], Nataša Anevska-Stojanovska[1], Jelena Lazova[1], Rumenka Petkovska[2], Liljana Anastasova[2], Natalija Nakov[2*]

[1]*ALKALOID AD Skopje, Blvd. Aleksandar Makedonski 12, 1000 Skopje, North Macedonia*
[2]*Institute of Applied Chemistry and Pharmaceutical Analyses, Faculty of Pharmacy, Ss. Cyril and Methodius University in Skopje, Mother Theresa 47, Skopje, North Macedonia*
natalijan@ff.ukim.edu.mk

A new eco-friendly RP-HPLC method for determining diazepam in coated tablets was developed and validated. The separation was achieved on a Lichrosper® 100 RP-18 (250 mm × 4 mm, 5 µm particle size) using the isocratic elution mode with a mobile phase composed of a mixture of water (H_2O) and ethanol (EtOH) in a 40:60 (v/v) ratio and a flow rate of 1.0 ml/min. The injection volume was 10 µl, and the detection wavelength was set at 254 nm. The column temperature was maintained at 35 ºC. The method was validated according to the ICH guideline, emphasizing selectivity/specificity, linearity, sensitivity, accuracy, precision, and robustness. The analytical method greenness score and Eco-scale approach were used for the method's greenness assessment. The method was applied to determine the assay of diazepam and the uniformity of dosage units by content uniformity.

Applying the method proposed in this study in the pharmaceutical industry is considered environmentally sustainable. It would bring benefits in terms of a safer working environment and reduction of toxic waste formation without compromising the reliability of the analytical results.

Keywords: green/eco-friendly analytical methods; reversed-phase liquid chromatography; ethanol; diazepam; pharmaceutical analysis

ЕКОЛОШКИ ПРИФАТЛИВ RP-HPLC МЕТОД ЗА ОПРЕДЕЛУВАЊЕ НА ДИАЗЕПАМ ВО ОБЛОЖЕНА ТАБЛЕТА

Развиен е и проверен нов, еколошки прифатлив RP-HPLC-метод за определување на диазепам во обложена таблета. Разделувањето беше извршено на Lichrosper® 100 RP-18 (250 mm × 4 mm, 5 µm) со изократско елуирање користејќи мобилна фаза составена од смеса на вода (H_2O) и етанол (EtOH) во сооднос 40:60 (*v/v*) и проток од 1,0 ml/min. Волуменот на инјектирање беше 10 µl, а брановата должина на детекција 254 nm. Температурата на хроматографската колона беше одржувана на 35 ºC. Методот беше валидиран во согласност со Водичот на ICH во однос на селективноста/специфичноста, линеарноста, осетливоста, точноста, прецизноста и робустноста. За евалуација на еколошката прифатливост на развиениот метод беа користени пристапите за процена на зелен индекс на аналитички метод (Analytical method greenness score approach) и процена на ранг на еколошка подобност (Eco-scale approach). Предложениот метод е еколошки и економски одржлив. Придобивките од примената на развиениот еколошки прифатлив RP-HPLC метод во фармацевтската индустрија се однесуваат на обезбедување поздрава работна средина, како и намалено формирање токсичен отпад без притоа да се компромитира валидноста на аналитичкиот резултат.

Клучни зборови: еколошки прифатливи аналитички методи;
реверзно-фазна течна хроматографија; етанол; диазепам; фармацевтски анализи

1. INTRODUCTION

Green analytical chemistry (GAC) focuses on minimizing the usage of toxic organic solvents, employing energy-efficient equipment, and generating minimal waste.[1] The implementation of the GAC principles in the process of green chromatographic method development, as well as the transfer of the conventional HPLC methods into eco-friendly solutions, is an essential part of the development strategy in the pharmaceutical industry.[2] In particular, GAC principles play a crucial role in the modern industry's development strategy. Reverse phase high-performance liquid chromatography (RP-HPLC) is the most commonly employed technique in pharmaceutical quality control. However, it requires significant quantities of organic solvents as eluents in the mobile phase. It is well known that methanol (MeOH) and acetonitrile (ACN), the most commonly used organic solvents in the RP-HPLC mobile phases, are toxic and lead to both acute and chronic harmful effects. Moreover, the negative ecological influence of these solvents shouldn't be neglected because of the enormous amount of chemical waste. Namely, the chemical waste generated annually by HPLC instruments worldwide is estimated at 34 million liters.[2,3]

Benzodiazepines play a crucial role in treating chronic psychiatric disorders, anxiety and insomnia. They have largely replaced other medications used for these indications. Diazepam (DZP) (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one) is a long-acting benzodiazepine with anxiolytic, sedative, muscle relaxant, anticonvulsant, and amnestic properties. This drug is manufactured by different pharmaceutical companies worldwide due to its designation as an essential drug on the World Health Organization Model List of Essential Medicines–22nd List, 2021.[4–6]

The literature review revealed several conventional ("non-green") HPLC methods that have been used for the separation and quantitative determination of different benzodiazepines in pharmaceutical formulations or biological samples.[7–11] There are few conventional RP-HLC methods for determining diazepam content in coated tablets, but their main disadvantage is the use of toxic organic solvents such as ACN and MeOH in the mobile phase.[12,13] To the author's knowledge, only two eco-friendly micellar liquid chromatography (MLC) methods have been reported to simultaneously determine diazepam with other benzodiazepines or other drugs in pharmaceutical dosage forms.[4,10] Both methods utilize a micellar mobile phase, which can have certain disadvantages, such as weak elution strength, especially when used with chromatographic columns of typical pore size and reduced efficiency.[14] Also, these methods require longer system equilibration time, and surfactant precipitation and crystal formation can occur around the pump or in the column.[15] Given the nature of the work in the pharmaceutical industry, applying an MLC method for drug quality control would be time-consuming. The European Pharmacopoeia (Ph. Eur) monograph for DZP active substance describes potentiometric titration as a method for assay determination.[16] On the other hand, the official United States Pharmacopeia (USP) monograph provides a conventional RP-HPLC method for the determination of diazepam content in coated tablets using a mobile phase composed of a mixture of ACN:H_2O:MeOH (40:40:20, *v/v/v* %).[17]

In the pharmaceutical industry, it is essential for quality control laboratories to align with current trends and implement the principles of green analytical chemistry (GAC). Considering the great demand in the market for this drug, the number of manufactured and controlled batches worldwide is substantial. Thus, the conventional methods for DZP determination generate significant amounts of toxic waste. Hence, there is a growing interest in greening the conventional reverse-phase high-performance liquid chromatography (RP-HPLC) methods for DZP analysis within the pharmaceutical industry. The aim is to safeguard the health of operators and protect the environment while maintaining chromatographic performance and avoiding the need for expensive equipment.[18]

Therefore, our study aimed to develop and validate a simple RP-HPLC method for determining DZP in coated tablets using a green (ethanol-based) mobile phase which could be used as an eco-friendly alternative in routine analysis of medicinal drug products. The "greenness" of the method was assessed using two approaches: the Analytical Method Greenness Score (AMGS) and the Eco-Scale Approach (ESA). The results were compared with the conventional USP method (used as a reference method) and the previously reported green MCL method for DZP determination.

2. MATERIALS AND METHODS

2.1. *Chemicals and standards*

Diazepam, a secondary reference standard, was provided by Alkaloid AD Skopje. HPLC grade acetonitrile, methanol, ethanol, sodium dihydrogen phosphate monohydrate, and ortho-phosphoric acid

(99.0%) were purchased from Merck (Darmstadt, Germany). Water was purified by a Werner water purification system, obtained in-house at Alkaloid AD Skopje. Regenerated cellulose membrane syringe filters (RC), pore size 0.45 μm, were purchased from Phenomenex (Torrance, CA. USA).

Diazepam 5 mg coated tablets produced by Alkaloid AD Skopje were used to test method applicability.

2.2. *Instrumentation*

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

A reverse phase column, Lichrosper® 100 RP-18 (250 mm × 4 mm, 5 μm particle size) (Agilent Technologies, USA), was used for method development and validation.

2.3. *Chromatographic conditions*

All analyses were carried out on a Lichrosper® 100 RP-18 (250 mm × 4 mm, 5 μm particle size) chromatographic column using isocratic elution with a mobile phase composed of a mixture of H_2O and ethanol (EtOH) in a ratio of 40:60 (*v/v* %) and a flow rate of 1.0 ml/min. The column temperature was maintained at 35 ºC, while the autosampler temperature was maintained at 5 ºC. The injection volume was set at 10 μl, and analyte peaks were monitored at a wavelength of 254 nm. The analysis time was 6 minutes.

2.4. *Standard and test solutions*

A standard solution of DZP at a concentration of 0.1 mg/ml was prepared in the mobile phase, composed of a mixture of H_2O and ethanol (EtOH) in a ratio of 40:60 (*v/v* %), as solvent. To prepare the test solution for assay determination, samples of 10 randomly selected tablets were transferred into volumetric flasks, dissolved, and diluted to the appropriate volume to get a final concentration of 0.1 mg/ml. To the test solution for content uniformity (CU), one randomly selected coated tablet was transferred, dissolved, and diluted to the appropriate volume to get a concentration of 0.1 mg/ml using the mobile phase as solvent. The same procedure was repeated for another nine tablets. The standard and test solutions for assay and content uniformity determination were filtered through 0.45 μm membrane filters (regenerated cellulose, RC).

2.5. *Reference method*

The eco-friendly RP-HPLC method developed in this study was compared with the method for determining DZP content in coated tablets in the official USP monograph as a reference method.[17]

The USP method was performed using a Lichrosper® 100 RP-18 (150 mm × 3.9 mm, 5 μm particle size) column with a mobile phase composed of H_2O:ACN:MeOH (40:40:20, *v/v/ %*) at a flow rate of 1 ml/min. The column temperature was maintained at 25 ºC. The injection volume was set at 20 μl, and analyte peaks were monitored at a wavelength of 254 nm. The autosampler temperature was maintained at 5 ºC. Under these proposed conditions, the analysis time was 12 minutes.

2.6. *Method validation*

The method was validated according to the ICH Guideline Q2A.[19] The method's robustness was evaluated by 11 experiments using the Plackett-Burman design, where three factors were analyzed at two levels (MODDE® software, Umetrics, Umea, Sweden). The most important factors that influenced the system analytical response were the mobile phase flow rate (in the range of 0.8–1.2 ml/min), the column temperature (in the range of 30–40 °C), and the ethanol percentage in the mobile phase (in range of 55–65, *v/v* %). The method's robustness was assessed by the following criteria: peak symmetry, retention time, and number of theoretical plates for the DZP peak. Additionally, the sweet spot diagram was used to examine the method's robustness.

2.7. *Assessment of the greenness of the method*

The greenness of the developed method in the study was evaluated using two assessment approaches, the Analytical Method Greenness Score (AMGS)[20] and the Eco-Scale Approach (ESA)[21]. The AMGS covers several factors, such as the type of technique used, analysis time, type and volume of organic solvents used, mobile phase composition, and other parameters relevant to the method and sample preparation. Lower values for the AMGS indicate that the method is eco-friendlier. Additionally, this online tool provides information

about the instrument energy score and solvent energy score as well as the solvent environmental, health, and safety (EHS) impact score. The Eco-Scale approach (ESA) evaluates the same parameters as the AMGS and calculates penalty points which are used for the eco-scale calculation according to the following formula:

Analytical Eco-Scale (AES) = 100 – total penalty points,

where the Analytical Eco-Scale (AES) is calculated by subtracting the total penalty points from 100. The lower the total penalty points, the higher the Analytical Eco-Scale score, indicating a greener method. When the result is closer to 100 points, it indicates a greener analytical method.[21] These results facilitate a straightforward comparison with the reference methods used in routine work.

3. RESULTS AND DISCUSSION

Considering the green chemistry fundamentals,[18] the focus of this study was to develop an eco-friendly RP-HPLC method for the determination of DZP in coated tablet aligned with the GAC concept. To achieve this, the first step of the method development was to replace the organic solvents (MeOH and ACN) in the mobile phase of the conventional (referent) USP method with EtOH, an environmentally friendly solvent.[2,3] Considering that the organic solvent content in the reference method was 60 % and EtOH has higher elution strength than ACN and MeOH, the preliminary investigations started with the mobile phase containing 50 % EtOH. Considering that the pK_a value for diazepam is 3.4,[5] and the pH value of the mobile phase (H_2O: EtOH, 50:50, *v/v* %) is 6.5, these chromatographic conditions allowed for ionization of the DZP molecule with acceptable retention.

The retention time of the DZP peak obtained with the above-mentioned eco-friendly mobile phase (H_2O:EtOH, 50:50, *v/v* %) is similar to the retention time obtained with the reference USP method, implying that the proposed "green mobile phase" has elution power comparable with the conventional mobile phase. Although the number of theoretical plates (*N*) was satisfactory, the peak symmetry was higher than 1.2 (1.27). Changes in ethanol content and the column temperature didn't improve the peak symmetry. In order to address the issue of peak symmetry, two other chromatographic columns were investigated: Waters Spherisorb (125 mm × 4.0 mm, 5 μm) and Lichrosper® 100 RP-18 (250 mm × 4.0 mm, 5 μm). The values for peak symmetry obtained using the Spherisorb column were above 1.2, similar to those obtained with the first column used. The values for DZP peak symmetry obtained using the Lichrosper® 100 RP-18 (250 mm × 4.0 mm, 5 μm particle size) fulfilled the pharmacopeia's system suitability criteria (peak symmetry below 1.2). Notably, despite the literature data suggesting that EtOH mobile phase may result in higher column backpressure,[2] in this study, the column pressure remained within acceptable limits (200 bar). The DZP peak had a comparable peak symmetry and number of theoretical plates as the reference USP method. Thefore, this column was used for further method development. Increasing the EtOH content in the mobile phase to 60 % (H_2O:EtOH, 40:60, *v/v* %) resulted in further improvement of the peak symmetry and an increase in the number of theoretical plates. The experiments indicated that EtOH exhibits chromatographic behavior similar to ACN. The column temperature was also optimized. Increasing the column temperature by 10 °C (from 25 °C to 35 °C) resulted in shorter retention time for DZP and reduced column backpressure.

A comparison among the system suitability criteria for the developed eco-friendly method in our study, the reference USP method, and the MLC method for determination of DZP described by Elmasi and Belal[4] are presented in Table 1.

T a b l e 1

Comparison of the system suitability criteria

Chromatographic parameters	System suitability criteria	Conventional USP method	EtOH-based method developed in our study	Green (MLC) method[4]
Retention time (min)	3–8	3.3	4.3	8.5
Peak symmetry	0.8–1.2	1.17	1.12	1.2
Number of theoretical plates (N)	≥ 2000	4586	4175	858

The results from the comparison revealed that all system suitability criteria of the EtOH-based, eco-friendly method comply with the criteria given by the reference USP method. Furthermore, method performance was compared with the "green" MLC method proposed by Elmansi & Belal.[4] The retention time of the DZP peak in the method developed in our study is shorter, which results in a shorter analysis time and reduced consumption of organic solvents. The peak symmetry values are comparable with the evaluated methods. Moreover, the MLC method is unsuitable for routine quality control in the pharmaceutical industry due to several drawbacks. One of the limitations is the longer analysis time required by the MLC method compared to the developed eco-friendly EtOH-based method. Longer analysis time can be impractical in a routine laboratory setting where efficiency and productivity are important factors. Additionally, the MLC method requires special considerations to prevent crystal formation and instrument damage. These considerations may involve additional steps or precautions that need to be taken during the analysis, which can increase complexity and introduce potential risks or complications.[15]

Given these limitations, the developed eco-friendly RP-HPLC method using an EtOH-based mobile phase is a more viable and practical option for routine drug quality control in the pharmaceutical industry. It provides results comparable to the reference method while offering the advantage of reduced organic solvent consumption and shorter analysis time. An overview of the key information for the proposed EtOH-based eco-friendly RP-HPLC method for determining DZP is given in Table 2.

T a b l e 2

Overall information for the proposed EtOH-based eco-friendly method

Parameters of interest	Proposed EtOH-based method
Retention time (min)	4.3
Peak symmetry	1.12
Theoretical plates	4175
Column backpressure (bar)	~ 200
Analysis time (min)	6
Instrument consumption of organic solvent for routine analysis	~ 40 ml
LOQ (mg/ml)	0.004
Eco-scale score	82
AMGS score	1432.4

3.1. *Method validation*

In this study, an EtOH-based eco-friendly method for determining DZP content in coated tablets was developed and validated with respect to selectivity/specificity, linearity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ). Special attention was given to investigating the method robustness, which is one of the most relevant parameters for method transfer and its potential application in routine analysis.

Selectivity/specificity. Representative chromatograms of solvent, placebo solution, standard solution of DZP, and test solutions (for assay determination and content uniformity determination) are presented in Figures 1A, 1B, 1C, 1D, and 1E, respectively. Comparing the representative chromatograms of solvent, placebo solution, standard solution of DZP, and test solutions revealed that there are no peaks from the solvent or the placebo that interfere with the elution of DZP.

Linearity and range. The linearity of the method was evaluated in the range between 0.05 and 0.15 mg ml^{-1} for DZP (50 % – 150 % of the working concentration). Each measurement was performed in triplicate, and the results were evaluated by linear regression analysis using the least squares regression method. The correlation coefficient was greater than 0.999 (y = 520.79x + 0.2507); thus, the results indicated very good linearity. This high correlation coefficient suggests that there is a strong linear relationship between the concentration of DZP and the corresponding peak area response.

Precision. The precision of the method was evaluated through repeatability (system and method repeatability) and intermediate precision. The system repeatability was evaluated by six determinations of the peak areas of the standard solution containing DZP. The RSD value obtained for system repeatability was 0.2 %. The method repeatability analysis demonstrated RSD values below 2.0 %, indicating that the method is precise. The intermediate precision, conducted on two consecutive days by two different analysts and on two different HPLC systems, showed that the RSD value of the entire determination was not more than 2%. This is acceptable for intermediate precision, thus indicating that the method is precise. The results for the linearity and the precision of the method are presented in Table 3.

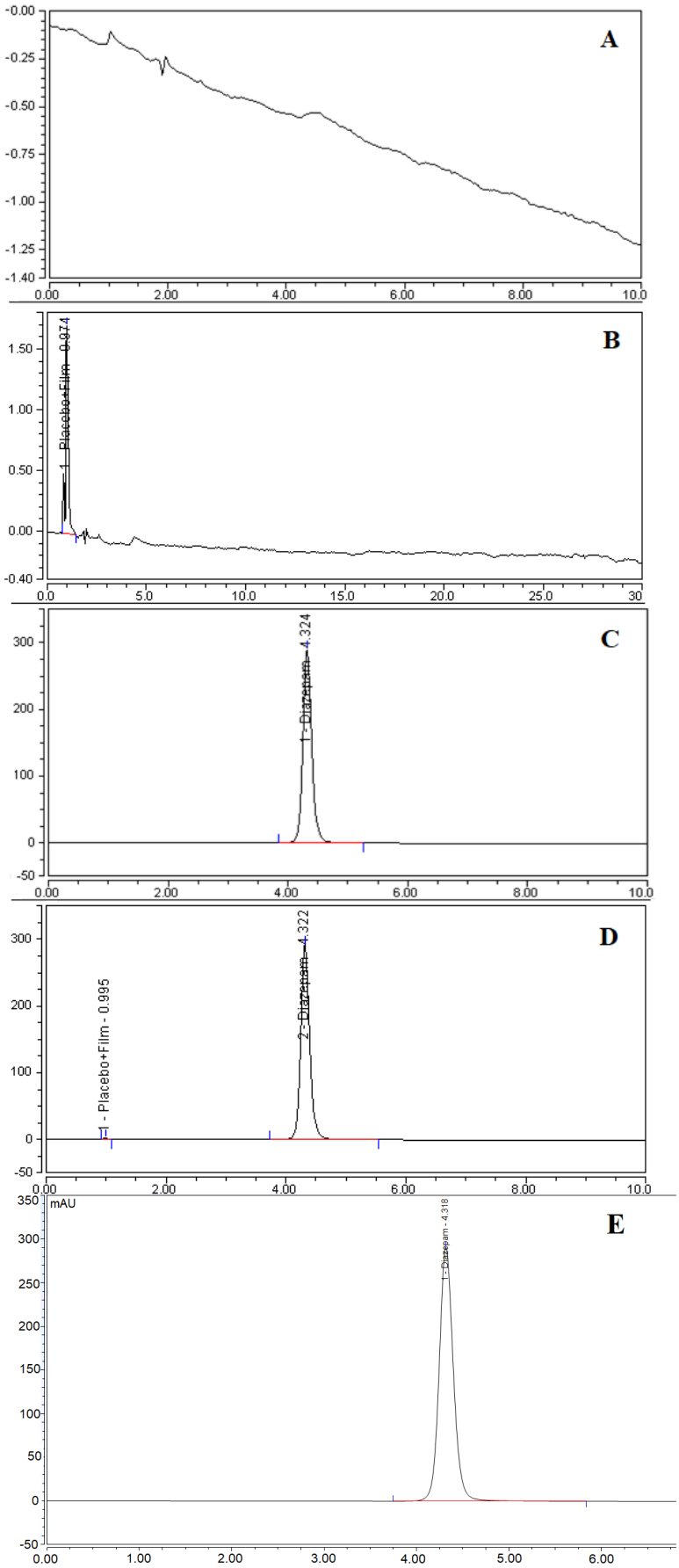

Fig. 1. Representative chromatograms of (A) Solvent, (B) Placebo, (C) Standard solution, (D) Test solution for assay determination, and (E) Test solution for content uniformity determination

T a b l e 3

Results from testing the linearity and precision of the method

Validation parameter	DZP
Linearity	
Concentration range (mg/ml)	0.05–0.15
Correlation coefficient	0.9999
Intercept	0.2507
Slope	520.79
Precision (RSD %)	
Repeatability	
100 % (n = 6)	1.0
Intermediate precision (n = 6)	
Analyst 1	1.0
Analyst 2	0.8

Accuracy. The accuracy of the method was evaluated through a study of analytical recovery at three concentration levels of DZP in spiked placebo from tablets. The results are shown in Table 4. The percentages of the recoveries are in the range of 99.73–100.3 %, indicating good accuracy of the method.

Limit of detection (LOD) and Limit of quantification (LOQ). These parameters were assessed through determination of the signal-to-noise (*S/N*) ratio by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected and quantified. From data obtained for the *S*/*N* ratio, it can be concluded that the LOD for the active compound is 0.002 mg/ml (*S/N* ratio 3:1). At the concentration level of 0.004 mg/ml (*S*/*N* ratio 11.3:1), it could be quantified to an acceptable degree of precision.

T a b l e 4

Accuracy of the developed "green" method for DZP determination in coated tablets

	Concentration level (%)	Added (mg/ml)	Determined (mg/ml)	Recovery (%) P = 95 %
DZP	50	0.05	0.05	100.03 ± 0.5
	100	0.10	0.10	100.3 ± 0.72
	150	0.15	0.15	99.73 ± 0.51

*Results are expressed as mean ± RSD (%) for three determinations at each level

Robustness testing. The Plackett-Burman design is one of the most used designs because it can handle a large number of factors in a relatively small number of experiments.[22] It is well suited to robustness testing, i.e., establishing whether the outcome of an analytical procedure is affected by changes in each relevant factor. The most important feature of Plackett-Burman designs is that it involves 4n experiments, where n = 1, 2, 3… In each case, the maximum number of factors that can be studied is 4n − 1, so an 8-experiment Plackett-Burman design can study not more than 7 factors, a 12-experiment design will handle up to 11 factors, and so on. Based on prior knowledge and literature evaluation, the content of ethanol in the mobile phase (%, *v/v*), flow rate, and column temperature were chosen as factors for robustness testing. The factors' lower and upper values were defined symmetrically around the nominal level. According to the Plackett-Burman design, the robustness evaluation involved 11 experiments. The matrix of the planned experiments, along with the results obtained for system suitability parameters, are presented in Table 5. The monitored chromatographic parameters under all deliberately varied chromatographic conditions were within the acceptance criteria for system suitability.

A sweet spot diagram was created to facilitate the visualization of the interactions between the investigated factors and their individual effect. This way of presentation provides a good, informative, and simple presentation of the experimental results. The sweet spot diagram was created by simultaneously changing the selected factors. The color of the sweet spots can vary from green to blue, whereby the green color indicates the area where all responses are within the selected range, the blue color indicates the areas where one of the answers is in the selected range, and the white color refers to areas where none of the answers are within the selected range. The obtained sweet spot diagram for the robustness testing (Fig. 2) is green in the entire range of tested conditions, demonstrating the method's robustness.

T a b l e 5

Plackett-Burman design for robustness testing of the "green" method for DZP determination

Experiment N°	Flow rate (ml/min)	Column temperature (°C)	EtOH content (%, *v/v*)	Rt (min)	Asymmetry factor	NTP
Nominal value	1.0	35	60	4.3	1.12	4175
N1	0.8	30	55	7.90	1.12	5066
N2	1.2	30	55	5.54	1.11	3803
N3	0.8	40	55	7.90	1.13	5292
N4	1.2	40	55	5.30	1.08	3971
N5	0.8	30	65	5.12	1.11	5033
N6	1.2	30	65	3.46	1.08	3775
N7	0.8	40	65	5.06	1.11	5316
N8	1.2	40	65	3.42	1.10	3965
N9	0.8	30	55	7.93	1.12	5068
N10	0.8	30	55	7.95	1.12	5071
N11	0.8	30	55	7.96	1.13	5081

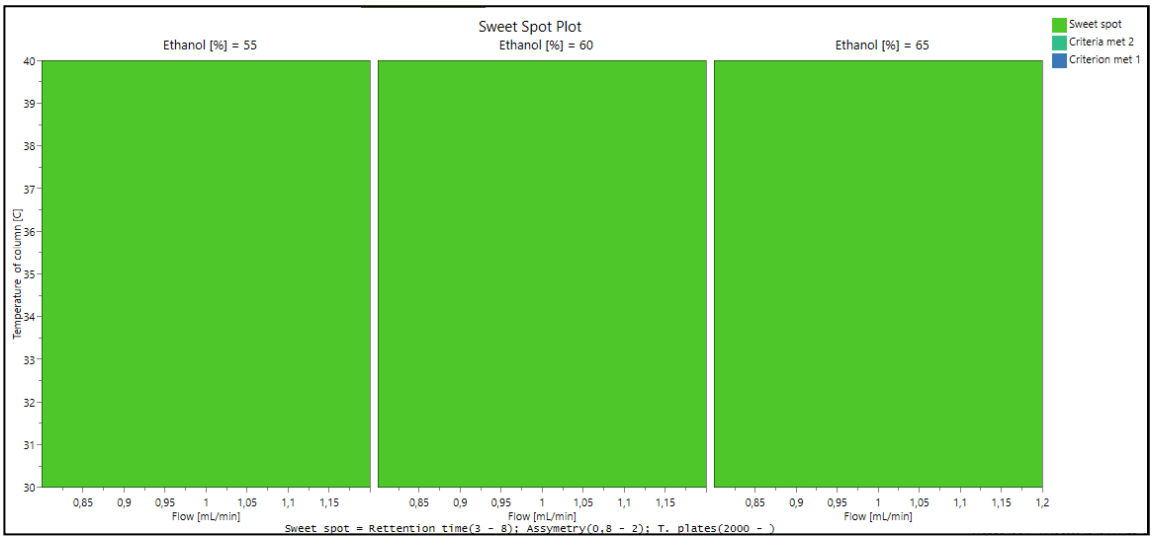

Fig. 2. Sweet spot diagram for robustness testing of EtOH-based RP-HPLC method for DZP determination in coated tablets

The model was validated by the analysis of variance (ANOVA). The statistical analysis indicated that the model was a good fit for the experimental data, allowing prediction of the effects of the factors on the selected responses. The percent of variation of the response explained by the model, known as R-squared *(R^2),* was greater than 0.5 in all cases, and the prediction ability of the model, *Q*-squared *(Q^2),* was also greater than 0.5, indicating that the model reasonably fits the experimental data. Overall, the validation through ANOVA demonstrated that the model adequately represents the relationship between the factors and responses, with a substantial portion of the response variation explained by the model and a good ability to predict responses.

3.2. *Application of the eco-friendly method*

The proposed method's applicability was assessed by determining the DZP content in manufactured batches of diazepam 5 mg coated tablets. According to the ICH *Q^2A* Guideline and common practice, a method's precision is assessed using six samples from the same industrial batch. The samples were analyzed using both the reference (USP) method and the proposed eco-friendly method (Table 6). The results obtained from both methods were compared and statistically evaluated. The statistical evaluation of the results using the Student T-test and the variance ratio F-test indicated that there was no statistically significant difference between the performance of

the reference and the eco-friendly method in terms of accuracy and precision (Table 6). This suggests that the proposed method is applicable to determining DZP content in the manufactured batches of diazepam coated tablets (Table 6).

T a b l e 6

Results from the determination of DZP in production batches of DZP coated tablets using the reference USP method and the newly developed EtOH-based "eco-friendly" RP-HPLC method

	DZP content (%) reference USP method		DZP content (%) "green" HPLC method
1	102.4		101.0
2	99.5		102.9
3	101.0		101.0
4	100.6		100.1
5	102.6		101.9
6	100.6		101.9
Average (%)	101.1		101.5
Variance	1.4		1.0
T-test		–0.5	
T-critical		2.6	
F-value		1.5	
F-critical		5.1	

T a b l e 7

Results from the determination of uniformity of dosage units by content uniformity in a production batch of DZP coated tablets using the newly developed EtOH-based "eco-friendly" RP-HPLC method

Sample	Content uniformity (%)
Tablet 1	102.0
Tablet 2	101.4
Tablet 3	102.2
Tablet 4	100.9
Tablet 5	100.6
Tablet 6	101.6
Tablet 7	101.6
Tablet 8	103.0
Tablet 9	100.0
Tablet 10	100.1
Average	101.3
RSD	0.9
AV	2.3

The proposed eco-friendly method, in accordance with the general monograph 2.9.40 Ph. Eur. 11,[16] was further applied to determine uniformity of dosage units by content uniformity in a production batch of DZP 5 mg coated tablets. The acceptance value (AV) obtained was 2.3. Thus, this production batch met the Ph. Eur. requirement (maximal value of AV = 15.0) for uniformity of dosage units (Table 7).

3.3. *Assessment of the greenness of the proposed method*

Alongside the eco-friendly method development, it is also important to evaluate the greenness of the developed method and compare it with the existing ones.[2] The results from comparing the proposed method and the reference USP method using the AMGS tool are presented in Figure 3A and 3B, respectively. The AMGS calculated for the reference USP method is 4759.01, whereas the AMGS calculated for the EtOH-based method proposed in our study is 1432.4, which implies that the newly developed method is far more eco-friendly compared to the reference method. Additionally, the solvent energy score of the reference USP method is not acceptable in terms of eco-friendliness.

The AMGS tool was not applicable for the assessment of the greenness score of the MLC method found in the literature[4] because the data for the solvent used for the MLC mobile phase (surfactant) could not be imported into the calculation tool. Therefore, a comparison of the greenness of the proposed EtOH-based method with the MLC method was assessed using the Eco-scale approach (Table 8). Considering the high value for the AMGS score of the reference method, the Eco-scale approach was not used for its assessment. The evaluation of results showed that both methods (ethanol-based and MLC-based) have excellent eco-friendliness (eco scale score > 75). The MLC method has a slightly higher eco-scale index which is due to the application of surfactant instead of EtOH in the mobile phase. However, the method proposed in our study has a shorter analysis time, and all system suitability criteria are fulfilled. This implies that this method can be routinely applied in the pharmaceutical industry to determine DZP content in coated tablets.

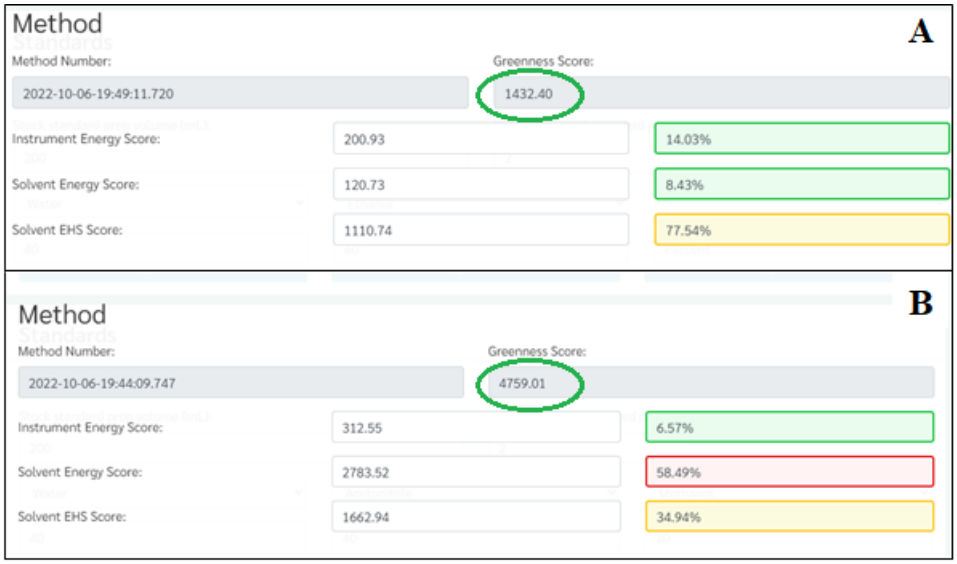

Fig. 3. Comparison of the developed method (A) and reference USP method (B) using the AMGS tool

T a b l e 8

Results from the analytical eco-scale approach for assessment of method greenness

Hazard	Penalty points	
Reagents	EtOH-based RP HPLC method proposed in our study	MLC-based RP HPLC method proposed by Elmansi & Belal, 2019
EtOH	12	0
Water	0	0
Polyoxiethilene(23)lauril ether	0	4
Energy consumption	0	1
Occupational hazard	0	0
Chemical waste	6	8
Total penalty points	18	13
Score	**82**	**87**

4. CONCLUSION

A simple, fast, and eco-friendly RP-HPLC method for determining DZP in coated tablets was developed and validated. The thorough evaluation of the method's robustness ascertained a simple method transfer among the pharmaceutical laboratories. A statistical comparison of the analytical results obtained with the eco-friendly and the reference (USP) method confirmed that the proposed method generates reliable analytical results. Assessment of the method's greenness was performed using two different approaches: AMGS and Eco-scale. Besides its low negative environmental impact, the proposed method has advantages such as short analysis time, great robustness, fulfilling system suitability criteria, and operation on conventional HPLC systems without a time-consuming conditioning step before analysis. Thus, it proves to be a better alternative than the reported method for DZP determination in coated tablets. This method can be used in routine analysis for the determination of assay of diazepam and uniformity of dosage units by content uniformity of DZP coated tablets in the pharmaceutical industry or other drug quality control laboratories.

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

REFERENCES

(1) Sajid, M.; Plotka-Wasylka, J., Green analytical chemistry metrics: review, *Talanta*, **2022,** 238 (2), 123046. DOI: 10.1016/j.talanta.2021.123046

(2) Nakov, N.; Acevska, J.; Brezovska, K.; Kavrakovski, Z.; Dimitrovska A., Green strategies towards eco-friendly HPLC methods in pharma analysis. In: *High perfor-*

mance liquid chromatography – recent advances and applications, IntechOpen Limited, London, **2023**. DOI: 10.5772/intechopen.110035

(3) Tobiszevski, M.; Namiesnik, J., Greener organic solvents in analytical chemistry, *Current Opinion in Green and Sustainable Chemistry*, **2017**, 5, 1–4. DOI: 10.1016/j.cogsc.**2017**.03.002

(4) Elmansi, H.; Belal, F.; Development of an eco-friendly HPLC method for the simultaneous determination of three benzodiazepines using green mobile phase, *Microchemical Journal*, **2019**, 145, 330–336. DOI: 10.1016/j.microc.2018.10.059

(5) Scriba, G. K. E.; Sean C. Sweetman (Ed.): Martindale: The Complete Drug Reference. *Chromatographia,* London, **2011**, 74, 647–648. DOI: https://doi.org/10.1007/s10337-011-2101-8

(6) World Health Organization (WHO): WHO model list of essential medicines, 22nd list, **2021.** Available at: https://www.who.int/publications/i/item/WHO-MHP-HPS-EML-2021.02. Accessed March 8, 2023.

(7) Szatkovska, P.; Koba, M.; Kośliński, J.; Wandas, J.; Bączek, T., Analytical methods for determination of benzodiazepines. A short review, *Central European Journal of Chemi*stry, **2014**, 12, 994–1007. DOI: https://doi.org/10.2478/s11532-014-0551-1

(8) Eldin, A. B.; Shalaby, A.; Abdallah, M. S.; Shaldam, M. A.; Abdallah, M.A., Applying green analytical chemistry (GAC) for development of stability indicating HPLC method for determination of clonazepam and its related substances in pharmaceutical formulations and calculating uncertainty, *Arabian Journal of Chemistry*, **2014**, 12 (9), 1212–1218. DOI: https://doi.org/10.1016/j.arabjc.2014.10.051

(9) El Mahjoub, A.; Staub, C., Simultaneous determination of benzodiazepines in whole blood or serum by HPLC/DAD with semi-micro column, *Journal of Pharmaceutical and Biomedical Analysis*, **2000**, 23 (2, 3), 447–458. DOI: https://doi.org/10.1016/S0731-7085(00)00325-3

(10) Gil-Agustí M.; Carda-Broch, S.; Garsía-Alvarez-Coque M.C.; Esteve-Romero, J., Use of micellar mobile phases for the chromatographic determination of clorazepate, diazepam, and diltiazem in pharmaceuticals, *Journal of Chromatographic Science*, **2000**, 38, 521–527. DOI: https://doi.org/10.1093/chromsci/38.12.521

(11) Capella-Peíro, M. E.; Bose, D.; Martinavaro-Domíngues, A.; Gil-Agustí M.; Esteve-Romero, J., Direct injection micellar liquid chromatographic determination of benzodiazepines in serum, *Journal of Chromatography B*, **2002**, 780 (2), 241–249. DOI: https://doi.org/10.1016/S1570-0232(02)00523-8

(12) Bakavoli, M.; Kaykhaii, M., Quantitative determination of diazepam, nitrazepam and flunitrazepam in tablets using thin-layer chromatography-densitometry technique, *Journal of Pharmaceutical and Biomedical Analysis*, **2003**, 31, 1185–1189. DOI: https://doi.org/10.1016/S0731-7085(03)00027-X

(13) Sruthi, A.; Tejaswi, P.; Thanuja, N.; Sudheer Kumar, D.; Vivek Sagar, P., Simple RR-HPLC method for estimation of diazepam in tablet dosage form, *Journal of Pharmacy Research*, **2013**, 6, 140–144. DOI: https://doi.org/10.1016/j.jopr.2012.11.029

(14) Djajić, N.; Krmar, J.; Rmandić, M.; Rašević, M.; Otašević, B.; Zečević, M.; Malenović, A.; Protič, A., Modified aqueous mobile phases: A way to improve retention behavior of active pharmaceutical compounds and their impurities in liquid chromatography, *Journal of Chromatography Open*, **2022**, 2, 1000023. DOI: https://doi.org/10.1016/j.jcoa.2021.100023

(15) Kamal, A. H; El-Malla, S. F., Mixed micellar liquid chromatographic method for simultaneous determination of norfloxacin and tinidazole in pharmaceutical tablets, *Microchemical Journal*, **2019**, 150, 104151. DOI: 10.1016/j.microc.2019.104151

(16) *European Pharmacopoeia. General monographs: diazepam*. 11th Ed. **2022–2023**. Strasbourg France: European Directorate for the Quality of Medicines & Healthcare, 2513–2514.

(17) *The United States Pharmacopoeia*. The National Formulary (USP43-NP 38), **2020**. Rockville, Maryland, United States Pharmacopeial Convention

(18) Yabré, M.; Ferey, L.; Touridomon Somé, I., Gaudin, K., Greening reversed phase liquid chromatography methods using alternative solvents for pharmaceutical analysis, *Molecules*, **2018**, 23, 1065. DOI: 10.3390/molecules23051065

(19) 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

(20) AMGS calculator link https://www.acsgcipr.org/amgs/

(21) Gałuszka, A.; Konieczka, P.; Migaszevski, Z. M.; Namieśnik, J., Analytical Eco-scale for assessing the greenness of analytical procedures, *Trends in Analytical Chemistry*, **2012**, 37, 61–72. DOI: https://doi.org/10.1016/j.trac.2012.03.013

(22) Mašković, M.; Jančić-Stojanović, B.; Malenović, A.; Ivanović, D.; Medenica, M., Assessment of liquid chromatographic method robustness by use of Plackett-Burman design*, Acta Chromatographica,* **2010,** 22, 281–296. DOI: 10.1556/AChrom.22.2010.2.10