

A novel HPLC method for the simultaneous determination of empagliflozin and dapagliflozin: Development, validation, robustness testing and greenness assessment

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Abstract

Empagliflozin (EMPA) and Dapagliflozin (DAPA) are mainly recommended for the treatment of type 2 diabetes mellitus and heart failure. Based on the principles of green analytical chemistry, a simple, rapid and robust HPLC method was developed for the determination of both analytes in bulk. An isocratic protocol was developed using a C18 column, with a mobile phase consisting of just 30% organic modifier (ACN), while the remaining 70% was HPLC water. The run was completed within 4 min, with a flow rate of 1.20 mL/min, while UV detection took place at 230 nm. The method was then validated according to the ICH Q2 (R1) guidelines, and all the parameters examined were within the specified limits. Furthermore, the robustness of the method was evaluated by employing a factorial experimental design. Finally, the environmental friendliness of the proposed method was assessed by using the Analytical GREENess (AGREE) metric tool. The proposed method can be used for analysis of EMPA and DAPA in bulk, with potential application to the relevant pharmaceuticals.

Key words: empagliflozin, dapagliflozin, AGREE tool, robustness testing, validation

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Introduction

One of the most common disorders in western countries is type 2 diabetes mellitus (T2DM) (1). Millions of people receive medication to manage its symptoms, and billions are spent for the research of new drugs. Empagliflozin - (2S,3R,4R,5S,6R)-2-[4-Chloro-3-[[4-[(3S)-oxolan-3-yl]oxyphenyl]methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol- (EMPA) and dapagliflozin - (2S,3R,4R,5S,6R)-2-[4-chloro-3-[(4-ethoxyphenyl)methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol- (DAPA) (Figure 1), two novel sodium-glucose co-transporter 2 (SGLT2) inhibitors, have been approved for its treatment (2). SGLT2 inhibitors have been demonstrated to significantly improve cardiovascular outcomes in patients with T2DM (3-5) as well. Furthermore, EMPA and DAPA have shown effective activity among patients with chronic kidney disease (6, 7).

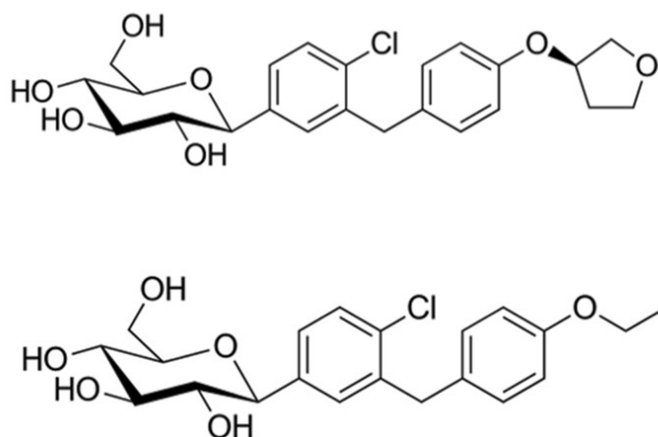


Figure 1. Structures of EMPA (top) and DAPA (down)

Slika 1. Struktura EMPA (gornja) i DAPA (donja)

Various methods have been described for the analysis of EMPA or DAPA in raw material, tablets and in stability studies, alone or in combination with other Active Pharmaceutical Ingredients (APIs). With regards to high performance liquid chromatography (HPLC) or ultra-high performance liquid chromatography (UPLC), a series of interesting methods have been proposed (8-41). To the best of our knowledge, there is only one available method (42) for the determination of this combination of APIs in pharmaceutical formulations with acetonitrile and 0.1% formic acid buffer, pH 3.7 (60:40 v/v) as the mobile phase.

In the current method, we attempted to develop a novel isocratic HPLC method that can determine EMPA and/or DAPA in bulk, with potential application to pharmaceuticals. The simplicity of the mobile phase was the main aim, and therefore various columns were tested in order to skip, if possible, the addition of salts and pH adjustment or gradient elution. Furthermore, the green assessment of the novel method was an additional aim of this study. Having in mind the principles of green chemistry

(43), ACN content was kept as low as possible, and thus the method can be considered eco-friendly, according to the Analytical GREENess (AGREE) metric tool. To this purpose, a very simple – in terms of mobile phase composition – and green HPLC method was developed and validated according to the ICH guidelines.

Experimental

Reagents and solvents

Standards of EMPA and DAPA were obtained from LGC Standards GmbH (Wesel, Germany). Acetonitrile and methanol (HPLC grade) were purchased from Fisher Chemical (ECELabs, Athens, Greece). HPLC grade water (Resistivity 18.2 MΩ x cm) was obtained via the Synergy UV water purification system (Merck Millipore, Athens, Greece).

Instrumentation and chromatographic conditions

Experiments for method development and validation were conducted on a GBC LC 1120 pump (Darmstadt, Germany) combined with a GBC LC 1210 UV–vis detector. Samples were injected via a Rheodyne injector valve with a 20 µL sample loop, while Empower software (Waters, Milford, MA, USA) was utilized for data acquisition and subsequent analysis. During method development, the following YMC columns (50 mm x 4mm, 5µm particle size) were used: ODS, C8, Cyanopropyl and Butyl (YMC Europe GMBH, Dinslaken, Germany). In all cases, the analysis was conducted under isocratic elution, with the flow rate set to 1.2 mL/min at 25 ± 2 °C. EMPA and DAPA were detected at 230 and 250 nm. Prior to use, all mobile phases were filtered by using a 0.45 µm nylon-membrane filter (Gelman Sciences, Northampton, UK) and degassed under vacuum.

Preparation of stock standard solutions and working standard solutions

Stock standard solutions for EMPA and DAPA (100 µg/mL) were prepared by accurately weighting 10.0 mg of both APIs and dissolving them by using a 50/50 v/v MeOH-H₂O diluent into two 100 mL volumetric flasks. The appropriate dilutions from each stock solution were made in order to obtain the working solutions (10 µg/mL) used during method development. All solutions were stored in a refrigerator at 5 °C.

Solutions for method validation

The final method was validated according to the ICH guidelines, and a series of experiments were conducted in order to evaluate the following parameters: Selectivity, Linearity, Limit of Detection (LOD), Limit of Quantitation (LOQ), Stability, Accuracy and Precision.

For selectivity, 50/50 v/v MeOH-H₂O diluent was injected, and the obtained chromatogram was compared with the one from a working solution containing both analytes. Regarding linearity, five solutions containing DAPA and EMPA were prepared, using the same diluent, over a concentration range of 2.5–7.5 µg/mL, which covers the range of 50%-150% (50%, 75%, 100%, 125% and 150%) of the nominal concentrations

for DAPA and EMPA (5.0 µg/mL-100%). In addition, LOD and LOQ for both EMPA and DAPA were evaluated by using the equations that follow: $LOD = 3.3 \times SD/a$ and $LOQ = 10 \times SD/a$, where SD is the standard deviation of the areas of the first calibrator and a is the slope of the relevant calibration curve. The stability of the working solutions was estimated under two different conditions (refrigerated and room temperature storage). Six independent solutions of 100% concentration for both APIs were prepared, three of which were stored in the refrigerator (4 °C) and three at room temperature (25 °C). Regarding accuracy, three test solutions were prepared with 50/50 v/v MeOH-H₂O, containing 4.0, 5.0 and 6.0 µg/mL, corresponding to 80, 100, and 120% concentration levels, respectively. Precision was assessed by using six replicate samples at 100% concentration (5.0 µg/mL for EMPA and DAPA) on three consecutive days.

Evaluation of Robustness

Robustness testing aims to assess the ability of the method to remain unaffected by small changes of the experimental conditions (44). To this purpose, an experimental design is often performed via a suitable software. In the present study, a factorial design was selected (2³) and the examined factors were the %ACN content, the flow rate of the mobile phase and the wavelength (λ). Each factor was examined at two levels (-1, +1), while 3 additional experiments with central (0) values were included (Table I).

Table I All the experiments proposed by the software for robustness testing

Tabela I Svi eksperimenti predloženi softverom za ispitivanje robusnosti

RUN	%ACN	Wavelength, λ	Flow rate (mL/min)
1	30	230	1.20
2	30	230	1.20
3	29	232	1.30
4	31	232	1.30
5	31	228	1.30
6	31	232	1.10
7	29	232	1.10
8	31	228	1.10
9	29	228	1.10
10	29	228	1.30
11	30	230	1.20

Software

Experimental design for robustness testing was planned and evaluated via Design-Expert® 13 trial version (Stat-Ease Inc., Minneapolis, USA). The AGREE metric approach and software (45) were utilized for the evaluation of the greenness of the method. All the other calculations were conducted via MS Office Excel 365 (Microsoft, Redmond, USA).

Results and Discussion

Method development

In the present study, we attempted to develop a novel isocratic HPLC method, as simple and as green as possible, which could determine EMPA and/or DAPA in pharmaceuticals. To this purpose, several columns and various organic modifier (ACN, MeOH): H₂O ratios in the mobile phase were tested. The lack of ionizable moieties in both EMPA and DAPA that can either protonate or deprotonate at different pH values enabled the avoidance of utilizing salts or adjusting pH values. From the beginning, only short columns (5 cm) were used, while the percentage of the organic modifier was kept as low as possible in an attempt to enhance the eco-friendly character of the method. Almost all the columns used (ODS, C8, Cyanopropyl and Butyl) were suitable for the analysis of DAPA, providing sharp peaks. However, despite structural similarity (both C-glycosyl compounds consisting of a beta-glucosyl residue different at p-benzyl substituent), EMPA was a more difficult case, and only the C18 (ODS) column provided sharp and symmetric peaks, with ACN proving to be the best organic modifier. An ACN content of 30% served the intended purpose, as EMPA and DAPA were separated efficiently, with a run time of just 4 min. EMPA and DAPA were eluted at 1.75 and 3.25 min (Figure 2B), respectively, with a flow rate of 1.20 mL/min. The detection of analytes took place initially at 2 wavelengths (230 and 250 nm); however, a 10-fold increase in the area was observed when 230 nm was utilized, and therefore this wavelength was kept for method validation.

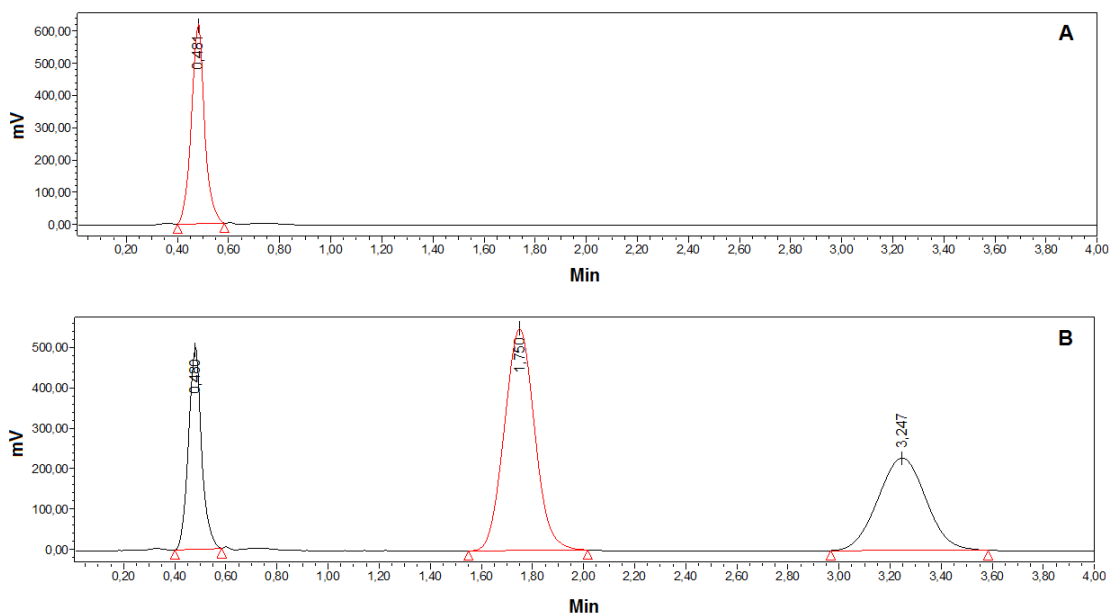


Figure 2. Representative chromatograms of A) Blank and B) solution in MeOH:H₂O 50:50, v/v, containing EMPA (1.75 min) and DAPA (3.25 min) at concentrations 10.0 and 5.0 µg/mL, respectively

Slika 2. Reprezentativni hromatogrami A) blanko uzorak i B) rastvor u MeOH:H₂O 50:50, v/v, koji sadrži EMPA (1,75 min) i DAPA (3,25 min) na koncentracionom nivou 10,0 µg/mL za EMPA i 5,0 µg/mL za DAPA

Validation

Selectivity

The assessment of selectivity revealed that EMPA and DAPA could be detected with no interferences from the diluent, as shown in Figure 2. The two peaks had efficient resolution and no signal was observed when the diluent was injected.

Linearity, LOD and LOQ

Two calibration curves were obtained for EMPA and DAPA, following the analysis of five standard samples (in triplicate).

An excellent linearity between the areas (y) and the concentrations (x) was revealed by R^2 values (≥ 0.9996) for both EMPA and DAPA. Furthermore, the LOD / LOQ values were estimated from calibration curves, as shown in Table II.

Table II Regression analysis, LOD and LOQ values

Tabela II Regresiona analiza, LOD i LOQ vrednosti

API	Calibration curve	R^2	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
EMPA	$y=215517x - 6030.07$	0.9998	0.0923	0.280
DAPA	$y=216656x - 713456$	0.9996	0.138	0.417

Stability

After conducting the stability tests, no issues were raised (%deviation $< 1\%$) for either of the APIs under environmental conditions during an 8 h period (short-term stability under working/analysis conditions), or under refrigerated conditions over two months (long-term stability).

Accuracy

In accordance with the ICH guidelines, the criterion for the mean % recovery (%R) of active compounds in pharmaceuticals should be within $98\% \leq \%R \leq 102\%$. In the present study, this criterion was fulfilled at all three levels (80%, 100% and 120%) for EMPA and DAPA, with %RSD values < 2.0 (Table III).

Table III Recovery results (mean values, $n=3$) obtained for three concentration levels for both APIs

Tabela III Recovery rezultati (srednja vrednost tri merenja) koji su dobijeni na tri koncentraciona nivoa za obe API

API	80% level (4.0 $\mu\text{g/mL}$)	100% level (5.0 $\mu\text{g/mL}$)	120% level (6.0 $\mu\text{g/mL}$)
EMPA	98.8	99.5	99.5
DAPA	99.7	98.2	98.8

Precision

Six replicate samples at 100% level were prepared for the assessment of repeatability and intermediate precision (IP). As far as repeatability is concerned, %RSD was ≤ 2 , proving the method to be precise. Table IV presents data for accuracy (%relative error) as well as the %RSD for the six replicate samples for both APIs. Regarding the IP, acceptable %RSDR values were obtained: 0.98 and 0.51 for EMPA and DAPA, respectively.

Table IV Repeatability of results for EMPA and DAPA

Tabela IV Ponovljivost rezultata za EMPA i DAPA

EMPA			
	Theoretical concentration ($\mu\text{g/mL}$)	Calculated concentration ($\mu\text{g/mL}$)	% Relative Error
1	5.0	5.10	1.99
2		5.09	1.75
3		5.06	1.16
4		4.99	-0.25
5		4.99	-0.25
6		5.04	0.88
Average		5.04	0.88
SD		0.048	
%RSD		0.95	
DAPA			
	Theoretical concentration ($\mu\text{g/mL}$)	Calculated concentration ($\mu\text{g/mL}$)	% Relative Error
1	5.0	5.04	0.72
2		5.02	0.38
3		5.03	0.67
4		5.04	0.79
5		5.04	0.76
6		5.04	0.72
Average		5.03	0.67
SD		0.0075	
%RSD		0.15	

Robustness testing

The following responses were assessed during robustness testing: the retention times (tR), the areas and the tailing factors (Tf) for the two analytes. For the assessment of the significant effects, the graphical approach was implemented (46-48), and to this purpose normal / half-normal graphs and Pareto charts were used (Figure 3).

Based on the graphs for all responses, the following common conclusions were reached for both analytes: a) For the retention times of EMPA and DAPA, the flow rate

and the % ACN were proven to be significant, b) as for the areas, the flow rate and wavelength were found to be statistically significant, c) while the Pareto charts showed that none of the examined factors was above the t-value limit for T_f , and thus they were considered non-significant. In conclusion, the analyst must pay special attention in order to reduce random errors and ensure method robustness.

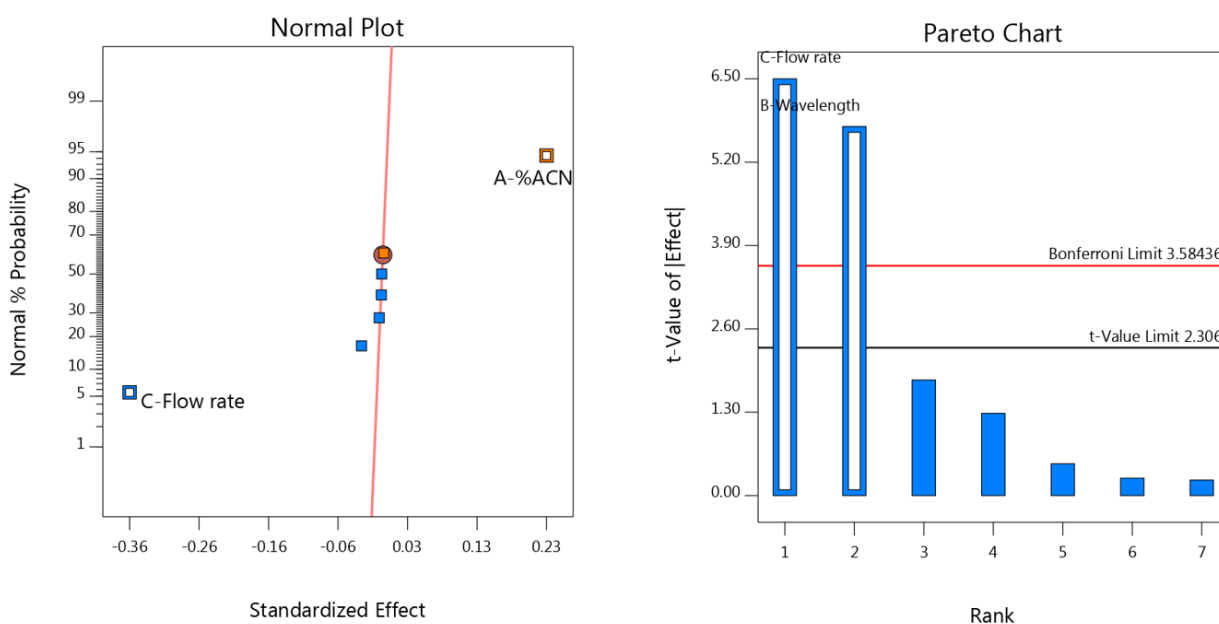


Figure 3. Representative normal plot graph for the response of EMPA t_R (left) and Pareto chart for the response of DAPA (Area) (right). A: %ACN; B: wavelength; C: flow rate (mL/min)

Slika 3. Reprezentativni grafici (*normal plots*) za odgovor t_R EMPA (levo) i DAPA (desno). A: %ACN; B: talasna dužina; C: protok (mL/min)

Greenness assessment

After the development and validation of the current method, the evaluation of its greenness was a critical aim. Among different tools, the AGREE metric tool via relevant software was utilized. This system was proposed in 2020 (49) and is based on the 12 basic principles of Green Analytical Chemistry, embedded in a clock-like diagram. Each of the 12 segments has a colour related to the score obtained between 0 and 1 (0-red, 1-green, value within this range-yellow). The final score represents the degree of greenness, and the value closest to one means that the method is green. In the current method, the overall score of 0.85, as shown in Figure 4 (the middle of the pictogram), indicates that the proposed method is green. This result is due to the low content of acetonitrile, lack of any salts or acids, and short run time. More specifically, the scores corresponding to GAC principles 2, 4, 6, 9-11 are excellent, while no low scores were obtained. Furthermore, it is obvious that low weights have been set to criteria 1 and 5.



Figure 4. Greenness results for the proposed method by the AGREE metric tool

Slika 4. Rezultati ekološke prihvatljivosti za predloženu metodu dobijeni AGREE alatom

Conclusions

A very simple, robust and eco-friendly HPLC method for the analysis of EMPA and DAPA in bulk, with potential application to pharmaceuticals, was developed and validated in accordance with the established guidelines. A major advantage of the method was the simplicity of the composition of the mobile phase, along with the isocratic conditions employed. Therefore, the repeated and time consuming equilibration of the system was omitted. Moreover, the method was proven to be green, as estimated by the AGREE metric tool. Robustness was also assessed via a factorial experimental design, indicating which experimental factors should be carefully controlled. To the best of our knowledge, this is the simplest validated HPLC method for the determination of both APIs. Thus, the novel method is expected to be useful in the quality control of pharmaceutical laboratories.

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N/A.

Declaration of Competing Interest

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

CRedit authorship contribution statement

Eleftheria Kladi: Methodology; Software; Validation; Roles/Writing - original draft;. **Maria Zerva:** Methodology; Software; Validation; Roles/Writing - original draft;. **Yannis Dotsikas:** Conceptualization; Software; Supervision; Writing - review & editing.

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Nova HPLC metoda za istovremeno određivanje empagliflozina i dapagliflozina: Razvoj, validacija, testiranje robusnosti i procena ekološke prihvatljivosti

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Kratak sadržaj

Empagliflozin (EMPA) i dapagliflozin (DAPA) se uglavnom preporučuju za lečenje dijabetesa tipa 2 i srčane insuficijencije. Na osnovu principa zelene analitičke hemije razvijena je jednostavna, brza i pouzdana HPLC metoda za određivanje oba analita u aktivnim supstancama. Razvijena je izokratska metoda, koja obuhvata primenu kolone C18, sa mobilnom fazom koja se sastoji od samo 30% organskog modifikatora (ACN), dok je preostalih 70% HPLC voda. Analiza je obavljena u roku od 4 min sa protokom od 1,20 mL/min, dok je talasna dužina detekcije 230 nm. Metoda je potom validirana u skladu sa smernicama ICH Q2 (R1) i svi ispitivani parametri bili su unutar propisanih granica. Potom je robusnost metode evaluirana primenom faktorskog eksperimentalnog dizajna. Na kraju, ekološka prihvatljivost predložene metode procenjena je korišćenjem alata za procenu analitičke „zelenosti“ (Analytical GREENess – AGREE). Predložena metoda može se koristiti za analizu EMPA i DAPA u aktivnim supstancama, sa potencijalnom primenom u relevantnim doziranim oblicima.

Ključne reči: empagliflozin, dapagliflozin, AGREE alat, ispitivanje robusnosti, validacija
