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Farmasötik Ürünlerde Empagliflozin Tayini İçin Yeşil bir HPLC Yönteminin Geliştirilmesi ve Validasyonu

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ÖZ

Bu çalışmada, empagliflozinin farmasötik ürünlerdeki miktar tayini için iki farklı kromatografik yöntem geliştirilmiştir. Bunlardan ilki mobil fazda organik modifier olarak asetonitril kullanılan klasik yöntem, ikincisi ise mobil fazda organik modifier olarak etanol kullanılan yeşil yöntemdir. Klasik yöntemde Extend C18 kolon (250 x 4,6 mm, 5 µm) kullanılmış ve sıcaklık 30°C'de sabit tutulmuştur. Mobil faz olarak formik asit çözeltisi (ultra saf suda %0,1) ve asetonitril (55/45, v/v) kullanılmış ve izokratik elüsyon uygulanmıştır. Mobil faz akış hızı 1,0 mL dk^{-1} ve enjeksiyon hacmi 10 µL dir. Dedeksiyon UV dedektör kullanılarak 223 nm de gerçekleştirilmiştir. Yeşil yöntemde organik modifier olarak etanol kullanılmıştır. Her iki kromatografik yöntem koşulları aynı olmasına rağmen, tek fark organik modifierdir. Her iki kromatografik yöntem de seçicilik, doğrusallık, doğruluk, kesinlik, saptama ve miktar belirleme limiti ve sağlamlık gibi çeşitli parametreler açısından İlaçlar için Teknik Gereksinimlerin Uyumlaştırılmasına İlişkin Uluslararası Konsey (ICH) kılavuzlarına göre valide edilmiştir. Kromatografik yöntemlerin 5-30 mg mL^{-1} gliklazid konsantrasyon aralığında korelasyon katsayıları 0.999'dan büyüktür. Geliştirilen kromatografik yöntemler farmasötik formülasyonlara uygulanmıştır. Elde edilen sonuçların ortalamalar açısından karşılaştırmaları Student (t) testi, standart sapmalar açısından karşılaştırmaları ise Fischer (F) testi kullanılarak yapılmıştır. Bu yöntemler arasında anlamlı bir fark bulunmamıştır. Her iki yöntemin çevresel etkisi AGREE ve GAPI yazılımları kullanılarak değerlendirilmiş ve sürdürülebilirlikleri teyit edilmiştir. Bu valide edilmiş metotlar, tablet formülasyonlarında Empagliflozinin kantitatif analizi için güvenilir ve çevre dostu yaklaşımlar sunarak farmasötik analizlerde daha güvenli ve sürdürülebilir laboratuvar uygulamalarını desteklemektedir. Farmasötik ürünlerdeki Empagliflozinin kantitatif analizi için yeni yaklaşım, şu anda kullanılan yöntemlere ekonomik ve ekolojik açıdan sorumlu bir alternatif olarak görülebilir.

Development and Validation of A Green HPLC Method for Determination of Empagliflozin in Pharmaceutical Products

Research Article

ABSTRACT

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In this study, two different chromatographic methods were developed for the quantification of empagliflozin in pharmaceutical products. The first was the classical method using acetonitrile as an organic modifier in the mobile phase, and the second was the green method using ethanol as an organic modifier in the mobile phase. In the classical method, an Extend C18 column (250 x 4.6 mm, 5 µm) was used and the temperature was kept constant at 30 °C. The mobile phase was the formic acid solution (0.1% in ultrapure water) and acetonitrile (55/45, v/v), and isocratic elution was applied. The flow rate of the mobile phase was 1.0 mL min^{-1} and the injection volume was 10 µL. Detection was performed using a UV detector at 223 nm. In the green method, ethanol was used as an organic modifier. The only difference between these methods was the organic modifier. All other conditions of the methods were identical. Both chromatographic methods were validated according to ICH guidelines for various parameters such as selectivity, linearity, accuracy, precision, limit of detection and quantification, and robustness. The coefficients of determination of the chromatographic methods were greater than 0.9990 in the concentration range of 5-30 mg mL^{-1} glyclazide. The developed chromatographic methods were applied to pharmaceutical formulations. Comparisons of the results obtained in terms of means were made using the Student (t) test and in terms of standard deviations using the Fischer (F) test. The methods used did not differ much from one another. The environmental impact of both methods was evaluated using AGREE and GAPI software, confirming their sustainability. These validated methods provide reliable and environmentally friendly approaches for the quantitative analysis of Empagliflozin in tablet formulations, supporting safer and more sustainable laboratory practices in pharmaceutical analysis. For the quantitative analysis of Empagliflozin in pharmaceutical products, the new approach can be viewed as an affordable and ecologically responsible alternative to the methods now in use.

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1.Introduction

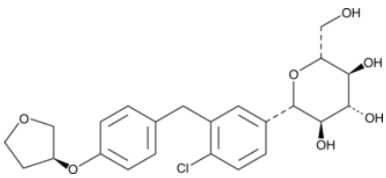
High-performance liquid chromatography (HPLC) is the most commonly used technique for drug analysis. It is extensively applied in the quality control of pharmaceuticals, stability testing of products, identification of drug degradation products, characterization of drug impurities, and analysis of biological samples. HPLC methods using reverse phase mode, typically involving a hydrophobic stationary phase and a polar mobile phase, are being developed in pharmaceutical labs. The Ultraviolet (UV)/Visible (VIS) detector mode is commonly employed in quality control laboratories. Consequently, while creating a pharmaceutical analysis method, the compatibility of the mobile phase with the detector is a crucial consideration. HPLC mobile phases generally consist of a mixture of water (with additives to adjust pH and ionic strength) and organic solvents like acetonitrile or methanol (Snyder, 2009). These solvents possess distinct chromatographic properties, making them preferred choices for HPLC analysis. They are available in the high purity necessary for HPLC applications and are miscible with water in any ratio. Their aqueous solutions exhibit low viscosity and a low UV cut-off wavelength. Additionally, they have minimal chemical reactivity with HPLC instruments, columns, and many types of samples (Snyder, 2009; Welch et al., 2010). Despite their excellent chromatographic properties, acetonitrile and methanol present certain health and

environmental concerns. Acetonitrile is a volatile, flammable, and toxic substance. While methanol is biodegradable and less toxic than acetonitrile, it is still classified as a hazardous solvent due to its toxicity and the significant challenges associated with waste disposal (ICH, 2005; Sheldon, 2012). Unfortunately, the amount of waste generated during HPLC analysis is significant. In a typical HPLC system, around 1-1.5 liters of waste are produced per day, amounting to approximately 500 liters per year (Welch et al., 2010). While this volume may seem small compared to the waste produced by large industrial manufacturers, this number is even higher in research laboratories where hundreds of liquid chromatographs are used in pharmaceutical companies. As a result, large amounts of toxic waste are generated daily. With technological advancements, the use of HPLC is becoming increasingly widespread, leading to a corresponding rise in waste production. These wastes, which contain significant amounts of acetonitrile and methanol, must be treated as chemical waste. This not only increases the environmental waste disposal burden in laboratories but also incurs high disposal costs. As a result, analytical chemists are searching for innovative ways to replace polluting analytical techniques with more ecologically friendly ones. The development of environmentally friendly HPLC methods has garnered significant attention from analytical chemists, who are actively seeking new alternatives to replace pollutive analytical techniques with cleaner options. Eliminating the use of hazardous chemicals has become essential in creating methods that are both environmentally and operator-friendly, without compromising analytical performance (Tobiszewski, 2010). All stages of the HPLC analysis process—ranging from sample preparation to separation and final determination—hold the potential for being made more environmentally and operator-friendly (Keith, 2007; Gałuszka, 2012; Tobiszewski, 2015; Tobiszewski, 2016).

In HPLC, a mixture of organic solvents and water-based additives form mobile phases to adjust pH and ionic strength. Acetonitrile and methanol are two common organic solvents used in HPLC. Unfortunately, both solvents are classified as hazardous due to their toxic effects and the significant importance of safe waste disposal. Because of this, they should be used as little as possible (Sheldon, 2012). Since developing an HPLC method without organic solvents is challenging, acetonitrile and methanol should be replaced with less hazardous alternatives to make the method more environmentally friendly and reduce its adverse effects on operator health (Capello, 2007). Ethanol is an environmentally friendly organic solvent (Płotka et al., 2013). Compared to acetonitrile and methanol, ethanol is less harmful. Its low vapor pressure reduces evaporation and minimizes the amount inhaled by operators. Additionally, ethanol is widely accessible and more affordable than other organic solvents, making it a practical choice for laboratories with limited resources, particularly in developing countries (Welch et al., 2010). Furthermore, ethanol's disposal costs are lower due to its eco-friendly waste properties, which is a significant advantage in regions where chemical waste disposal is costly. In chromatography, ethanol exhibits properties similar to those of acetonitrile and methanol (Miyabe et al., 1999). Column-filling materials and adsorption mechanisms are quite similar. It has similar separation mechanisms when using different solvents. When ethanol is used instead of

acetonitrile or methanol, chromatographic separation of a mixture containing basic and neutral compounds yields similar peak results (Ribeiro et al., 2002). According to organic solvent classifications, ethanol belongs to the same selectivity group as methanol (Shen et al., 2015). However, using ethanol in HPLC has two primary disadvantages (Mohamed, 2015). The first is that ethanol has a higher UV cut-off (210 nm) compared to methanol and acetonitrile. This can increase background noise and significantly shift the baseline, reducing sensitivity in gradient elution systems (Snyder, 2009). However, ethanol's high UV cut-off value may not pose an issue if the target compounds have strong UV chromophores. The second disadvantage is related to the viscosity of ethanol/water mixtures, which is higher at room temperature compared to methanol/water and acetonitrile/water mixtures. Increased viscosity leads to higher column pressure, limiting the use of ethanol in conventional chromatography systems (Shaaban and Górecki, 2015). Column temperature plays a crucial role in selectivity, yield, and mobile phase viscosity. Raising the column temperature lowers the mobile phase viscosity, thereby reducing column pressure. Empagliflozin, a new-generation oral antidiabetic drug, promotes renal glucose excretion and is used for treating type 2 diabetes and heart failure (Wittich and Beckman, 2020). The physicochemical properties of empagliflozin are detailed in Table 1 (https://go.drugbank.com/drugs/DB09038).

Table 1. The physicochemical properties of empagliflozin

Property	Value
Name	(2S,3R,4R,5S,6R)-2-[4-chloro-3-[[4-[(3S)-oxolan-3-yl]oxyphenyl]methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol
Formula	$C_{23}H_{27}ClO_7$
Structure	
Molecular weight	450.90
Melting point	151-153 ^{0}C
Log P	1.79
pKa (Strongest Acidic)	12.57
pKa (Strongest Basic)	-3.00
Solubility	Soluble in methanol, ethanol, dimethyl sulfoxide, and dimethylformamide. Insoluble water.

Several analytical techniques for measuring empagliflozin (EPG) in biological matrices and pharmaceutical products have been documented in the literature. These include spectrophotometric methods (Padmaja and Veerabhadram, 2015; Ayoub, 2016; Ayoub, 2017; Elnadi et al., 2022), spectrofluorometric methods (Abdel-Ghany et al., 2018), high-performance thin-layer chromatography (HPTLC) (Thakor et al., 2019; Abbas et al., 2021; Patel et al., 2022), high-performance liquid

chromatography (HPLC) (Abdel-Ghany et al., 2017; Abdel-Ghany et al., 2018; Badragheh et al., 2018; Hassib et al., 2019; Mabrouk et al., 2020; Manoel et al., 2020; Pandya and Shah, 2020; Sharif et al., 2020; Moussa et al., 2021; El-Kafrawy et al., 2022; Gollu and Gummadi, 2022; Marie et al., 2022), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Ayoub and Mowaka, 2017; Dias et al., 2019; Shah et al., 2019a; Shah et al., 2019b; Van der Aart-van et al., 2020; Burin et al., 2021). However, many of these methods are highly complex and demand costly equipment, specialized chemicals, and toxic organic solvents. The sample preparation process is often intricate, involving lengthy run times and gradient elution. Therefore, the current study aimed to develop and validate an environmentally and analyst-friendly liquid chromatography method in which ethanol is used as a mobile phase organic solvent for EPG quantification in pharmaceutical products by a simple extraction procedure.

2.Materials and Methods

2.1. Instruments

An Agilent 1260 series HPLC system (Wilmington, DE, USA) was used in the analysis equipped with a degasser, quaternary pump, autoinjector, ultraviolet detector, and a column oven set to 30°C. ChemStation software was utilized for system control and data analysis. An Extend C18 column (5 µm, 250 × 4.6 mm; Agilent, USA) was used for chromatographic separation. A Mettler Toledo pH meter with a glass electrode was employed for pH measurements. Millipore Milli-Q water purification system was used to obtain ultrapure water with a conductivity of less than 0.05 µS cm^{-1} (Milford, MA, USA).

2.2. Reagents

Empagliflozin (EPG) United States Pharmacopeia (USP) Reference Standard, ethanol (≥99.9%), acetonitrile (≥99.9%), and trifluoroacetic acid (≥99.0%) were procured from Sigma-Aldrich Chemie GmbH (Istanbul, Turkey). All other chemicals used were of analytical reagent grade. EPG tablets (Jardiance, 10 mg) were obtained from a local pharmacy in Afyonkarahisar, Türkiye.

2.3. EPG standard solutions

25 mg of the EPG reference standard was precisely weighed and transferred to a volumetric flask with a volume of 50 mL. After adding 30 mL of ethanol to the EPG standard, the solution was subjected to ultrasonic treatment until it became completely clear. Once it reached room temperature (25°C), its volume was adjusted to 50 mL using ethanol. This prepared stock solution was then further diluted with methanol to create working standard solutions at concentrations of 5, 10, 15, 20, 25, and 30 µg mL^{-1}.

2.4. EPG sample solution

Ten tablets containing EPG were precisely weighed and crushed in a clean and dry mortar to a fine powder. A 25 mg amount of EPG powder was transferred into a 50 mL volumetric flask, followed by the addition of 30 mL of methanol. The solution was then agitated on a rotary shaker for 20 minutes to achieve complete dissolution. The volume was then adjusted with ultra-pure water. The resulting mixture was sonicated for 10 minutes and subsequently filtered through a 0.45 mm membrane filter. The sample solution was diluted with methanol from the prepared stock solution to achieve a concentration of 20 μg mL^{-1}.

2.5. Determination of λ_{max} for EPG

Standard solutions of EPG in methanol, with concentrations ranging from 5 to 30 μg mL^{-1}, were analyzed using a UV spectrophotometer (Shimadzu UV-1800). The scanning was performed over a wavelength range of 200 to 400 nm. The overlapping spectra of the standard solutions are presented in Figure 1.

2.6.Method validation

Chromatographic methods were validated following the guidelines provided by the International Conference on Harmonization (2005) and the Center for Drug Evaluation and Research (CDER, 1994). Several validation parameters, including specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness, were examined. Based on these parameters, a concentration range of 5 to 30 μg/mL was chosen for the validation process. Initially, the selectivity of the chromatographic methods was evaluated by injecting standard, sample, and mobile phase solutions into the chromatographic system. Chromatograms were analyzed to identify any interference peaks in the region corresponding to EPG peak. The linearity of the methods was determined by injecting six standard solutions within the 5 to 30 μg/mL range into the HPLC system. A calibration curve was constructed by plotting the peak area against EPG concentration, with three replicates performed on three different days. The regression equation, slope, and intercept were calculated using linear regression analysis. Linearity was further assessed by evaluating the absolute mean recovery, relative standard deviation (RSD), and the coefficient of determination (R^2) of the calibration curve. To check the system suitability, an EPG standard solution (20 μg/mL) was injected six times, and values for peak area, retention time (t_R), tailing factor (T_n), and theoretical plate number (N) were recorded. The relative standard deviation (RSD%) for both peak areas and retention times was calculated based on these six injections. The accuracy of the method was tested by adding different amounts of EPG standard to the sample solution. The sample solution (20 mg/mL) was spiked with EPG at concentrations of 80%, 100%, and 120% of the expected content. These solutions were injected into the chromatographic system, and the recovery percentages of the added standard amounts were calculated. Triplicate tests were conducted for each concentration level to ensure

reliability. The precision of the chromatographic methods was evaluated through intra-day and inter-day reproducibility. Intra-day precision was determined by calculating the RSD% of the peak areas from three injections of the standard solution (20 μg/mL) on the same day. For inter-day precision, the standard solution was injected three times daily over three consecutive days under the same experimental conditions, and the RSD% of the resulting peak areas was determined to ensure consistent results over time. LOD was calculated using a specific formula 1.

$$LOD=3.3\sigma/S \quad (1)$$

LOD was determined based on the standard deviation of the calibration curve in the developed chromatographic methods. Similarly, LOQ was calculated using the same approach, relying on the standard deviation of the calibration curve based on formula 2. In these calculations, σ represents the standard deviation of the intercept of the calibration curve, and S denotes the slope of the calibration curve.

$$LOQ=10\sigma/S \quad (2)$$

LOQ was determined with the standard deviation of the calibration curve in the same methods. In these formulas, σ represents the standard deviation of the intercept of the calibration curve, while S denotes the slope of the calibration curve. To evaluate the robustness of the chromatographic methods, intentional minor changes were made to the method conditions, and their impact on system suitability parameters was examined.

The flow rate of the mobile phase: (±0.1 mL min^{-1}),
Organic solvent content in the mobile phase: (±2%),
Column temperature: (±5 °C)
pH value of the mobile phase: (±0,05)

For each of these modifications, a standard solution with a concentration of 20 μg mL^{-1} was injected in triplicate into the system, first under normal conditions and then under the altered conditions, to evaluate the impact on the chromatographic system's performance.

2.7. Evaluation of the greenness of methods

The greenness of both chromatographic methods was assessed using the AGREE-Analytical GREEnness metric software. AGREE is a metric system designed to evaluate the greenness of analytical procedures. It is a user-friendly and easily implementable program that incorporates 12 key principles for greenness assessment. The software allows for flexible weight assignment, provides an easy-to-interpret color pictogram output that highlights strengths and weaknesses, and is simple to use. The greenness score, displayed at the center of the colored pictogram, represents the weighted average

of the benchmark scores. This score ranges from 0.0 (lowest score) to 1.0 (perfect score). The graph visually represents the overall score, benchmark scores, and the associated benchmark weights (Płotka-Wasylka, 2018; Pena-Pereira et al., 2020).

3. Results and Discussion

3.1. Analytical method development and optimization

All conditions were optimized to develop and validate an efficient chromatographic method for the quantification of EPG in pharmaceutical preparations.

Firstly, to determine the wavelength at which empagliflozin can absorb UV radiation maximum, standard solutions in the range of 5-30 µg mL^{-1} were scanned against ultra-pure water in the 200-400 nm wavelength range in a spectrophotometer. Upon examining the spectra, it was observed that EPG absorbs UV rays maximally at a wavelength of 223 nm. Additionally, baseline noise was minimal at this wavelength. It was also found that there was no interference from drug additives or filler materials at this wavelength in pharmaceuticals. The overlapping spectrum of the standard solutions is shown in Figure 1.

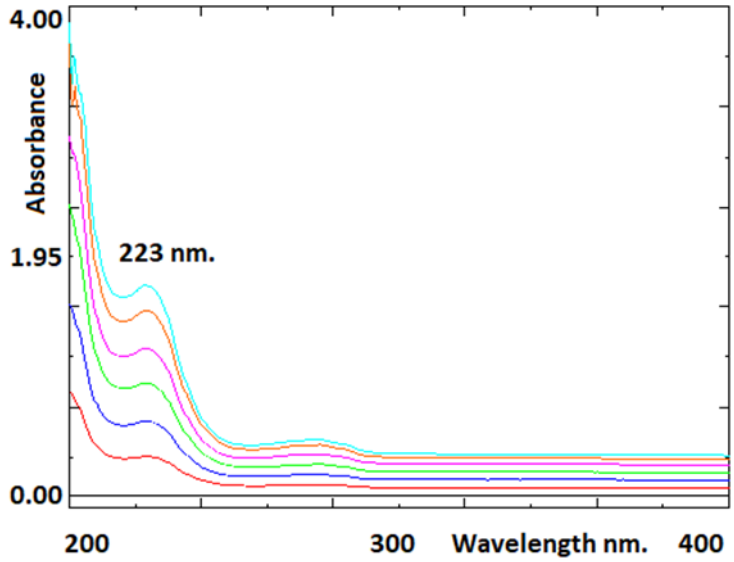


Figure 1. Overlapping spectra of EPG standard solutions in the concentration range of 5-30 µg mL^{-1}.

The chromatographic conditions were optimized to achieve good peak parameters, including an ideal peak shape, a low tailing factor, a short retention time, and a high theoretical plate number. Various ratios of water/methanol, water/acetonitrile, and methanol/acetonitrile mixtures were tested as mobile phases. Initially, acetonitrile and ultrapure water were used as mobile phases in a volume ratio of 20/80 (v/v), but this resulted in a very long analysis time. The water component of the mobile phase was then acidified with formic acid. Good peak parameters were achieved when a solution of formic acid (0.1% in water) and acetonitrile (55/45, v/v) was used as the mobile phase. C18 columns of

different lengths were tested and good peak parameters were obtained using an Agilent C18 (250 mm x 4.6 mm, 5 µm) column. Chromatographic analyses were performed at 30 °C, which proved to be cost-effective and offered many advantages, such as high column efficiency, low column pressure, and favorable chromatographic peak shape. The injection volume was set to 10 µL, as a high tailing factor was observed when the injection volume was 20 µL.

3.2. The conditions of the developed chromatographic method

Two different chromatographic methods have been developed for the quantitative analysis of EPG: the first method and the green method. Below are the detailed conditions for each method:

First Method: An Agilent C18 column (250 x 4.6 mm, 5 µm) was used, with the temperature maintained at 30 °C. The mobile phase consisted of a formic acid solution (0.1% in water) and acetonitrile (55/45, v/v), with isocratic elution applied. The flow rate of the mobile phase was set to 1.0 mL min^{-1}, and the injection volume was 10 µL. Detection was performed using a UV detector at a wavelength of 223 nm.

Green Method: An Agilent C18 column (250 x 4.6 mm, 5 µm) was used, with the temperature maintained at 30 °C. The mobile phase consisted of a formic acid solution (0.1% in water) and ethanol (55/45, v/v), with isocratic elution applied. The flow rate of the mobile phase was set to 1.0 mL min^{-1}, and the injection volume was 10 µL. Detection was performed using a UV detector at a wavelength of 223 nm.

3.3. Results of validation studies

Standard, sample, and mobile phase solutions were injected into the chromatographic system to evaluate the selectivity of the chromatographic methods. The three chromatograms were compared, and the presence of any interfering peak(s) around the analyte peak was examined. No peak interfering with EPG retention time was observed in either method (Figures 2, 3). Three replicate standard solutions (5, 10, 15, 20, 25, and 30 µg mL^{-1}) were prepared by diluting the stock standard solution (500 µg mL^{-1}) with ethanol. These standard solutions were injected into the chromatographic system, and the peak areas and retention times of the analyte in the chromatograms were recorded. Average peak areas were calculated for each concentration level. A calibration graph was constructed by plotting the peak area values against the concentration of the standard solution. The linearity of the chromatographic methods was evaluated through regression analysis. The regression equation, slope, and intercept were calculated using linear regression analysis based on the least squares method. The linearity of the method was assessed using the absolute mean recovery, RSD, and R^2 of the resulting calibration curve. The results are presented in Table 2.

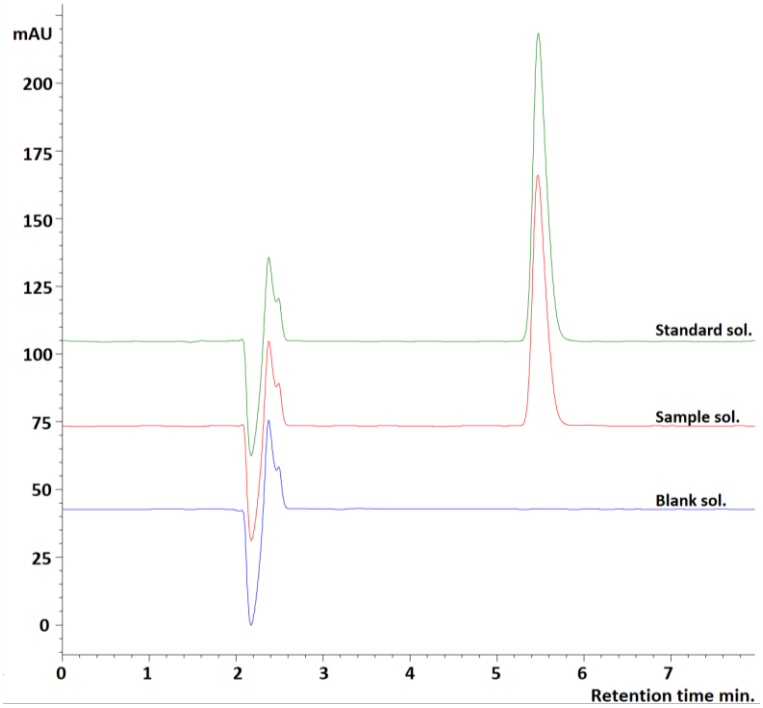


Figure 2. Chromatograms of classical method (Standard solution with a concentration of 25 µg mL^{-1}, Sample solution with a concentration of 20 µg mL^{-1}, Blank solution)

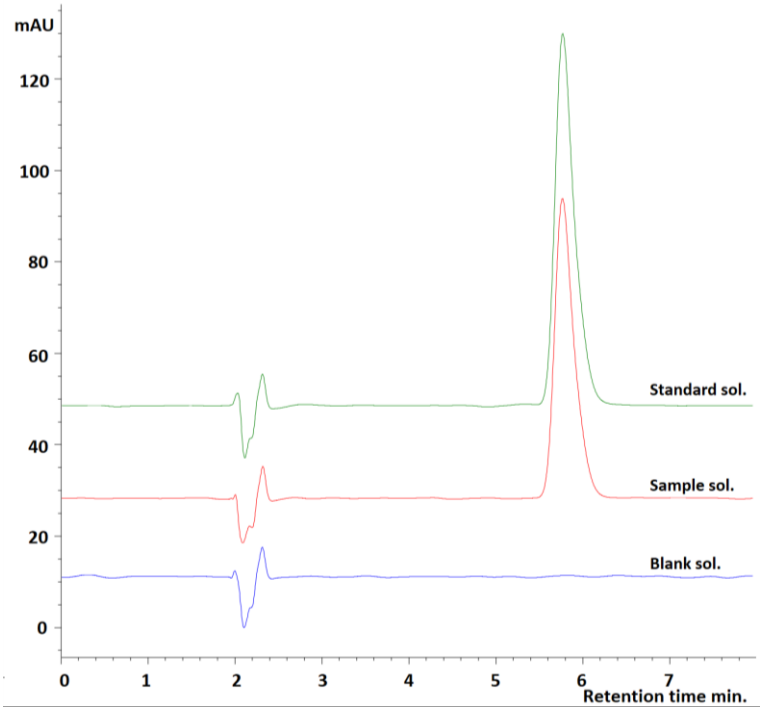


Figure 3. Chromatograms of green method (Standard solution with a concentration of 25 µg mL^{-1}, Sample solution with a concentration of 20 µg mL^{-1}, Blank solution)

Table 2. Regression data of chromatographic methods

Parameter	First method	Green method
Concentration range [μg mL^{-1}] [n = 6]	5–30	5–30
The slope of the regression equation	49.739	50.992
The intercept of the regression equation	4.9985	10.7050
Correlation coefficient	0.9999	0.9998
Retention time [min.]	5.470	5.727
LOD [μg mL^{-1}]	0.50	0.60
LOQ [μg mL^{-1}]	1.40	1.90
Recovery % [n = 3]	99.25–100.33	99.31–100.22

The accuracy of the chromatographic methods was assessed by spiking three different amounts of EPG standard into the sample solution. The standard was added to the sample solution (20 mg mL^{-1}) at concentrations corresponding to 80%, 100%, and 120% of the EPG content. The prepared solutions were then injected into the chromatographic system, and the percentage recovery of the added standard was calculated. Each concentration was tested in triplicate. The recovery percentages ranged from 99.66% to 99.83% for the classical method and from 99.49% to 99.72% for the green method. The highest relative standard deviation values were 0.309 for the first method and 0.466 for the green method. The results of the recovery study are presented in Table 3.

Table 3. Accuracy data of chromatographic methods

Method	Spiked level %	Amount added (μg mL^{-1})	Average recovery (%)	SD	RSD (%)
First method	80	16	99.66	0.247	0.309
	100	20	99.73	0.175	0.175
	120	24	99.83	0.147	0.176
Green method	80	16	99.49	0.373	0.466
	100	20	99.63	0.236	0.236
	120	24	99.72	0.153	0.184

Intraday precision was assessed by calculating RSDs of both the retention times and peak areas of empagliflozin from three injections of the standard solution (20 μg mL^{-1}) conducted on the same day. In both chromatographic methods, the RSD values for peak areas and retention times were found to be under 1.00%. The intraday precision results are provided in Table 4. For interday precision, the same standard solution was injected three times daily over three consecutive days. The RSDs for both the retention times and peak areas of the resulting peaks were calculated and analyzed. In both methods,

the RSD values for these parameters remained below 1.00%. The interday precision results are outlined in Table 5. These findings confirm that the methods satisfy the necessary validation criteria.

Table 4. Intraday precision results of chromatographic methods

	First method			Green method		
Sample No	**Retention time min.**	**Peak Area**	**Assay %**	**Retention time min.**	**Peak Area**	**Assay %**
1	5.470	1002.53	99.97	5.758	1037.36	100.04
2	5.467	1003.02	100.02	5.764	1036.40	99.95
3	5.473	1002.86	100.01	5.767	1036.93	100.00
Mean	5.470	1002.80	100.00	5.763	1036.90	100.00
S.D.	0.003	0.2499	0.025	0.005	0.4785	0.046
R.S.D. %	0.055	0.0249	0.025	0.080	0.0461	0.046

Table 5. Interday precision results of chromatographic methods

		First method			Green method		
Day	**Injection No**	**Retention time min.**	**Peak Area**	**Assay %**	**Retention time min.**	**Peak Area**	**Assay %**
First	1	5.470	1002.53	99.82	5.758	1037.36	99.98
	2	5.467	1003.02	99.87	5.764	1036.40	99.89
	3	5.473	1002.86	99.86	5.767	1036.93	99.94
Second	4	5.471	1003.81	99.95	5.708	1037.31	99.98
	5	5.468	1004.32	100.00	5.713	1037.92	100.03
	6	5.466	1004.17	99.99	5.709	1037.24	99.97
Third	7	5.473	1005.22	100.09	5.710	1038.47	100.09
	8	5.470	1006.41	100.21	5.708	1037.57	100.00
	9	5.469	1006.33	100.20	5.709	1038,83	100.12
Mean		5.366	5.470	1004.30	100.00	5.727	1037.56
SD		0.003	0.002	1.4371	0.143	0,027	0.7517
RSD %		0.056	0.037	0.1431	0.143	0,471	0.0724

To evaluate the system's suitability, key parameters were determined using a standard solution at a concentration of 20 µg mL^{-1}. The results, summarized in Table 6, showed that the EPG peak displayed excellent symmetry and minimal variability in both peak areas and retention times. Additionally, the correlation coefficient of the calibration curve was greater than 0.9990, indicating that the method is highly reliable for analyzing samples, from simple to complex matrices.

Table 6. The results of system suitability tests for chromatographic methods (n=6)

System suitability parameter	First method	Green method
Symmetry factor	0.6524	0.7490
Peak Purity Index	1.02	1.04
Tailing factor	1.3707	1.3907
Teoric plate count (N)	5855	4252
RSD % for peak areas	0.0249	0.0461
RSD % for retention times	0.055	0.080

The robustness study data indicate that the chromatographic methods used for analyzing empagliflozin are stable and reliable under variations in critical method parameters. Specifically, small changes in column temperature, flow rate, organic solvent content, and the pH value of the mobile phase do not significantly impact the linearity, recovery, or accuracy of the methods. As detailed in Table 7, the average recovery across all robustness tests ranged from 99.68% to 100.42%, which demonstrates that the methods consistently yield results close to the true value. Furthermore, the RSD % for these recovery results was less than 1.00%.

Table 7. The results of robustness tests

Method	Parameters	Values	Recovery %	RSD %
	The flow rate of the mobile phase	0.90 mL min^{-1}	100.42	0.21
		1.10 mL min^{-1}	99.76	0.14
	Column temperature	25 ^{0}C	99.95	0.16
First		35 ^{0}C	100.10	0.26
method	Acetonitrile content of the mobile phase	43 %	99.92	0.78
		47 %	100.38	0.52
	The pH of the mobile phase	1.95	100.33	0.60
		2.05	99.83	0.52
	The flow rate of the mobile phase	0.90 mL min^{-1}	100.32	0.23
		1.10 mL min^{-1}	99.81	0.16
	Column temperature	25 ^{0}C	99.98	0.18
Green		35 ^{0}C	99.87	0.30
method	The ethanol content of the mobile phase	43 %	99.96	0.81
		47 %	100.40	0.56
	The pH of the mobile phase	1.95	100.26	0.62
		2.05	99.68	0.52

3.4. Application of chromatographic methods to pharmaceutical formulations and comparison of results

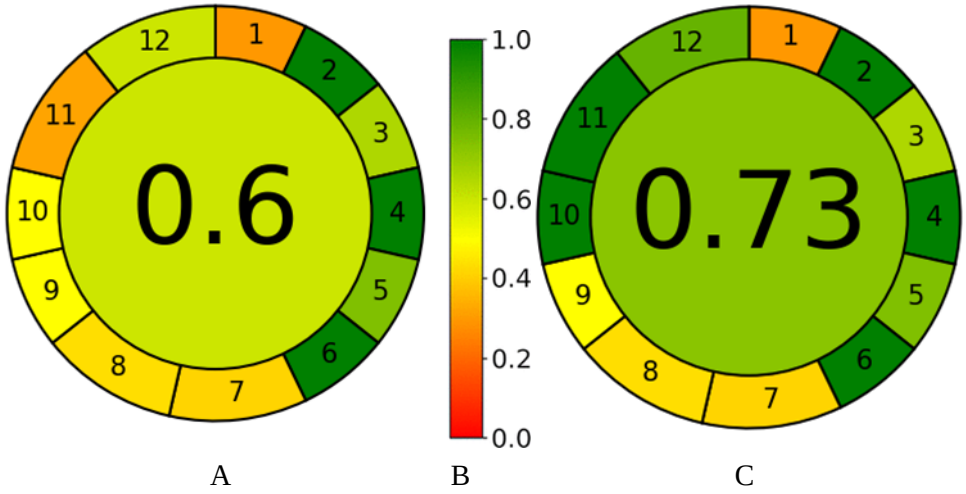
Six tablets of Jardiance, each containing 10 mg of EPG, were analyzed using both chromatographic methods. The results are shown in Table 8. To compare the mean values from both methods, Student's t-test was applied, and the standard deviations were compared using Fischer's F-test. Analyzing the data reveals no significant differences between the two methods in terms of accuracy and precision. This conclusion is further supported by the fact that the calculated t and F values, based on a 95% confidence interval and six repetitions, were lower than the corresponding critical values found in standard reference tables.

Table 8 Statistical evaluation of analysis results of EPG tablets (Jardiance, 10 mg)

Sample	**First method**		**Green method**	
	mg/tablet	**%**	**mg/tablet**	**%**
1	9.924	99.14	10.086	100.83
2	9.874	98.64	9.978	99.75
3	10.054	100.44	10.044	100.41
4	10.126	101.16	9.992	99.89
5	9.978	99.68	10.003	100.00
6	10.106	100.96	9.914	99.11
Average	10.010	100.00	10.003	100.00
SD	0.1015	1.0141	0.0587	0.5870
RSD %	1.0141	1.0141	0.5870	0.5870
t_{value} / t_{table}	0.1352/2.5706			
F_{value} / F_{table}	2.9891/5.0503			

3.5. Evaluation of the Greenness of chromatographic methods

The greenness assessment pictograms for the chromatographic methods are displayed in Figure 4. The classical method achieved a greenness score of 0.60, while the green method scored higher at 0.73. In the pictogram for the classical method (Figure 4A), poor performance was observed for principles 1 and 11 of green analytical chemistry, while excellent performance was noted for principles 2, 4, and 6. The color scale corresponding to the pictogram references is shown in Figure 4B. On the other hand, the pictogram for the green method (Figure 4C) indicated poor performance for principles 1, 7, and 8, but excellent performance for principles 2, 4, 6, 10, and 11. Based on this evaluation, it is concluded that the green method is likely to be more environmentally friendly compared to the classical method.



Basic principles of green analytical chemistry (GAC)

1. Direct analytical techniques.
2. Minimum sample size and number of samples.
3. In-situ measurements.
4. Integration of analytical procedures.
5. Automated and miniaturized methods.
6. Derivatization.
7. High-volume waste generation.
8. Multi-analyte
9. Energy use.
10. Reagents from renewable resources.
11. Toxic reagents.
12. Operator safety.

Figure 4. A: AGREE pictogram of the classical method, **B**: color scale for reference, **C**: AGREE pictogram of the green method

Figure 5 illustrates a comparison of two green analytical chemistry pictograms labeled A (74) and B (79), which appear to represent GAPI (Green Analytical Procedure Index) assessments for two solvents: a classic and a green method for determining EPG. Both pictograms share a common structure, featuring pentagon shapes surrounding a central red hexagon, all within a green circular outline. Visually, the main difference between the two is the presence of an additional two yellow segments in pictogram A compared to pictogram B. The numerical values (74 and 79) likely correspond to greenness scores, with the ethanol (B) achieving a higher score, indicating it may be marginally more sustainable or environmentally friendly according to GAPI standards. These pictograms offer a straightforward visual representation for comparing the relative environmental impact of the two analytical methods. They allow for a quick assessment of the methods' compliance with green analytical chemistry principles, highlighting differences in sustainability and eco-friendliness at a glance.

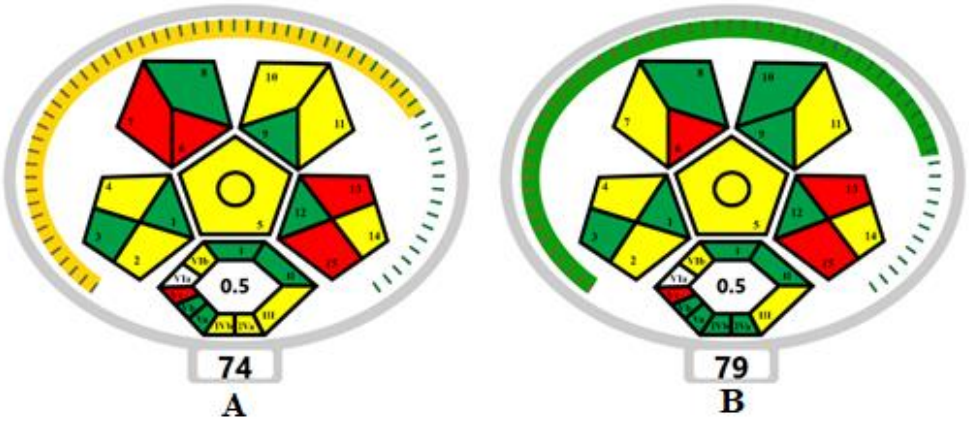


Figure 5. A. GAPI pictogram of the classical method, **B**: GAPI pictogram of the green method

This investigation aimed to assess the chromatographic behavior of active pharmaceutical ingredients using a mobile phase that is both environmentally friendly and operator-safe. Utilizing ethanol in the mobile phase has offered a novel approach to environmentally conscious analysis, diverging from traditional chromatographic solvents. The greenness of the methodology was assessed comprehensively, encompassing the entire process from sample preparation to detection. In the developed method, no toxic solvents were required for extraction, significantly enhancing its environmental profile. Sample preparation was streamlined, employing only ultrapure water, which is a more sustainable alternative to commonly used solvents like acetonitrile. The chromatographic analysis was conducted with ethanol, reinforcing the method's commitment to operator safety and environmental responsibility. Importantly, the waste generated from this analysis was non-toxic, highlighting a crucial advantage of the proposed methodology. Throughout the study, EPG was analyzed with a high degree of sensitivity, repeatability, accuracy, linearity, and robustness. LOD and LOQ for the developed HPLC method were notably low, further attesting to its efficacy. Additionally, the system suitability parameters confirmed that the chromatographic performance was satisfactory and met all verification requirements. This research underscores the significance of greening initiatives in HPLC, particularly given its widespread use in the pharmaceutical industry, which helps mitigate toxicity at each stage of analysis. The results demonstrate that environmentally friendly mobile phases, based on ethanol and water, can be effectively utilized in pharmaceutical analyses. Such advancements are likely to inspire analysts to adopt more sustainable practices in their laboratories. Comparative analysis with previously published methods indicated that the LOD and LOQ values, along with improved greenness metrics, exceeded those reported in the literature. The findings affirm that this green approach to pharmaceutical analysis for EPG was executed without compromising chromatographic quality, effectively minimizing the hazardous effects associated with traditional analytical methodologies.

4.Conclusions

The development of environmentally friendly methods to prevent pollution, reduce energy consumption, and enhance waste management has become increasingly vital for humanity's future. In this regard, a green HPLC method has been established for the quantification of EGP in pharmaceutical products, designed to be environmentally friendly and safe for operators while ensuring high chromatographic performance. This newly developed green HPLC method meets all verification criteria outlined by ICH guidelines, demonstrating linearity, accuracy, sensitivity, robustness, and repeatability. Safe and cost-effective organic solvents, such as ethanol, were employed in both the sample preparation and detection stages of the method. Furthermore, the foliage profile score of this method surpasses that of previously published chromatographic methods. Thus, this developed method can be considered an environmentally friendly and economical alternative to existing methods, ensuring the safety of analysts and the environment in the quantitative analysis of EPG in pharmaceutical products.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Authors' Contributions

The authors contributed equally to this study.

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