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New green HPLC and TLC methods for the determination of dapagliflozin, metformin hydrochloride, and its two official impurities, melamine and cyanoguanidine, in their quaternary mixture

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ABSTRACT

Two environmentally friendly, simple, and accurate chromatographic methods were developed for the quantitative measurement of quaternary mixtures of dapagliflozin, metformin hydrochloride, melamine, and cyanoguanidine in both pure forms and pharmaceutical formulations. Recently, the development of new analytical methods requires taking into account green aspects. The principal objectives of green chemistry are the reduction and elimination of hazardous substances and their harmful effects on the environment and human health. The green HPLC method uses the C_{18} column (250 × 4.6 mm × 5 µm particle size) and a mobile phase consisting of methanol: water in a ratio of (90:10, by volume) with pH adjusted to 3.5 using *o*-phosphoric acid. The flow rate was 1.2 mL/min detected at 225 nm. Retention time (R_t) values were found to be 5.75, 2.06, 2.49 and 3.01 min for dapagliflozin, metformin hydrochloride, melamine, and cyanoguanidine, respectively, while the proposed green TLC method uses a silica gel plate $60F_{254}$ and ethanol: ethyl acetate (1:9, by volume) as a developing system detected at 225 nm. The R_f values were found to be 0.66, 0.74, 0.51 and 0.83 for the four components, respectively. Good linearity was shown through concentration ranges of 1-30, 2-70, 0.5-25, and 1-25 µg/mL for the four components, respectively, for the proposed HPLC method and 0.1-1.5, 0.2-3.0, 0.1-1.5 and 0.1-1.2 µg/band for the four components, respectively, for the proposed TLC method. The proposed methods were successfully applied to diaflozimet 10/1000® tablets containing dapagliflozin and metformin hydrochloride and the results were statistically compared to a published HPLC method and no significant differences were found.

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1. Introduction

Dapagliflozin (DAPA) is (2*S*,3*R*,4*R*,5*S*,6*R*)-2-(4-Chloro-3-(4-ethoxy benzyl) phenyl)-6-(hydroxy methyl) tetrahydro-2*H*-pyran-3,4,5-triol with its chemical formula $C_{21}H_{25}ClO_6$ [1,2], as shown in Figure 1a. DAPA is an inhibitor of sodium-glucose cotransporter-2 (SGLT2). The transporter is responsible for the re-absorption of glucose in the kidney. Therefore, inhibition of SLGT2 has been proposed as a new strategy in the treatment of diabetes. It is indicated for the treatment of diabetes mellitus type 2, and improves glycemic control in adults when combined with diet and exercise. It was later approved to reduce kidney failure, cardiovascular death, and hospitalization for heart failure in adults [1,3,4]. Metformin hydrochloride (MET HCl) is a 1,1-dimethylbiguanide hydrochloride with its chemical formula $C_4H_{12}ClN_5$ [5,6], as shown in Figure 1b. It reduces hepatic glucose output by inhibiting hepatic gluconeogenesis. Additionally, MET HCl increases peripheral glucose uptake and utilization while slowing intestinal sugar absorption. It has the ability to reduce hyperlipidemia. Low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) cholesterol concentrations fall and high-density lipoprotein (HDL) cholesterol increases [7]. Melamine (MEL) is 1,3,5-triazine-2,4,6-triamine [5,6,8]. It is a potential impurity of MET HCl with the chemical formula $C_3H_6N_6$, as shown in Figure 1c. The limit of MEL in MET HCl should not be greater than (0.1 %) according to the British Pharmacopeia [5,9]. There have been many uses for MEL in industry, such as molding compounds, in fire retardant products, adhesives for woods, and fertilizer urea mixtures [8]. Cyanoguanidine (CYGN) is a dimer of cyanamide. It is also called dicyandiamide. CYGN is an official impurity of MET HCl (related compound A), as shown in Figure 1d, its limit should not exceed (0.02%) according to the British Pharmacopeia [5]. CYGN has an irritant effect on the skin and eyes [10].

In the literature review, DAPA was determined only using different HPLC methods [11-15] or spectrophotometric methods [16,17]. Various chromatographic methods for the determination of MET HCl alone were reported by HPLC methods [18,19] and the TLC method [20] or spectrophoto-

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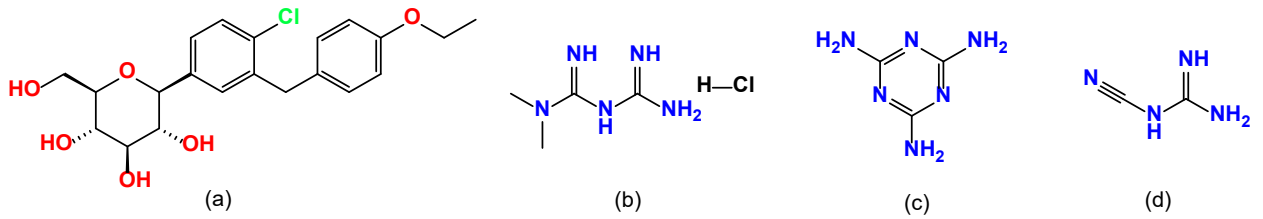

Figure 1. Chemical structure of the four proposed components, (a) DAPA; (b) MET HCl; (c) MEL; and (d) CYGN.

metric methods [19,21,22]. Different chromate-graphic methods for the determination of MET HCl with its two potential impurities, MEL and CYGN [9,23,24] have been reported. DAPA and MET HCl were determined in their binary mixtures by various HPLC methods [25-28]. On the other hand, UV spectrophotometric methods have been published to determine the binary mixture of DAPA and MET HCl [29,30]. The objective of this study is to validate and develop new environmentally friendly chromatographic methods that can separate the primary active ingredients of diaflozimet 10/1000® tablets (dapagliflozin and metformin HCl) in the presence of official impurities of metformin HCl, cyano-guanidine, and melamine. These methods will be more environmentally friendly than the previously reported one. The first method uses the green HPLC method, which uses a less toxic green mobile phase and has less waste generated than the reported HPLC method. The proposed thin-layer chroma-tography (TLC) method is also the first one developed for the determination of the four components under study and offers the benefits of simplicity, low costs, and high separation speed with green mobile phase to avoid environmental hazards.

2. Experimental

2.1. Pure samples

DAPA was kindly supplied by AstraZeneca-Egypt, New Cairo, Egypt. Its purity was 99.4% as certified by the company. MET HCl was kindly supplied by El-Nasr Company, El Gomhoria Street, Abu Zaabal area, Egypt. Its purity was 99.2% as certified by the company. MEL was purchased from Cornell Lab Company, Sarayat Elmaadi, Egypt. Its purity was 99% as certified by the company. CYGN was purchased from Alfa Aesar. The agent of Alpha Aesar in Egypt is Cornell Lab Company, Sarayat Elmaadi, Egypt. It was labeled with a purity of 99%.

2.2. Tablet formulation

Diaflozimet 10/1000 ® tablets Batch No:2307801 labeled with 10 mg of DAPA and 1000 mg of MET HCl, was marketed by Eva Pharma, 176 El Sadat Street, Kafr El Gabal, Haram, Giza 12561, Egypt.

2.3. Materials and reagents

Methanol and ethanol HPLC grade were obtained from Fisher Scientific, Loughborough, United Kingdom. o-Phosphoric acid and ethyl acetate were purchased from El-Nasr Company, located on El Gomhoria Street in the Abu Zaabal area, Egypt.

2.4. Instrumentation

2.4.1. For the HPLC method

Chromatographic separation was achieved using a Shimadzu HPLC system, equipped with a PDA detector with a C_{18} Zorbax column (250 × 4.6 mm × 5 µm particle size) maintained at 25 °C.

2.4.2. For the TLC method

Scanning was performed using a TLC scanner, model 3 S/N Camag (Muttenz, Switzerland), connected and controlled with winCATS software with a scanning speed of 20 mm/s and a spraying rate of 10 mL/s. TLC plates made of aluminum with dimensions of 20 × 20 cm coated with silica gel $60F_{254}$ with a thickness of 0.25 mm and a particle size of 5 µm (Merck, Germany) were used. Applications with a Camag Linomat IV applicator were performed using a 100 mL syringe.

2.5. Sample preparation and standard solutions for both HPLC and TLC methods

Stock solution (1 mg/mL): For the preparation of stock solutions, 25 mg each of DAPA, MET HCl, MEL, and CYGN were weighed and transferred to four separate 25 mL volumetric flasks, then a few mLs of methanol were added and shaken to dissolve. Volumes were made up to the mark with methanol to produce a stock solution containing 1 mg/mL of each component.

Working solutions (100 µg/mL): Working methanolic solutions containing 100 µg/mL of each of DAPA, MET HCl, MEL and CYGN were prepared by transferring 2.5 mL of each of the stock solutions of each component to 25 mL volumetric flasks and completing the mark with methanol.

2.6. Chromatographic conditions

2.6.1. For the HPLC method

Chromatographic elution was achieved isocratically on the C_{18} Zorbax column (250 × 4.6 mm × 5 µm particle size) maintained at 25 ° C using a mobile phase consisting of methanol: water in a ratio of 90:10 (by volume) with a pH adjusted to 3.5 using o-phosphoric acid. The run time was 6 minutes and the flow rate was 1.2 mL/min. The injection volume was 5 µL with the UV detector adjusted at 225 nm.

2.6.2. For the TLC method

The Camag Linomat IV applicator was used to apply samples to TLC plates, with a slit of 6×0.3 mm. The application volume of the samples was 10 µL. A mixture of ethanol and ethyl acetate was used as a developing system in the ratio of 1:9 (by volume), then was added to the jar to be saturated for 15 min. The plates were then placed inside the jar until the developing system reached the front line of the plate. Finally, the plates were scanned using a UV scanner, at 225 nm.

2.7. Construction of calibration curves and linearity

2.7.1. For the HPLC method

Accurate aliquots were transferred from each component working solution (100 µg/mL) into four separate series of 10 mL volumetric flasks and the volumes were completed with

methanol, to obtain concentrations of 1-30, 2-70, 0.5-25 and 1-25 µg/mL for DAPA, MET HCl, MEL and CYGN, respectively. Different chromatograms were recorded at 225 nm. Calibration graphs were constructed relating the peak areas of each of DAPA, MET HCl, MEL, and CYGN versus the corresponding concentrations in µg/mL. Linear correlations were obtained for the four components and the corresponding regression Equations (1-4) were found to be the following:

$Y_1 = 0.0017 \times X_1 + 0.0067$, r_1=0.9999 for DAPA (1)

$Y_2 = 0.0042 \times X_2 + 0.0322$, r_2=0.9999 for MET HCl (2)

$Y_3 = 0.0035 \times X_3 + 0.0140$, r_3=0.9999 for MEL (3)

$Y_4 = 0.0025 \times X_4 + 0.0066$, r_4=0.9997 for CYGN (4)

where r is the correlation coefficient, X is the concentration in µg/mL and Y is the peak area $\times 10^{-4}$.

2.7.2. For the TLC method

Accurate aliquots equivalent to 100-1500 µg for DAPA, 200-3000 µg for MET HCl, 100-1500 µg for MEL and 100-1200 µg for CYGN were transferred from their working and stock solutions into four separate series of 10 mL volumetric flasks and the volumes were completed with methanol. Then 10 µL was applied in triplicate to the TLC plates (20 × 10 cm) using a Camag Linomat IV applicator. The procedure was applied under chromatographic conditions (Section 2.6.2) to obtain concentrations of 0.1-1.5, 0.2-3.0, 0.1-1.5 and 0.1-1.2 µg/band for DAPA, MET HCl, MEL and CYGN, respectively.

Different chromatograms were recorded at a UV detection wavelength of 225 nm. Calibration graphs were constructed that relate the peak areas obtained from each of DAPA, MET HCl, MEL, and CYGN versus the corresponding concentrations in µg/band. Linear correlations were obtained for the four components and the corresponding regression Equations (5-8) were found to be:

$Y_1 = 0.0136 \times X_1 - 0.0009$, r_1=0.9997 for DAPA (5)

$Y_2 = 0.0285 \times X_2 + 0.0032$, r_2=0.9999 for MET HCl (6)

$Y_3 = 0.0167 \times X_3 - 0.0007$, r_3=0.9999 for MEL (7)

$Y_4 = 0.0296 \times X_4 - 0.0020$, r_4=0.9998 for CYGN (8)

where r is the correlation coefficient, X is the concentration in µg/band and Y is the peak area $\times 10^{-5}$.

2.8. Application to pharmaceutical formulation (Diaflozimet 10/1000® tablets)

Ten tablets were carefully weighed and finely powdered. An amount equivalent to 10 mg of DAPA and 1000 mg of MET HCl was weighed from the powdered dosage form, dissolved in 100 mL of methanol, sonicated for 10 min and filtered to obtain a tablet solution containing 100 µg/mL for DAPA and 10000 µg/mL for MET HCl.

2.8.1. For the HPLC method

For the determination of DAPA: A suitable dilution with methanol was performed from the prepared DAPA working solution to obtain a tablet solution that contains 10 µg/mL of DAPA. The linearity procedure (Section 2.7.1) was applied and the concentrations of DAPA were calculated from the calculated regression equations. Then, the mean recoveries and standard deviations (SD) were calculated.

For the determination of MET HCl: One mL of the tablet stock solution was diluted to 100 mL using methanol to obtain a working solution of tablets containing 100 µg/mL MET HCl, then the appropriate dilution was made to obtain a tablet dilution used for the determination of MET HCl (20 µg/mL).

The linear procedure (Section 2.7.1) was followed, and the concentrations of MET HCl were calculated using the respective regression equation. Then, the mean recoveries and standard deviations (SD) were calculated.

2.8.2. For the TLC method

For the determination of DAPA: Accurate aliquots equivalent to 500 µg DAPA were transferred from its working solution (100 µg/mL) into a 10 mL volumetric flask and the volume was completed to the mark with methanol. Then 10 µL was applied in triplicate to the TLC plate to obtain a tablet solution that contains 0.5 µg/band of DAPA. The procedure under linearity (Section 2.7.2) was applied and concentrations of DAPA were calculated from the computed regression equations. Then, the mean recoveries and standard deviations (SD) were calculated.

For the determination of MET HCl: Five mL of tablet stock solution (10000 µg/mL) was diluted to 50 mL using methanol to obtain a working solution of tablets containing 1000 µg/mL MET HCl then accurate aliquots equivalent to 2000 µg MET HCl were transferred from its working solution (1000 µg/mL) into 10 mL volumetric flask and the volume was completed to the mark with methanol. Then 10 µL was applied in triplicate to the TLC plate to obtain a tablet solution containing 2 µg/band of MET HCl.

The procedure under linearity (Section 2.7.2) was followed and concentrations of MET HCl were calculated using respective regression equation. Then, the mean recoveries and standard deviations (SD) were calculated.

2.9. Standard addition technique

Different concentrations of pure DAPA and pure MET HCl were added separately to the dosage form solutions to perform the standard addition technique, then applying the linear procedure (Section 2.7.1) and (Section 2.7.2) to evaluate the precision of the methods.

3. Results and Discussion

3.1. Development of chromatographic methods and their optimization

The main aim of this work was to develop simple, accurate, and environmentally friendly less toxic green analytical HPLC and TLC methods for the quantitative determination of DAPA and MET HCl and its two potential impurities, MEL and CYGN, in pure forms and in pharmaceutical formulations, with high resolution and selectivity. Moreover, three greenness assessment tools were applied to evaluate the greenness criteria of the developed HPLC and TLC methods; national environmental method index (NEMI), analytical eco-scale and assessment of greenness profile (AGP).

3.1.1. For the HPLC method

To optimize various HPLC parameters such as resolution, peak shape, run time, and retention times, several trials were made.

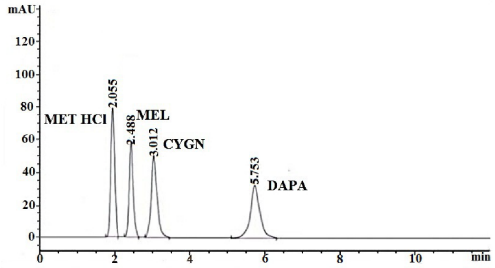

Figure 2. Green HPLC separation of a mixture of 25 µg/mL of each of dapagliflozin, metformin hydrochloride, melamine and cyanoguanidine using methanol:water in the ratio of 90:10(by volume), pH adjusted to 3.5 using *o*-phosphoric acid.

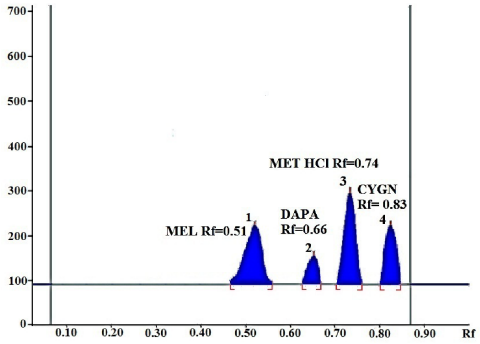

Figure 3. Two-dimensional green TLC chromatogram of a mixture of dapagliflozin (R_f =0.66), metformin hydrochloride (R_f = 0.74), melamine (R_f = 0.51) and cyanoguanidine (R_f = 0.83) using a developing system consisting of ethanol: ethyl acetate (1:9, by volume) at 225 nm.

Mobile phase: Various trials were carried out using different mobile phases, including methanol: buffer (triethanolamine adjusted to pH = 3.5 with orthophosphoric acid in a ratio of 80:20% (by volume) [25], acetonitrile:water (75:25%, by volume) [26] and buffer: acetonitrile (60:40%, by volume) [27] which resulted in poor separation. Different ratios of the mobile phase components consisting of methanol: water were tried as 60:40 (by volume) and 70:30 (by volume). It was found that the peaks of the three components (MET HCl, MEL and CYGN) exhibited a single peak, but when the ratio of the mobile phase was changed to 90:10 (by volume), the peaks of the mentioned three components were separated with tailing. Therefore, the pH was adjusted to 3.5 using o-phosphoric acid to provide optimal separation, resolution of the four peaks without tailing and maintaining the eco-friendliness of the solvents, Figure 2.

Effect of pH: Various pH values were tested, including (acidic and alkaline pH). An acid pH adjusted to 3.5 using o-phosphoric acid was found to be the best for the optimal separation of the four peaks without tailing.

Scanning wavelength: Different wavelengths were studied for detection of the effluent, including 245, 285, 240, 266 and 225 nm; it was found that detection at 225 nm was found to be the best scanning wavelength that shows optimum sensitivity for the four components.

Flow rate: Various flow rates were tested, including 0.8, 0.5, 1 and 1.2 mL/min to provide good separation and resolution of the peaks. The best flow rate used was 1.2 mL/min, which resulted in proper separation of the four components in a short run time (6 minutes).

The R_t values of DAPA, MET HCl, MEL and CYGN were found to be 5.75, 2.06, 2.49 and 3.01 min, respectively, with no interference among the peaks, Figure 2.

3.1.2. For the TLC method

To optimize various TLC parameters such as resolution, peak shape, and R_f, several trials were made.

Developing system: The optimum developing system was determined by performing various trials, including methanol: chloroform with different ratios and with or without ammonia, where MEL was close to the baseline. Ethanol:ethyl acetate:ammonia (1:9:0.1, by volume) was tested where CYGN was very close to the front line. Finally, optimum separation and good resolution between the four components, the best R_f values, and eco-friendliness were obtained when ethanol: ethyl acetate (1:9 by volume) was used as a developing system, as shown in Figure 3.

Scanning wavelength: Different wavelengths were tried to achieve the maximum sensitivity for the four components, including 245, 285, 240, 266 and 225 nm. It was found that 225 nm was the best scanning wavelength for reaching the maximum possible sensitivity among the four components. The R_f values were found to be 0.66, 0.74, 0.51 and 0.83 for DAPA, MET HCl, MEL and CYGN respectively, with no overlap of the peaks as shown in Figure 3.

3.2. Method validation

It was performed in accordance with the ICH guidelines for analytical method validation [31].

3.2.1. Linearity

The good linearity of the proposed HPLC and TLC methods was evident from the closeness of the correlation coefficient to 1 and the low values of the intercepts, as shown in Table 1.

3.2.2. Repeatability

Repeatability was determined by analyzing three concentrations three times on the same day. The assayed concentrations were (1, 10, 25 µg/mL), (2, 25, 50 µg/mL), (0.5, 10, 20 µg/mL) and (1, 10, 20 µg/mL) for DAPA, MET HCl, MEL and CYGN, respectively, using the HPLC method and (0.1, 0.6, 1.5 µg/band), (0.2, 1, 3 µg/band), (0.1, 0.8, 1.5 µg/band) and

Table 1. Results of the validation parameters of the proposed HPLC and TLC methods for the determination of dapagliflozin, metformin HCl, melamine, and cyanoguanidine.

Parameters	RP-HPLC method				Parameters	TLC method			
	DAPA	MET HCl	MEL	CYGN		DAPA	MET HCl	MEL	CYGN
Linearity range (µg/mL)	1-30	2-70	0.5-25	1-25	Linearity range (µg/band)	0.1-1.5	0.2-3	0.1-1.5	0.1-1.2
Slope	0.0017	0.0042	0.0035	0.0025	Slope	0.0136	0.0285	0.0167	0.0296
Intercept	0.0067	0.0322	0.0140	0.0066	Intercept	-0.0009	0.0032	-0.0007	-0.0020
Correlation coefficient	0.9999	0.9999	0.9999	0.9997	Correlation coefficient	0.9997	0.9999	0.9999	0.9998
LOD *	-	-	0.158	0.339	LOD *	-	-	0.034	0.028
LOQ *	-	-	0.479	1.026	LOQ *	-	-	0.102	0.086
Accuracy (Mean recovery %)	100.98	99.79	100.70	101.16	Accuracy (Mean recovery %)	100.19	99.84	100.33	100.17
Precision repeatability [a]	0.698	1.052	0.527	0.436	Precision repeatability [a]	0.531	0.699	0.579	0.784
Intermediate precision [b]	0.903	1.343	0.762	0.686	Intermediate precision [b]	0.917	0.964	0.839	1.044

[a] The intraday precision (n = 9), an average of three different concentrations repeated three times within one day. The assayed concentrations for DAPA, MET HCl, MEL and CYGN were (1,10, 20 µg/mL), (2, 25, 50 µg/mL), (0.5, 10, 20 µg/mL) and (1,10, 20 µg/mL) for the HPLC method and (0.1, 0.6, 1.5 µg/band), (0.2,1, 3 µg/band), (0.1, 0.8, 1.5 µg/band)and (0.1, 0.6, 1.2 µg/band) for the TLC method.
[b] Interday precision (n = 9), an average of three different concentrations repeated three times on three successive days.
* The detection and quantification limits are determined by calculations (LOD=3.3×SD of the response / slope, LOQ=10×SD of the response/slope).

Table 2. Results of the determination of dapagliflozin and metformin HCl in their dosage form by the proposed HPLC and TLC methods and application of the standard addition technique.

Dosage form	HPLC method					TLC method				
	Taken µg/mL	Found (%±SD)	Standard addition (mean± SD)			Taken µg/band	Found (%±SD)	Standard addition (mean± SD)		
			Pure added	Pure found	Found (%±SD)			Pure added	Pure found	Found (%±SD)
DAPA	10	101.28±0.558	2	2.002	100.10±0.091	0.5	101.33±0.650	0.1	0.101	101.00±0.147
			5	5.056	101.12±0.099			0.3	0.302	100.67±0.086
			10	10.046	100.46±0.134			0.5	0.513	102.60±0.215
					100.56±0.422					101.42±0.843
MET HCl	20	100.98±0.719	2	2.048	102.40±0.299	2	100.63±0.921	0.2	0.205	102.50±0.497
			5	5.065	101.30±0.082			0.6	0.614	102.33±0.446
			10	10.180	101.80±0.216			0.8	0.796	99.50±0.455
					101.83±0.450					101.44±1.376

Table 3. Results of the study of robustness of the proposed HPLC and TLC methods (RSD%) for dapagliflozin, metformin HCl, melamine, and cyanoguanidine *.

Robustness parameters in HPLC	DAPA	MET HCl	MEL	CYGN	Robustness parameters in TLC	DAPA	MET HCl	MEL	CYGN
Mobile phase ratio methanol (90±1%)	0.542	0.612	0.316	0.254	Ethyl acetate (9 mL±10%)	0.355	0.476	0.389	0.692
Flow rate (1.2±5 %)	0.346	1.004	0.651	0.583	Saturation time 15±5 min (33.33%)	0.784	0.735	0.644	0.837
Detection wavelength 225±0.5 nm (0.22%)	0.733	0.968	0.453	0.475	Detection wavelength 225±0.5 nm (0.22%)	0.461	0.614	0.498	0.581

* The results in the table represent (RSD%) for AUC.

(0.1, 0.6, 1.2 µg/band) for DAPA, MET HCl, MEL and CYGN, respectively, using the TLC method. Table 1 shows the calculated RSD% values.

3.2.3. Intermediate precision

The intermediate precision was determined by assaying the previous concentrations chosen for the two proposed methods three times on three successive days. Table 1 shows the calculated RSD% values.

3.2.4. LOD and LOQ

The limit of detection (LOD) and limit of quantitation (LOQ) are shown in Table 1**.** The slope of the calibration curve and the standard deviation of the response were used to calculate the limits of detection and quantitation. The formula (LOD = 3.3 × SD of the response/slope, LOQ = 10 × SD of the response/slope) was used to determine them [31].

3.2.5. Accuracy

The accuracy of the proposed methods was estimated by calculating the mean recoveries of different blind samples of pure components, Table 1. The accuracy was also verified through the application of the standard addition technique to the pharmaceutical formulation Table 2.

3.2.6. Selectivity

The selectivity of the proposed methods was shown by the good separation of the four components in the green HPLC and TLC chromatograms, as shown in Figures 2 and 3, and the good results of the dosage form analysis suggest good selectivity, since there is no interference from additives.

3.2.7. Robustness

The robustness of the proposed HPLC method measures its ability to be unaffected by small variations in method parameters and provides an indication of its reliability during normal usage, including changes in the mobile phase ratio (methanol±1%), flow rate ±5% and detection wavelength ±0.22%. While the robustness of the proposed TLC method was evaluated by measuring its ability to be unaffected by small changes in the method parameters such as the change in the ratio of the developing system (ethyl acetate±10%), saturation time (15 min±33.33%) and the detection wavelength ±0.22% [31], as shown in Table 3.

3.2.8. System suitability

The suitability test for the proposed HPLC and TLC methods was established to confirm the performance of the system using the following parameters: capacity factor (K), tailing factor (T), column efficiency (N), selectivity factor (α), resolution (R) and symmetry factor, and the system was found to be suitable [32,33], as shown in Tables 4 and 5.

Table 4. System suitability testing parameters of the HPLC method for the determination of dapagliflozin, metformin HCl, melamine, and cyanoguanidine.

Parameters	RP-HPLC method				
	MET HCl	MEL	CYGN	DAPA	Reference [32]
Resolution (R)	1.665	1.564	3.916		> 1.5
Selectivity factor (α)	1.371	1.327	2.290		> 1
Tailing factor (T)	0.968	1.000	1.048	1.008	~ 1
Capacity factor (K´)	1.317	1.805	2.396	5.486	1-10 acceptable
Column efficiency (N)	17297.510	21737.669	14515.430	8472.834	Increase with the efficiency of separation
HETP [a]	0.014	0.012	0.017	0.030	-

[a] HETP, height equivalent to theoretical plate (mm/plate). The smaller the value, the higher the column efficiency.

Table 5. System suitability testing parameters of the TLC method for the determination of dapagliflozin, metformin HCl, melamine, and cyanoguanidine.

Parameters	TLC method				
	MEL	DAPA	MET HCl	CYGN	Reference [33]
Resolution (R)	2.235	1.714	1.867	-	> 1.5
Selectivity factor (α)	1.866	1.467	1.712	-	> 1
Symmetry factor	0.929	0.968	1.000	1.029	~ 1
Capacity factor (K´)	0.961	0.515	0.351	0.205	0 < K´ < 10

Table 6. Statistical comparison of the results obtained by the proposed HPLC and TLC methods and the reported method [25] for the determination of dapagliflozin and metformin HCl in their pharmaceutical preparation.

Diaflozimet 10/1000®tab	Items	HPLC method		TLC		Reported method [c]	
		DAPA	MET HCl	DAPA	MET HCl	DAPA	MET HCl
	Mean	101.28	100.98	101.33	100.63	101.41	101.44
	SD	0.558	0.719	0.650	0.921	0.458	0.672
	Variance	0.374	0.620	0.507	1.019	0.251	0.542
	n	6	6	6	6	6	6
	Student's t-test (2.228) [a]	0.696	0.319	0.827	0.143	-	-
	F-value (5.050) [b]	1.490	1.144	2.020	1.880	-	-

[a] The figures in parentheses represent the corresponding tabulated t value at p = 0.05.
[b] Figures in parentheses represent the corresponding tabulated F value at p = 0.05
[c] The HPLC method [25] uses a mobile phase consisting of methanol: Buffer (Triethanolamine adjusted to pH = 3.5 with orthophosphoric acid in a ratio of 80:20 % (by volume) at a flow rate of 0.8 mL/min. At 245 nm, a photodiode array detector was used to monitor the analytes.

Table 7. Analytical eco-scale penalty points of the proposed HPLC method for simultaneous determination of the proposed components.

Solvents	RP- HPLC method			
	Reagents			
	No of pictograms	Amount PP	Hazard [a] PP	Total penalty points [b]
Methanol	3	90 mL (10-100) = 2	Danger (more severe hazard) 3×2 = 6	2×6 = 12
Water	none	10 mL (10-100) = 2	0	0
o-Phosphoric acid	1	<10 mL = 1	Danger (more severe hazard) 1×2 = 2	1×2 = 2
The instruments	-		-	-
Energy used	≤1.5 k Wh/ sample		-	1
Occupational hazard	-		-	0
Waste [c]	1.2× 6 = 7.2 (1-10 mL)		-	3
Total penalty points	-		-	∑18
Eco-scale score	-		-	82

[a] Hazard penalty points=No. of pictograms×signal. The signal may be warning=1 or danger=2.
[b] The total penalty points=the amount of penalty points × hazard penalty points.
[c] For the HPLC method: Waste=flow rate × run time.

3.2.9. Statistical comparison

When the suggested methods (green HPLC and TLC methods) and the published method (HPLC method) were applied to DAPA and MET HCl in their pharmaceutical formulation Diaflozimet 10/1000® tablets (Batch No: 2307801), a statistical comparison of the results produced was performed, where the reported HPLC method used a mobile phase consisting of Methanol: Buffer (Triethanolamine adjusted to pH = 3.5 with orthophosphoric acid in a ratio of 80:20 %, by volume) at a flow rate of 0.8 mL/min. A photodiode array detector set to 245 nm was used to monitor the analytes [25]. The advantage of the proposed methods over the reported method is the quantification of four analytes; DAPA, MET HCl, MEL, and CYGN, while the reported method quantifies only DAPA and MET HCl. The calculated values of t and F were smaller than the theoretical ones, indicating that there is no significant difference between the proposed methods and the reported method with respect to accuracy and precision (Table 6).

3.2.10. Greenness profile of the proposed HPLC and TLC methods

The greenness profile is used to evaluate the greenness of any analytical method using four criteria for the solvents used: not to be PBT (persistent, bioaccumulative, or toxic), not to be hazardous or corrosive (pH is between 2 and 12), and not to generate waste >50 g/sample (NEMI assessment method).

For the HPLC method, the solvents used in the mobile phase satisfy the four greenness criteria (NEMI assessment tool) since water is the best green solvent and methanol is in the second category of recommended green solvents. They are not listed as PBT. The pH of the mobile phase is about 3.5. The generated waste is 7 mL/ run, which determines the four components in Figure 4. The analytical eco-scale is the second assessment tool [34] used to demonstrate the excellent green analysis for the proposed HPLC method. Table 7 shows the eco-scale score that was determined. The results showed that the proposed method had an eco-scale score equal to 82 points, indicating the greenness of the method, where the Globally Harmonized System of Classification and Labeling of Chemicals [34] states that the procedure is considered green when the final score is greated than 75 points. The assessment of the greenness profile (AGP) method is the third greenness assessment tool [35]. A pentagram divided into five parts (environmental, health, safety, energy, and waste) expresses the green assessment tool. The AGP pentagram is indicated by three colors: green, yellow, or red, as shown in Figure 5.

Table 8. Analytical eco-scale penalty points of the proposed TLC method for simultaneous determination of the proposed components.

Solvents	TLC method			
	Reagents			
	No of pictograms	Amount PP	Hazard [a] PP	Total penalty points [b]
Ethanol	2	3 mL (<10 mL) = 1	Danger (more severe hazard) 2×2 = 4	1×4 = 4
Ethyl acetate	2	27 mL (10-100) =2	Danger (more severe hazard) 2×2 = 4	2×4 = 8
The instruments	-		-	-
Energy used	≤1.5 k Wh/ sample		-	1
Occupational hazard	-		-	0
Waste [c]	30/10 = 3 (1-10 mL)		-	3
Total penalty points	-		-	∑16
Eco-scale score	-		-	84

[a] Hazard penalty points=No. of pictograms×signal. The signal may be warning=1 or danger=2.
[b] The total penalty points=the amount of penalty points × hazard penalty points.
[c] For the TLC method: Waste=volume of the developing system / no spots per plate.

Table 9. Analytical eco-scale penalty points of the reported HPLC method [25] for the simultaneous determination of the proposed components.

Solvents	RP-HPLC method			
	Reagents			
	No of pictograms	Amount PP	Hazard [a] PP	Total penalty points [b]
Methanol	3	80 mL (10-100) = 2	Danger (more severe hazard) 3×2 = 6	2×6 = 12
Triethanolamine	4	20 mL (10-100) = 2	Danger (more severe hazard) 4×2 = 8	2×8 = 16
o-Phosphoric acid	1	<10 mL = 1	Danger (more severe hazard) 1×2 = 2	1×2 = 2
The instruments	-		-	-
Energy used	≤1.5 k Wh/ sample		-	1
Occupational hazard	-		-	0
Waste [c]	0.8× 10 = 8 (1-10 mL)		-	3
Total penalty points	-		-	∑34
Eco-scale score	-		-	66

[a] Hazard penalty points=No. Of pictograms×signal. The signal may be warning=1 or danger=2.
[b] The total penalty points=the amount of penalty points × hazard penalty points.
[c] For the HPLC method: Waste=flow rate × run time.

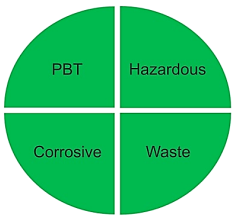

Figure 4. The greenness profile of the proposed HPLC and TLC methods using the NEMI assessment tool.

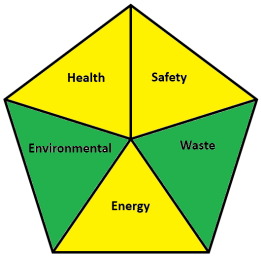

Figure 5. Assessment of the greenness profile (AGP pentagram) of the green proposed HPLC and TLC methods.

For the TLC method, ethanol and ethyl acetate, which were used as the developing system in the proposed TLC method, are not listed as PBTs, as ethanol is the best green solvent (NEMI assessment tool). Figure 4 shows that the resulting waste is 3 mL/sample. The results of the analytical ecoscale proved that the proposed TLC method had an eco-scale score equal to 84 points (Table 8), ensuring that the TLC method is greener than the HPLC method. The pentagram (AGP) of the developed TLC method is shown in Figure 5.

From a greenness point of view, the proposed green HPLC and TLC methods (Tables 7 and 8) were compared to the reported one [25] regarding the analytical eco-scale assessment tool (Table 9). It is obvious that the reported method is not considered green, as its final score is below 75. Hence, the proposed methods improve upon non-green reported one in that they were developed to minimize waste for routine analysis without endangering the environment and to neither use nor produce hazardous chemicals. Short run times and green mobile phases were used to accomplish this, as presented in Tables 7-9.

4. Conclusions

The proposed ecofriendly methods present new green, selective, rapid, and precise HPLC and TLC methods for the determination of dapagliflozin, metformin hydrochloride, melamine, and cyanoguanidine in their pure forms and in their dosage form. The present work shows the ability to resolve and separate the chromatographic peaks with optimum retention times and R_f values. Hence, the precision, reliability, and selectivity of the two proposed methods make them advantageous to the other reported methods.

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