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DEVELOPMENT AND VALIDATION OF GREEN HPTLC METHOD FOR SIMULTANEOUS DETERMINATION OF DAPAGLIFLOZIN AND LINAGLIPTIN IN COMBINED DOSAGE FORM

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A simple, accurate, rapid, sensitive and green high performance thin layer chromatography (HPTLC) technique was developed for the simultaneous determination of Dapagliflozin (DAPA) and Linagliptin (LINA) in pure and pharmaceutical dosage form. Chromatographic separation of both the drugs was performed on precoated silica gel aluminium plate 60 F 254, (10 X 10 cm), 100 µm thickness; using the solvent system chloroform: methanol: ethyl acetate: 1 % formic acid (3:4:3:0.5, v/v). Short-wave ultraviolet light at 224 nm was used to view the chromatographic bands. The proposed technique revealed compact bands with retention factor (Rf) values of 0.66 and 0.20, for DAPA and LINA, respectively. Calibration curves were polynomial in the concentration range of 3000-9000 ng/band for DAPA and 1500-4500 ng/band for LINA, with correlation coefficients of 0.9951 and 0.9969, respectively. The proposed method was validated with respect to ICH guideline. Green assessment of the developed HPTLC method was assessed using different green analytical chemistry metrics such as Analytical GREEness (AGREE), and Green Analytical Procedure Index (GAPI). The proposed method was applied for the analysis of drug in tablet formulation, and it can be used for routine quality control analysis.

Keywords: *Dapagliflozin, Linagliptin, HPTLC, Green assessment, Validation*

INTRODUCTION

Dapagliflozin (DAPA), also known as SGLT2, is a protein that belongs to the sodium-glucose transporter subtype 2. The compound is designated as (2S,3R,4R,5S,6R).2–[4-chloro-3–[(4–ethoxyphenyl) methyl]phenyl] -6-(hydroxymethyl)oxane3,4,5-trio **(Fig. 1(a))** with the molecular weight of 408.873g/mol and the empirical formula $C_{21}H_{25}ClO_6$, It acts as an inhibitor by preventing the kidney's proximal tubule site from containing sodium glucose cotransporter 2 (SGLT2). Being an anti-diabetic, it lowers blood sugar by stopping the kidneys from reabsorbing glucose[1]. The IUPAC nomenclature for Linagliptin (LINA) is 8-[(3R)-3-aminopiperidin-1-yl].4-methylquinazolin-2-yl [methyl [2-ynyl-3-methyl-7-but-1-]] purine-2,6-dione **(Fig. 1 (b))**. In addition, the molecular weight is 472.5 g/mol and the chemical formula is $C_{25}H_{28}N_8O_2$. Dipeptidyl peptidase 4 is inhibited by it, which raises insulin production and decreases glucagon release, giving rise to its anti-glycemic action[2]. There are two antidiabetic ingredients in this DAPA and LINA combined formulation. LINA has been reported to be quantified alone by different techniques like-HPTLC and by analytical methods likeRP-HPLC when combined with Metformin and Empagliflozin[11-20]. It is beneficial and practical to take the two drugs in combination to treat Type 2 Diabetes since they complement each other and perform well together. 3. Bulk and dosage form alone or along with others. These methods include UV Spectroscopy[4], RP-HPLC method [5], and the use of analytical techniques such as RP-HPLC, HPTLC, UPLC, etc. in conjunction with other medications such as metformin and saxagliptin[6–10]. Upon doing a

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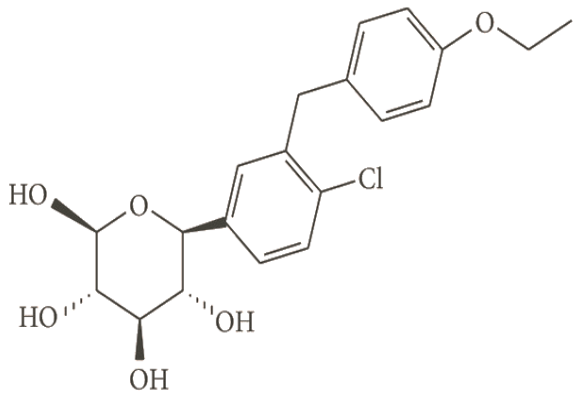
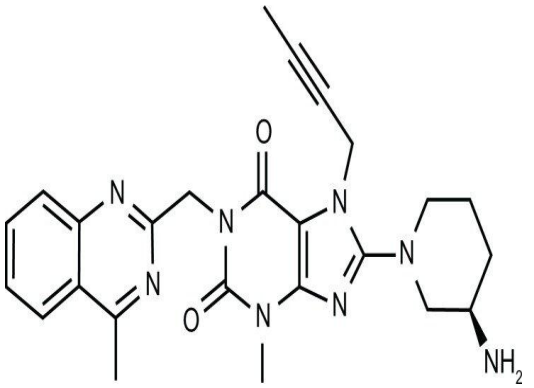
comprehensive literature study, it was found that only one article has been published with RP-HPLC technique for this combination [21]. Hence, an exact, accurate, sensitive, repeatable, and green HPTLC technique was used to quantify the 1:2 fixed dose combination of DAPA and LINA in bulk and mixed dosage form.

High-performance thin-layer chromatography (HPTLC) is an automated variation of thin-layer chromatography (TLC) with smaller sorbent particles and pores, shorter analysis times, and more efficient development chambers that use less mobile phase. Sample application in HPTLC is automated, and a UV/Visible/Fluorescence scanner—a sophisticated kind of densitometer—scans the whole chromatogram both qualitatively and quantitatively. The pharmaceutical industry has come to rely more and more on it due to its advantages, which include better separation efficiency and detection limits, reduced analysis costs, shorter analysis times, no need for solvent pre-treatment (such as filtration and degassing), lower mobile phase consumption per sample, and the removal of interference from previous analyses because each analysis uses a fresh stationery phase and mobile phase. Further more it allows application up to 18 samples in same plate to be determined in single run.[22, 23].

The method was designed with the goal of minimizing or completely doing away with the usage and production of dangerous materials such as solvents, reagents, and by products that are harmful for the environment or human health is known as "green chemistry." Paul Anastas and John Warner published the twelve principles of green analytical chemistry (GAC) in 1998. These design guidelines serve as a foundation for sustainable design[24]. In a nutshell, the three main ideas of green chemistry are as follows: I). It designs for every stage of the chemical life cycle. II) It aims to minimize the inherent hazards of chemical processes and products by designing their intrinsic character. Thirdly III, it functions as a logical set of guidelines or standards for design. Many metrics have been created to evaluate how environmentally friendly analytical techniques are. While some are only applicable to certain analytical methods, others are more frequently used and have a wide range of uses [25]. The Green Analytic Procedure Index (GAPI)[26,27] and Analytical GREEness (AGREE) measurements[28, 29] were among the metrics used to evaluate the novel HPTLC technique. Thus, the invention and validation of a rapid, environmentally friendly, sensitive, and precise HPTLC technique for the simultaneous measurement of DAPA and LINA in the pure and commercial formulation would be the main goals of this work.



(a)



(b)

Fig. 1: Chemical structure of (a) DAPA and (b) LINA.

Experimental

MATERIAL AND METHODS

Instruments

The HPTLC apparatus consists of a 100 µl syringe (Hamilton, Bonaduz, Switzerland) and a Linomat V sample applicator manufactured by CAMAG (Muttenz, Switzerland). 10 x 10 cm aluminum HPTLC plates precoated with silica gel F254 (E. Merck, Darmstadt, Germany; provided by Anchrom Technologists, Mumbai, India) were used for chromatography. The absorbance mode of HPTLC densitometric scanning was operated using the TLC scanner 4 and VisionCATS software version 1.4.8 (CAMAG, Muttenz, Switzerland). The deuterium lamp was the source of the radiation. The sample was applied while a steady stream of nitrogen gas was drying it off.Using a Camag Twin trough glass chamber, the solvent solution was created by mixing. The ATX 200 electronic balance, manufactured by Shimadzu Corp. in Japan, was utilized for the weighing of chemicals and pharmaceuticals.

Chemicals and Reagents

Analytically pure DAPA and LINA were received as gift samples from Ajanta Pharma Limited in Mumbai, India, and Ami Life Sciences Pvt. Ltd. in Vadodara, Gujrat, India. Merck India Ltd. provided the methanol (HPLC grade) and chloroform. We purchased AR-grade formic acid and ethyl acetate from S.D. Fine Chemicals, Ahmedabad. LINA 5 mg and 10 mg DAPA in the marketed formulation were purchased from a nearby drugstore.

Chromatographic System

Sample application

On the HPTLC plates, standards and formulation samples of DAPA and LINA were applied as narrow bands, each measuring 6 mm in length. The bands were applied with a gap of 10.4 mm between them, 15 mm from the left and 8 mm from the bottom borders. The samples were put under a continuous stream of nitrogen gas drying at a constant application rate of 150 nL/s.

Mobile Phase and Migration

A mobile phase comprising of Chloroform: methanol: ethyl Acetate: 1 % formic acid (3:4:3:0.5, v/v) was used to develop the plates. The development process was linear rising in a twin-trough chamber that was in equilibrium with the mobile phase. At 28 ± 2◦C, the ideal chamber saturation time for the mobile phase was 20 minutes. For each development, ten millilitres of the mobile phase (5 mL in the trough holding the plate and 5 mL in another trough) were utilized, and it took 11 minutes for the mobile phase to migrate 70 mm. The TLC plates were allowed to dry at room temperature for two minutes following development.

Densitometric Analysis

VisionCATS software was used to manage the absorbance mode of densitometric scanning. The deuterium lamp served as the radiation source, and the bands were scanned at 224 nm. The slit measured 5 mm in length and 0.45 mm in width, and it scanned at a speed of 20 mm per second. For every established band, peak area and peak height data were acquired, and a regression equation was created by graphing peak areas against concentration.

Preparation of standard stock solution of DAPA and LINA

Validation

For the purpose of validating analytical methods, the International Council on Harmonization (ICH) released guideline Q2 (R1)[30]. The process that was established was verified in terms of robustness, linearity, accuracy, precision, specificity, and sensitivity.

Linearity and Calibration curves

The linearity of the method was assessed by creating calibration curves for DAPA and LINA at five different concentration levels, spanning a range of 3000- 9000 ng/band and 1500-4500 ng/band, respectively. Plotting peak area vs concentration (n=6) led to the development of the calibration curves.

Accuracy

By calculating the recoveries of DAPA and LINA using the standard addition technique, the correctness of the procedure was ascertained. A prequantified sample solution was spiked with known concentrations of DAPA and LINA at 80,100, and 120%. By using the TLC plate under the previously stated

chromatographic conditions, the resultant solutions were examined. The peak area was determined, and the regression equation was used to estimate the quantities of DAPA and LINA.

Precision

Repeatability

The scanner's repeatability was tested by scanning the same area six times, calculating the peak area and % RSD.The reproducibility of injection was assessed by administering DAPA (6000 ng/band) and LINA (3000 ng/band) six times on the same plate in order to estimate the repeatability of peak area measurement.

Intermediate Precision

Both intraday and interday accuracy were assessed. By analyzing sample solutions of DAPA (3000, 6000, and 9000 ng/band) and LINA (1500, 3000, and 4500 ng/band) at three levels covering low, medium, and high concentrations of the calibration curves three times on the same day and over a period of three days, respectively,

Specificity

The new method's specificity was evaluated by contrasting tablet formulation samples with reference medications. By overlaying the peak purity spectra and comparing the Rf with the standard, the band for DAPA and LINA in the sample were verified. By comparing the spectra at three distinct levels—the peak start (S), peak apex (M), and peak end (E) location of the band—the peak purities of DAPA and LINA were verified.

Sensitivity

The lowest concentration of analyte that can be detected was established to be the limit of detection (LOD), and the lowest quantity of analyte that can be quantified by the technique was determined to be the limit of quantification (LOQ). In accordance with ICH recommendations, LOD and LOQ were computed using the following formula.

LOD is 3.3 X σ/s.

LOQ is 10 X σ/s.

where, on the calibration curve, s is the slope and σ is the standard deviation of the y-intercept.

Robustness

The impact of slight modifications to the chamber saturation time, migration distance and mobile phase ratio on the outcomes were investigated. The samples were applied three times, and the % RSD was computed.

Analysis of pharmaceutical dosage form

After being weighed (average weight: 203.025 mg), twenty tablets were finely powdered. In a 25 ml volumetric flask, tablet powder equivalent to 50 mg of DAPA and 25 mg of LINA were transferred. 15 ml of methanol was added to the flask and allowed to sonicate for 5 minutes. The flask was then cooled at room temperature and the volume was adjusted with the methanol. The solution was filtered through Whatman filter paper (No. 42) to obtain concentrations of 2000 µg/ml of DAPA and 1000 µg/ml of LINA. 1000 µg/ml of DAPA and 500 µg/ml of LINA were obtained by transferring an aliquot of 12.5 ml from the aforesaid solution into a 25 ml volumetric flask and adding methanol to bring the volume up to the required level. Using a Hamilton syringe and continuous nitrogen gas steam, 6 µl of solutions were applied to precoated HPTLC plates. This resulted in a band with 6000 ng/band for DAPA and 3000 ng/band for LINA, which were then analyzed using the suggested methodology. After being produced under ideal chromatographic conditions, the stationery phase plates were scanned. After identifying the peak locations, the quantification process involved inserting this value into the regression equation.

RESULTS AND DISCUSSION

Results

The polarity of the solvent was taken into consideration while choosing the mobile phase for the development of an HPTLC technique for the simultaneous measurement of DAPA and LINA. This development required bands that were both compact and well-resolved. To ensure that both medications could be properly resolved, a variety of solvents were explored in varying ratios, including methanol, toluene,

chloroform, hexane, butanol, acetone, and ethyl acetate alone and in combination with other solvents **(Table 1)**. The mobile phase was chosen to be a combination of Chloroform: methanol: ethyl acetate: 1 % formic acid (3:4:3:0.5, v/v) which gave a distinct and compact band of DAPA and LINA with Rf value of 0.66 and 0.22, respectively **(Fig. 2)**. Densitometric measurements in scanning mode were performed on the produced plate in the UV region between 200 and 400 nm in order to determine the detection wavelength. A TLC scanner was then used to capture the overlay spectrum. Both drugs were significantly absorbed at 224 nm, as seen by the superimposed spectra. It was therefore chosen as the detecting wavelength. The ideal solvent migration distance was 70 mm, while the ideal mobile phase chamber saturation duration was 20 minutes.

Validation

Linearity and calibration curves

For DAPA and LINA, the calibration curves were drawn in the range of 3000-9000 ng per band and 1500-4500 ng per band, respectively, indicating a linear correlation coefficient (r2) of 0.9951 and 0.9969, respectively. The analytical method's applicability is demonstrated by the regression data, which are displayed in **Table 2** and indicate a strong linear connection throughout the concentration range under investigation. In **Fig. 4**, an overlaying three-dimensional densitogram is shown, whereas **Fig. 3** displays the regression curves.

Table 1: Different trials for optimization of mobile phase.

Trial No	Mobile Phase (% v/v)	Inference
1	Methanol: ethyl acetate: acetonitrile (2.5:5.5:2, v/v)	No separation
2	Methanol: ethyl acetate: acetonitrile (3:4.5:2.5, v/v)	No separation
3	Methanol: ethyl acetate: acetonitrile (2:7:1, v/v)	No separation
4	Chloroform: methanol (9:1, v/v)	No separation
5	Hexane: toluene: ethyl acetate (4:2:4, v/v)	No separation
6	Chloroform: methanol: ethyl acetate (3:4:3, v/v)	Good resolution but slight tailing in DAPA
7	Chloroform: methanol: ethylacetate:1% formic acid (3:4:3:0.5, v/v)	Both drugs show good resolution and no tailing

Table 2: Regression analysis data for calibration curve.

Parameters	**DAPA**	**LINA**
Method linearity (ng/band)	3000-9000	1500-4500
Correlation co-efficient, r^2	0.9951	0.9969
Slope of regression equation (mean)	0.1487	0.0676
Intercept of regression equation	376	393
SD of Intercept	9.70	6.037

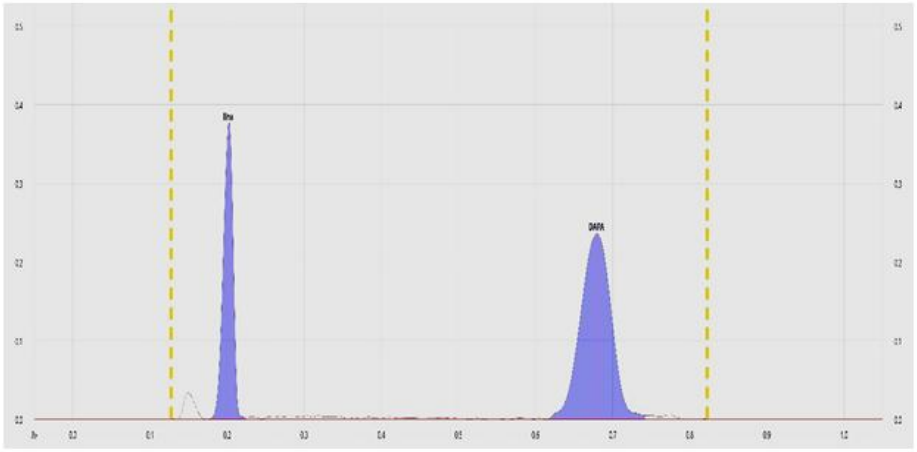
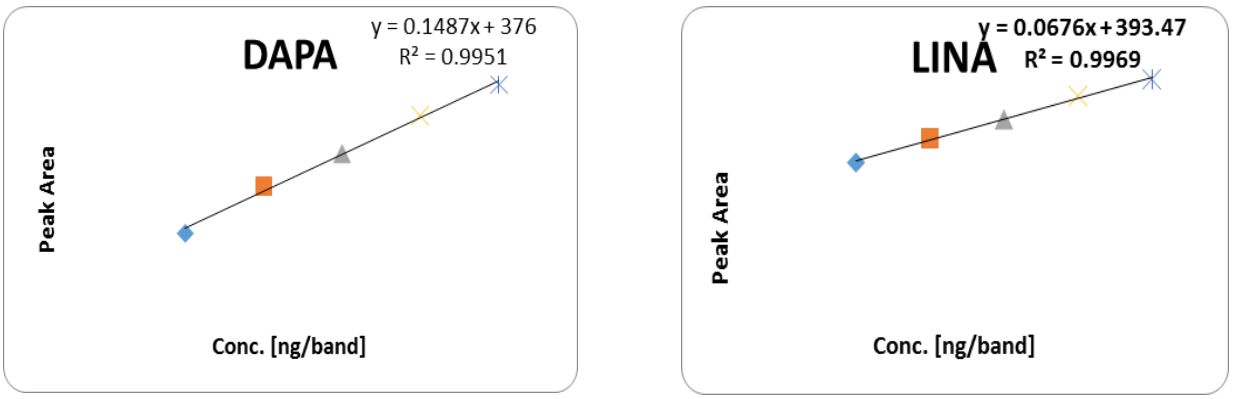


Fig. 2: The HPTLC densitogram displays Rf values of 0.22 for LINA (3000 ng/band) and 0.66 for DAPA (6000 ng/band), under optimal conditions.



(a) **(b)**

Fig. 3: Calibration curves of (a) DAPA (3000-9000 ng/band) (b) LINA (1500-4500 ng/band).

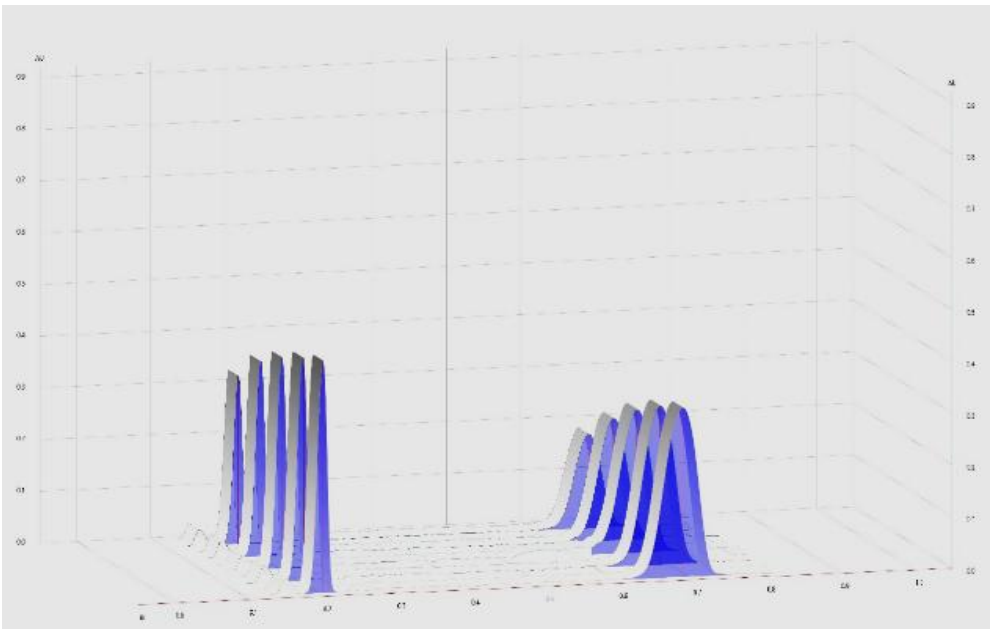


Fig. 4: Overlaid 3-D densitogram of DAPA and LINA under optimized chromatographic conditions.

Accuracy

% Recovery of DAPA and LINA was found 99.77 % and 99.95 % , respectively. The closeness of result to the true value (100 %) indicate that the method is accurate **(Table 3)**.

Intermediate Precision

For intra-day precision %RSD value for DAPA and LINA were estimated to be 0.74 – 0.77 and 0.42 – 0.95 %, respectively whereas inter-day precision % RSD values were found to be 1.20 – 1.78% for DAPA and 0.32-1.55% for LINA.

Repeatability

Scanner repeatability and injection repeatability were investigated by applying and analysingDAPA (6000 ng/band) and LINA (3000 ng/band). It was discovered that the % RSD for both drugs was less than 1%.

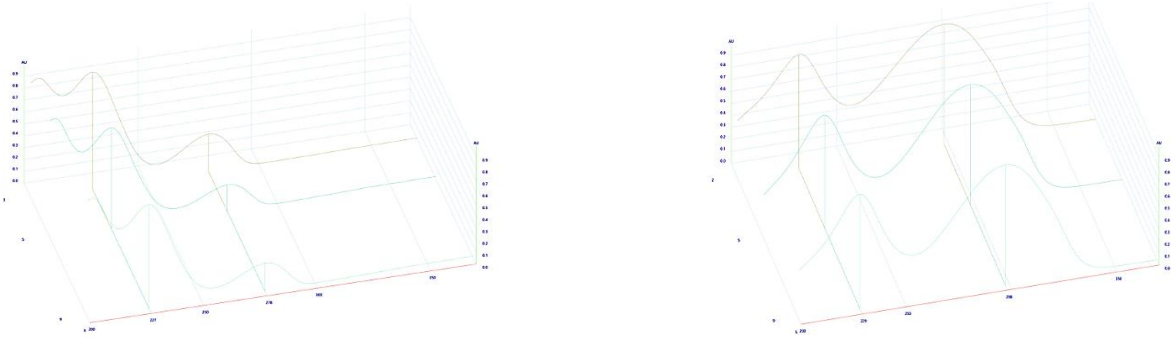
Specificity

There were just two peaks in the chromatogram of the pharmaceutical formulations of DAPA and LINA that were produced using the developed technique. These peaks were determined to be the same Rf for both standard pharmaceuticals, DAPA and LINA, with Rf of 0.66 and 0.20, respectively. By comparing the superimposed spectra at the peak start, peak apex, and peak end positions of the band, the peak purity of both pharmaceuticals in combined form was verified. **Table 2**'s findings revealed that the purity for every peak surpassed 0.999, demonstrating the method's specificity even in the presence of different excipients **(Fig. 5)**.

Table 3: Summary of Validation parameters of DAPA and LINA**.**

Parameters	**DAPA**	**LINA**
Linearity (ng/band)	3000-9000	1500-4500
Limit of detection (LOD) (ng/band)	215.38	226.16
Limit of quantitation (ng/band)	652.69	685.36
Accuracy (%) (n=9)		
80 %-120%	99.77 ± 1.13	99.95 ± 1.47
Intermediate Precision %RSD		
Precision (Intra day, n=3)	0.74 – 0.77	0.42 – 0.95
Precision (Inter day, n=3)	1.20 – 1.78	1.06 – 1.54
Instrument Precision (Repeatability) %RSD		
Repeatability of scanner, n=6	0.25	0.72
Repeatability of injection, n=6	0.19	0.62
Specificity		
r (S,M)	0.9998	0.9997
r (E, M)	0.9998	0.9996

nrepresents the number of determinations, while RSD stands for relative standard deviation.



(a) **(b)**

Fig. 5: The dosage form's peak purity spectra with the corresponding standards, (a) DAPA and (b) LINA.

Sensitivity

It was discovered that the LOD for DAPA and LINA were, respectively, 215.38 ng/band and 226.16 ng/band. The approach is sensitive and can precisely and reliably measure the nomogram amount of drug, as demonstrated by the LOQ of DAPA and LINA, which were determined to be 652.69 ng/band and 685.36 ng/band, respectively **(Table 3)**.

Robustness

The robustness of the approach was demonstrated by the %RSD of the peak area of less than 2% that resulted from a slight purposeful modification in many parameters, such as the solvent system's acetone level, the chamber saturation time, and the distance travelled **(Table 4)**.

Analysis of Pharmaceutical dosage form

Good recovery was seen in the study of the tablet formulation comprising 10 mg DAPA and 5 mg LINA. By examining the tablet formulation, the approach may be utilized for routine quality control, as evidenced by the percentages of 99.88 ± 1.48 for DAPA and 100.46 ± 1.73 for LINA.

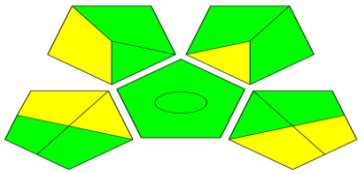
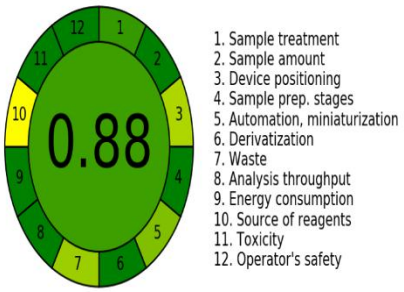
Greenness evaluation

Two distinct green analytical chemistry criteria, such as AGREE and GAPI, were used to assess how green the developed HPTLC technology was. The environmental friendliness of an analytical process, from sample collection to completion, may be evaluated using GAPI. The GAPI metric assesses how green each step of an analytical process is using a pictogram that has three colour levels: green, yellow, and red. The suggested HPTLC-densitometric techniques' GAPI pentagram showed that it had less of an influence on the environment because it produced more green fields (9), yellow fields (6), and no red fields. Analytical Greenness AGREE metrics are a further method that offers a readily interpreted and useful result. It is a thorough, adaptable, and uncomplicated evaluation methodology. The evaluation standards are computed into a 0–1 scale using the 12 principles of green analytical chemistry. The ultimate evaluation result is the sum of the assessment outcomes for each principle. The outcome is a clock-like graph with the overall score and a color representation in the middle.An Agree score of 0.88 was projected for the sustainable HPTLC method, indicating an outstanding green analytical approach for quantitative measurement of DAPA and LINA. A report and an automatically created graph can be produced by the program used to conduct the evaluation **(Table 5).**

Table 4: Robustness study of developed HPTLC method.

Drugs	**Ratio (v/v)**	**Peak area ± SD**	**%RSD**
Change in mobile phase ratio (Chloroform: methanol: ethyl acetate: 1 % formic acid, 3: 4: 3: 0.5, v/v, ± 0. 5 ml in methanol content)[a], saturation time 20 ± 5 min, migration distance 70 ± 5 mm			
DAPA	3: 3.5: 3.5: 0.5, v/v	1259 ± 5.56	0.42
	3: 3.5: 3.5: 0.5, v/v	1279 ± 5.13	0.38
LINA	3: 3.5: 3.5: 0.5, v/v	599 ± 3.78	0.32
	3: 3.5: 3.5: 0.5, v/v	592 ± 4.72	0.41
DAPA	15 min	1261±10	0.75
	25 min	1279 ±8.62	0.42
LINA	15 min	601±10.96	0.94
	25 min	594 ± 4.61	0.40
DAPA	65	1253± 3.05	0.23
	75	1262±6.02	0.45
LINA	65	598 ±2.88	0.25
	75	593 ± 14.97	1.29

Table 5: Greenness assessment of Proposed HPTLC method.

Proposed Method: HPTLC **Mobile Phase:** Chloroform: methanol: ethyl acetate: 1% formic acid 3: 4: 3: 0.5, v/v	
GAPI	**AGREE**
	

Conclusion

For measuring DAPA and LINA in pharmaceutical dosage forms, an eco-friendly, quick, accurate, precise, sensitive, and cost-effective HPTLC approach has been developed. The suggested approach requires less time and less mobile phase, making it both cost-effective and environmentally friendly. The stationary phase silica gel F254 and the solvent system chloroform: methanol: ethyl Acetate: 1% formic acid (3:4:3:0.5, v/v) were used to create the technique. The approach underwent validation in compliance with ICH recommendations Q2 (R1). The created method's greenness was evaluated by the use of AGREE and GAPI measures. The technique may be applied to the routine examination of in-process quality control of dosage forms and samples.

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نشرة العلوم الصيدليـــة
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تطوير والتحقق من صحة طريقة كروماتوجرافيا الضغط العالى ذات الطبقة الرقيقة الخضراء (HPTLC) لتعيين متزامن للداباغليفلوزين والليناجليبتين مجتمعين في شكل جرعات صيدلية

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تم تطوير تقنية بسيطة ودقيقة وسريعة وحساسة وخضراء لكروماتوجرافيا الطبقة الرقيقة (HPTLC)عالية الأداء لتقدير متزامن لداباغليفلوزين (DAPA) ولیناغلیبتین (LINA) في شكل أشكال صيدلية نقية ومجتمعة . وتم إجراء الفصل الكروماتوجرافي لكلا الدواءين على ألواح ألومنيوم مصنوع من هلام السيليكا المطلي مسبقًا ٦٠ F 254 ، (١٠ × ١٠ سم)، بسمك ١٠٠ ميكرومتر؛ باستخدام نظام المذيبات الكلوروفورم: الميثانول: أسيتات الإيثيل: ١% حمض الفورميك (٣:٤:٣:٠.٥، حجم/حجم) . و تم استخدام الضوء فوق البنفسجي قصير الموجة عند ٢٢٤ نانومتر لعرض النطاقات الكروماتوغرافية . و كشفت التقنية المقترحة عن نطاقات مدمجة ذات قيم عامل الاحتفاظ (Rf) تبلغ ٠.٦٦ و٠.٢٠، لـ (DAPA) و (LINA)، على التوالي. وكانت منحنيات المعايرة متعددة الحدود في نطاق التركيز من ٣٠٠٠-٩٠٠٠ نانوغرام/النطاق لـ DAPA و١٥٠٠-٤٥٠٠ نانوغرام/النطاق للينا، مع معاملات الارتباط ٠.٩٩٥١ و٠.٩٩٦٩ على التوالي. و تم التحقق من صحة الطريقة المقترحة فيما يتعلق بقواعد (ICH) . وتم تقييم التقييم الأخضر لطريقة (HPTLC) المطورة باستخدام مقاييس مختلفة للكيمياء التحليلية الخضراء مثل GREEness التحليلية (AGREE)، ومؤشر الإجراءات التحليلية الخضراء (GAPI). وقد تم تطبيق الطريقة المقترحة لتحليل الدواء في شكل أقراص، ويمكن استخدامها لتحليل مراقبة الجودة الروتينية.