

AQbD-Assisted Development and Validation of a Stability-Indicating and Greener HPTLC-Densitometric Method for Simultaneous Determination of Dapagliflozin and Sitagliptin in Pharmaceutical Formulations

Priyanka Malani

priyankamalani2000@gmail.com

Krishna School of Pharmacy & research, KPGU

Zeel Shah

Krishna School of Pharmacy & research, KPGU

Lakshmi Radhika G

Rajiv Gandhi University

Salaj Khare

CVM University

Kautuk P Shah

Krishna School of Pharmacy & research, KPGU

Archana Barot

Gujarat Technological University

Imtiyaz Bagban

Krishna School of Pharmacy & research, KPGU

Bansari Patel

Krishna School of Pharmacy & research, KPGU

Ashim Kumar Sen

Sumandeep Vidyapeeth Deemed to be University

Hamza Shaikh

Krishna School of Pharmacy & research, KPGU

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AQbD-Assisted Development and Validation of a Stability-Indicating and Greener HPTLC-Densitometric Method for Simultaneous Determination of Dapagliflozin and Sitagliptin in Pharmaceutical Formulations

Priyanka Malani ¹, Zeel Shah², Lakshmi Radhika G³, Salaj Khare⁴, Kautuk P Shah⁵, Archana Barot⁶, Imtiyaz Bagban⁷, Bansari Patel⁸, Ashim Kumar Sen⁹, Hamza Shaikh¹⁰

¹Assistant professor, Department of Quality Assurance, Krishna School of Pharmacy & research, KPGU, Vadodara-Mumbai National Highway 8, Vadodara, Gujarat 391240, India

²Student, Department of Quality Assurance, Krishna School of Pharmacy & research, KPGU, Vadodara-Mumbai National Highway 8, Vadodara, Gujarat 391240, India

³Assistant Professor, Department of Pharmaceutics, SJM College of Pharmacy, Rajiv Gandhi University, SJM Campus, Chitradurga 577502, Karnataka, India

⁴Associate Professor, Department of Pharmacology Indukaka Ipcowala college of Pharmacy, CVM University, New V V Nagar, Anand, Gujarat India

⁵Professor, Department of Pharmacognosy, Krishna School of Pharmacy & research, KPGU, Vadodara-Mumbai National Highway 8, Vadodara, Gujarat 391240, India

⁶Assistant Professor, Department of Quality Assurance, GTU Campus, near Visat Three Roads, Visat-Gandhinagar Road, Chandkheda, Ahmedabad, 382424, Gujarat, India.

⁷Assistant Professor, Department of Pharmacology, Krishna School of Pharmacy & research, KPGU, Vadodara-Mumbai National Highway 8, Vadodara, Gujarat 391240, India

⁸Assistant professor, Department of Quality Assurance, Krishna School of Pharmacy & research, KPGU, Vadodara-Mumbai National Highway 8, Vadodara, Gujarat 391240, India

⁹Professor, Department of Pharmacy, Sumandeep Vidyapeeth Deemed to be University, Piparia, Vadodara-391760, Gujarat, India.

¹⁰Assistant Professor, Department of Quality Assurance, GTU Campus, near Visat Three Roads, Visat-Gandhinagar Road, Chandkheda, Ahmedabad, 382424, Gujarat, India.

Corresponding Author

Priyanka Malani

Krishna School of Pharmacy & research, KPGU,

Vadodara-Mumbai National Highway 8, Vadodara, Gujarat 391240, India

Mob No: 9924860850

priyankamalani2000@gmail.com

Abstract

A robust, stability-indicating HPTLC method was developed and validated for the simultaneous determination of dapagliflozin propanediol monohydrate (DAPA) and sitagliptin phosphate monohydrate (SITA) in combined pharmaceutical dosage forms using an Analytical Quality by Design (AQbD) approach. A two-factor Central Composite Design (CCD) was employed to systematically optimize ethyl acetate (X_1) and methanol (X_2), while the R_f values of DAPA and SITA were selected as critical analytical responses. The optimized mobile phase was ethyl acetate:methanol:formic acid (7.5:2.0:0.5, v/v/v), with a chamber saturation time of 30 min and densitometric detection at 210 nm. The optimized method produced R_f values of 0.74 ± 0.02 for DAPA and 0.31 ± 0.02 for SITA. The quadratic models demonstrated good predictive performance, with R^2 values of 0.9886 and 0.9452 for DAPA and SITA, respectively. The optimized condition was experimentally verified, showing close agreement between predicted and observed responses. The method was validated according to ICH Q2(R2). DAPA and SITA demonstrated linear responses over 200–1000 and 2000–10,000 ng/band, respectively, with R^2 values of 0.9980 and 0.9991. The method exhibited satisfactory precision, with %RSD values below 2%, and recoveries of 99.49–100.91% for DAPA and 99.96–100.06% for SITA. The LOD/LOQ values were 17.31/52.47 ng/band for DAPA and 184.00/557.33 ng/band for SITA. Forced degradation studies demonstrated degradation under acidic, alkaline, oxidative, thermal, and photolytic conditions, with the greatest degradation observed under alkaline stress. The method successfully separated degradation products from the parent analytes, confirming its stability-indicating capability. Application to SITASON-D tablets yielded assay values of $98.79 \pm 0.70\%$ for DAPA and $100.01 \pm 0.60\%$ for SITA. Green analytical assessment further indicated a more favorable environmental profile than the reported RP-HPLC procedure. The developed AQbD-assisted HPTLC method therefore provides a simple, reliable, sensitive, and comparatively greener approach for routine quality control and stability assessment of DAPA–SITA pharmaceutical formulations.

Keywords: Dapagliflozin; Sitagliptin; HPTLC; Analytical Quality by Design; Central Composite Design; Stability-indicating method; Green analytical chemistry.

1. INTRODUCTION

1.1 Clinical and pharmaceutical background

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycaemia resulting from impaired insulin secretion, insulin action, or both [1,2]. Type 2 diabetes mellitus (T2DM) represents the predominant form of diabetes and constitutes a growing global health burden. The International Diabetes Federation estimated that approximately 589 million adults were living with diabetes in 2024, with this number projected to reach 853 million by 2050 [1]. Persistent hyperglycaemia is associated with serious microvascular and macrovascular complications, including diabetic kidney disease, neuropathy, cardiovascular disease, and premature mortality [2–4].

When glycaemic control is inadequately achieved with monotherapy, combination pharmacotherapy is frequently required [5,6]. Dapagliflozin (DAPA), a sodium–glucose cotransporter-2 (SGLT2) inhibitor, reduces renal glucose reabsorption and promotes urinary glucose excretion, whereas sitagliptin (SITA), a dipeptidyl peptidase-4 (DPP-4) inhibitor, enhances glucose-dependent insulin secretion and suppresses glucagon release [7–9]. Their complementary mechanisms support their combined use in T2DM. Consequently, reliable analytical methods for the simultaneous determination of DAPA and SITA are essential for pharmaceutical quality control and stability assessment.

1.2 Existing analytical methods and research gap

Several analytical methods have been reported for DAPA and SITA, including RP-HPLC [10–14,18–20], HPTLC [15,19], and LC–MS [21], both individually and in combination with other antidiabetic drugs [16,17,22–24]. HPLC-based methods for simultaneous DAPA–SITA determination have also been reported [25], along with stability-indicating HPTLC [26,27] and HPLC [28] procedures. However, conventional analytical method development may rely on empirical or one-factor-at-a-time experimentation, which provides limited information regarding factor interactions and method robustness.

1.3 AQbD rationale

Analytical Quality by Design (AQbD) offers a systematic, risk-based approach for identifying critical method parameters (CMPs), evaluating their effects on critical analytical attributes (CAAs), and establishing robust operating conditions [29,30]. Design of Experiments (DoE), particularly Central Composite Design (CCD), enables simultaneous evaluation of linear, interaction, and quadratic effects while reducing experimental requirements [31,32]. HPTLC is particularly attractive for pharmaceutical analysis because of its simplicity, low solvent consumption, high sample throughput, and suitability for simultaneous analysis.

Despite these advantages, an **AQbD-assisted, CCD-optimized, stability-indicating HPTLC method for simultaneous determination of dapagliflozin propanediol monohydrate and sitagliptin phosphate monohydrate in combined pharmaceutical dosage forms has not been comprehensively reported**. Therefore, the present study aimed to develop and validate a robust HPTLC method using an AQbD–CCD framework. The optimized method was validated according to ICH Q2(R2) [33] and evaluated through forced degradation studies to establish its specificity and stability-indicating capability. In addition, the environmental sustainability of the developed method was assessed using complementary Green Analytical Chemistry (GAC) tools.

1.4 Green analytical chemistry rationale

Green Analytical Chemistry aims to minimize the environmental and health impacts of analytical procedures while maintaining analytical performance by reducing hazardous chemicals, solvent consumption, waste generation, and energy requirements [34]. In the present study, four complementary greenness assessment tools were employed. The **Analytical Eco-Scale** provides a semi-quantitative score based on penalty points associated with reagents, hazards, energy, and waste [35]. **AGREE** evaluates the analytical procedure according to the

12 principles of GAC and provides an overall greenness score, whereas **AGREEprep** specifically evaluates the sustainability of sample-preparation procedures [37]. **GAPI** provides a visual assessment of the overall analytical workflow using a colour-coded pictogram. Collectively, these tools were used to provide a comprehensive evaluation of the greenness and sustainability of the proposed HPTLC method.

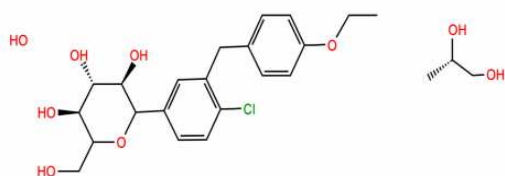


Fig: 1 Structure of DAPA

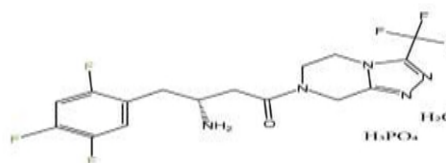


Fig: 2 Structure of SITA

1.5 Study objective

Therefore, the present study aimed to develop and validate an AQbD-assisted, stability-indicating HPTLC-densitometric method for simultaneous determination of DAPA and SITA in pharmaceutical formulations. A CCD-based experimental design was employed to optimize the mobile-phase composition, followed by comprehensive validation according to ICH Q2(R2)[36], forced-degradation studies, application to a marketed formulation, and comparative assessment of the environmental performance using multiple green analytical chemistry metrics.

MATERIALS AND METHODS

2.1 Chemicals, Reference Standards and Pharmaceutical Formulation

Certified reference standards of dapagliflozin propanediol monohydrate (DAPA; purity, 99.82%) and sitagliptin phosphate monohydrate (SITA; purity, 99.72%) were obtained as gift samples from Sun Pharmaceutical Industries Ltd. (India). Ethyl acetate, methanol, and formic acid of analytical-reagent grade were purchased from Merck Life Science Pvt. Ltd. (Mumbai, India). The solvents were used without further purification. A marketed tablet formulation, SITASON-D (Unison Pharmaceuticals Pvt. Ltd., Ahmedabad, India), labelled to contain 10 mg of DAPA and 100 mg of SITA per tablet, was used for assay evaluation.

2.2 Instrumentation and Chromatographic Equipment

Chromatographic analyses were performed using a CAMAG HPTLC system (CAMAG, Muttenz, Switzerland) equipped with a Linomat 5 automated sample applicator, TLC Scanner 4, Automatic Developing Chamber 2 (ADC 2), Reprostar 3 documentation system, and

visionCATS software (version 3.2). Samples were applied as 6-mm bands onto precoated silica gel 60 F₂₅₄ aluminium plates (10 × 10 cm; 200 µm layer thickness; Merck KGaA, Darmstadt, Germany) using a 100-µL Hamilton microsyringe under a nitrogen stream.

Chromatographic development was performed using ethyl acetate: methanol: formic acid (7.5:2.0:0.5, v/v/v) in a twin-trough chamber. The chamber was saturated for 30 min at 25 ± 2 °C, and development was performed to a migration distance of 70 mm. Densitometric scanning was carried out in absorbance–reflectance mode at 210 nm using a deuterium lamp. The slit dimension was 6.00 × 0.45 mm, the scanning speed was 20 mm s⁻¹, and the spectral resolution was 100 µm step⁻¹.

Densitometric analysis was performed in absorbance–reflectance mode at 210 nm using a deuterium lamp. The scanner was operated with slit dimensions of 6.00 × 0.45 mm (micro), a scanning speed of 20 mm s⁻¹, and a data resolution of 100 µm step⁻¹. Peak integration and chromatographic evaluation were carried out using visionCATS software.

Method optimization was performed following an Analytical Quality by Design (AQbD) approach using Central Composite Design (CCD). Ethyl acetate and methanol concentrations were selected as critical method parameters, while the *R_f* values of Dapagliflozin Propanediol Monohydrate and Sitagliptin Phosphate Monohydrate were considered critical analytical attributes. Experimental design, statistical analysis, response surface modelling, and numerical optimization were performed using Design-Expert® software (Version 12.0, Stat-Ease Inc., Minneapolis, MN, USA).

2.3 Preparation of Standard Stock Solution

2.3.1 Standard stock solution

Accurately weighed 10 mg of Dapagliflozin Propanediol Monohydrate (DAPA) and 100 mg of Sitagliptin Phosphate Monohydrate (SITA) reference standards were transferred separately into 10 mL volumetric flasks. The drugs were dissolved in methanol and the volume was adjusted to the mark with the same solvent to obtain stock solutions containing 1000 µg mL⁻¹ of DAPA and 10000 µg mL⁻¹ of SITA.

2.3.2 Working standard solution

Working standard solutions were prepared by transferring 1.0 mL aliquots of each stock solution into a 10 mL volumetric flask and diluting to volume with methanol to obtain final concentrations of 100 µg mL⁻¹ of DAPA and 1000 µg mL⁻¹ of SITA. The solutions were freshly prepared and filtered, where applicable, prior to chromatographic analysis.

2.4 Preliminary Chromatographic Development

A range of mobile-phase systems containing ethyl acetate, methanol, chloroform, acetonitrile, toluene, hexane, ammonia, and formic acid was initially screened for simultaneous chromatographic separation of DAPA and SITA. The systems were evaluated with respect to analyte migration, band compactness, peak shape, and separation. The preliminary screening identified ethyl acetate:methanol:formic acid as a promising solvent system; however, further optimization of the solvent proportions was required to achieve reproducible migration of both

analytes. Therefore, an AQbD-based Design of Experiments (DoE) approach was subsequently employed to optimize the mobile-phase composition.

2.5 Selection of Detection Wavelength

The UV absorption spectra of DAPA and SITA were recorded over 200–400 nm. Based on the absorption characteristics of both analytes and the suitability of the wavelength for simultaneous densitometric detection, 210 nm was selected for subsequent HPTLC analysis. The corresponding spectra are presented in Figure 3.

2.6 AQbD-Based Method Optimization:

2.6.1 Analytical Target Profile (ATP)

The analytical target profile (ATP) was defined as the development of a reliable, reproducible and stability-indicating HPTLC-densitometric procedure capable of simultaneous quantification of DAPA and SITA in pharmaceutical formulations. The method was required to provide well-defined and reproducible analyte bands, adequate chromatographic discrimination, acceptable precision and accuracy, and robustness against small deliberate variations in critical chromatographic parameters.

2.6.2 Risk Assessment and Selection of Variables

Preliminary chromatographic studies identified ethyl acetate (X_1) and methanol (X_2) as critical method parameters (CMPs) with a potential influence on analyte migration. The R_f values of DAPA (Y_1) and SITA (Y_2) were selected as the analytical responses for optimization. Formic acid was maintained at 0.5 mL throughout the experimental design based on preliminary screening and was therefore not included as an experimental factor. The selected variables were subsequently investigated using a two-factor central composite design (CCD).

Table 1. Risk assessment of critical method parameters affecting HPTLC performance

Method parameter	Potential analytical impact	Risk	Treatment
Ethyl acetate	R_f /migration	High	Included in CCD
Methanol	R_f /migration	High	Included in CCD
Formic acid	Migration/band characteristics	Medium	Fixed after preliminary screening
Chamber saturation time	R_f /peak characteristics	Medium	Robustness
Migration distance	R_f /separation	Medium	Robustness
Detection wavelength	Response/sensitivity	Low–medium	Spectral selection

2.6.3 Central Composite Design

A Central Composite Design (CCD) was employed to systematically investigate the effects of ethyl acetate (X_1) and methanol (X_2) on the chromatographic responses. The factorial levels were established at 7.0 and 8.0 mL for ethyl acetate and 1.0 and 3.0 mL for methanol, with centre-point levels of 7.5 mL ethyl acetate and 2.0 mL methanol. A rotatable CCD with an axial distance (α) of 1.414 was employed.

Accordingly, the axial points extended beyond the factorial region to approximately 6.79 and 8.21 mL for ethyl acetate and 0.59 and 3.41 mL for methanol, respectively. The design comprised four factorial points, four axial points, and five replicated centre points, resulting in a total of 13 experimental runs ($4 + 4 + 5 = 13$).

The coded and actual levels of the independent variables used in the CCD are presented in Table X. The complete CCD design matrix, including the design points, coded factor levels, and corresponding actual factor values, is presented in Table 2.

Table 2. Independent variables and their levels used in the Central Composite Design

Factor	Variable	Low (−1)	Centre (0)	High (+1)
X_1	Ethyl acetate (mL)	7.0	7.5	8.0
X_2	Methanol (mL)	1.0	2.0	3.0

Note: The −1, 0, and +1 values represent the coded factorial levels. The axial points at ± 1.414 extend beyond the factorial region according to the selected axial distance (α).

Table 3 Central Composite Design matrix showing coded and actual factor levels

Run	Design point	Coded X_1	Coded X_2	Ethyl acetate (mL)	Methanol (mL)	DAPA R_f	SITA R_f
1	Axial	0	−1.414	7.50	0.59	0.829	0.240
2	Factorial	−1	−1	7.00	1.00	0.798	0.260
3	Axial	−1.414	0	6.79	2.00	0.661	0.390
4	Factorial	+1	−1	8.00	1.00	0.816	0.270
5	Centre	0	0	7.50	2.00	0.740	0.310
6	Centre	0	0	7.50	2.00	0.741	0.311
7	Centre	0	0	7.50	2.00	0.739	0.309
8	Centre	0	0	7.50	2.00	0.740	0.310
9	Centre	0	0	7.50	2.00	0.742	0.312
10	Factorial	−1	+1	7.00	3.00	0.681	0.365
11	Axial	+1.414	0	8.21	2.00	0.829	0.250
12	Axial	0	+1.414	7.50	3.41	0.690	0.350
13	Factorial	+1	+1	8.00	3.00	0.720	0.330

The R_f values of DAPA (Y_1) and SITA (Y_2) were selected as the analytical responses for optimization. The experimental data generated from the CCD were analyzed using Design-Expert® software. Quadratic polynomial models were fitted to evaluate the linear, interaction, and quadratic effects of the independent variables on the analytical responses. Analysis of variance (ANOVA) was performed to assess the statistical significance of the fitted models and individual model terms.

Model adequacy was evaluated using the model F-value, p -value, coefficient of determination (R^2), adjusted R^2 , predicted R^2 , coefficient of variation (CV), adequate precision, and lack-of-fit. Response-surface and contour plots were generated to visualize the effects of the independent variables on the analytical responses. Numerical optimization using the desirability function was employed to identify the optimum chromatographic conditions. The optimized conditions were subsequently verified experimentally by comparing the predicted and experimentally observed R_f values, and the prediction error was calculated.

2.6.4 Statistical Analysis

Statistical analysis of the experimental design was performed using Design-Expert® software (Version 12.0). The CCD data were subjected to regression analysis and analysis of variance (ANOVA) to evaluate the significance of the fitted quadratic models and the individual linear, interaction, and quadratic terms. Model adequacy was assessed using the F-value, p -value, coefficient of determination (R^2), adjusted R^2 , adequate precision, and coefficient of variation (CV). The influence of the formulation variables on the chromatographic responses was further evaluated using response-surface analysis. Numerical and graphical desirability functions were employed to identify the optimum chromatographic conditions for simultaneous control of the R_f values of DAPA and SITA. The optimized condition was experimentally verified using replicate analyses by comparing the predicted and observed responses, and the prediction error (%) was calculated to assess the reliability of the model. Replicate measurements were evaluated using mean, standard deviation (SD), and percentage relative standard deviation (%RSD). Statistical significance was considered at $p < 0.05$.

2.7 Method validation

Selection of Detection Wavelength

The developed HPTLC method was validated in accordance with the International Council for Harmonisation (ICH) Guideline Q2(R2) for analytical procedure validation. The validation parameters evaluated included specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness to demonstrate the reliability, reproducibility, and suitability of the proposed analytical method for routine pharmaceutical analysis. In addition, forced degradation studies were performed under acidic, alkaline, oxidative, thermal, and photolytic stress conditions to establish the stability-indicating capability of the method.

2.7.1 Specificity

The specificity of the developed method was assessed by comparing the chromatographic profiles of blank, standard, and sample solutions to confirm the absence of interference from excipients or other matrix components at the *R_f* values of DAPA and SITA. Peak purity was evaluated by overlaying the UV spectra recorded at the start, apex, and end of each chromatographic peak. The purity of the analyte peaks was confirmed using the spectral correlation coefficients, $r(s,m)$ and $r(e,m)$, indicating the absence of co-eluting impurities or degradation products.

2.7.2 Linearity and Range

The linearity of the developed HPTLC method was evaluated by analyzing five concentration levels in the ranges of 200–1000 ng band⁻¹ for Dapagliflozin Propanediol Monohydrate (DAPA) and 2000–10000 ng band⁻¹ for Sitagliptin Phosphate Monohydrate (SITA). Aliquots corresponding to 2–10 µL of the working standard solutions were applied onto the HPTLC plates using a CAMAG Linomat 5 sample applicator. Each concentration level was analyzed in triplicate ($n = 3$). Calibration curves were constructed by plotting the mean peak area against the corresponding analyte concentration, and linear regression analysis was performed to determine the calibration equation and correlation coefficient.

2.7.3 Precision

The precision of the developed method was evaluated in terms of repeatability, intra-day precision, and inter-day precision, and the results were expressed as percentage relative standard deviation (%RSD).

2.7.3.1 Repeatability: Instrument repeatability was assessed by applying six replicate bands containing 600 ng band⁻¹ of DAPA and 6000 ng band⁻¹ of SITA on the same HPTLC plate. Scanner repeatability was evaluated by scanning the same chromatographic band six consecutive times under identical operating conditions.

2.7.3.2 Intra-day precision: Intra-day precision was determined by analyzing three concentration levels of DAPA (200, 600, and 1000 ng band⁻¹) and SITA (2000, 6000, and 10000 ng band⁻¹), each in triplicate, on the same day at 3 h intervals. Peak areas were recorded at 210 nm, and %RSD values were calculated.

2.7.3.3 Inter-day precision: Inter-day precision was evaluated by analyzing the same concentration levels on three consecutive days under identical experimental conditions. The precision of the method was expressed as %RSD of the measured peak areas.

2.7.4 Accuracy

The accuracy of the method was evaluated using the standard addition technique at three concentration levels corresponding to 80%, 100%, and 120% of the target concentration. Standard solutions equivalent to 320, 400, and 480 ng band⁻¹ of DAPA and 3200, 4000, and 4800 ng band⁻¹ of SITA were prepared by spiking pre-analyzed samples with the appropriate amounts of reference standards. Each level was analyzed in triplicate, and the percentage recovery was calculated from the measured peak areas.

2.7.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The sensitivity of the method was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ) based on the standard deviation of the regression response (σ) and the slope (S) of the calibration curve, according to ICH Q2(R2).

$$LOD = 3.3 * \frac{\sigma}{Slope}$$
$$LOQ = 10 * \frac{\sigma}{Slope}$$

where σ represents the standard deviation of the response and S represents the slope of the corresponding calibration curve.

2.7.6 Robustness

The robustness of the proposed method was investigated by introducing deliberate variations in critical chromatographic parameters, including the mobile phase composition, chamber saturation time, and migration distance. The effect of these changes on chromatographic performance was evaluated by monitoring variations in R_f values and peak areas. The method was considered robust when no significant changes in chromatographic performance were observed.

2.8 Forced Degradation Studies

The stability-indicating capability of the proposed HPTLC method was evaluated by subjecting Dapagliflozin Propanediol Monohydrate (DAPA) and Sitagliptin Phosphate Monohydrate (SITA) to acidic, alkaline, oxidative, thermal, and photolytic stress conditions in accordance with the recommendations of ICH Q1A(R2).

2.8.1 Acidic degradation: An aliquot (1.0 mL) of the mixed standard stock solution containing DAPA (1000 $\mu\text{g mL}^{-1}$) and SITA (10000 $\mu\text{g mL}^{-1}$) was transferred into a 10 mL volumetric flask, followed by the addition of 1.0 mL of 0.1 N hydrochloric acid. The solution was refluxed at 60 °C for 2 h, neutralized with 1.0 mL of 0.1 N sodium hydroxide, and diluted to volume with methanol.

2.8.2 Alkaline degradation: An aliquot (1.0 mL) of the mixed standard stock solution was treated with 1.0 mL of 0.1 N sodium hydroxide and refluxed at 60 °C for 1 h. After cooling, the solution was neutralized with 1.0 mL of 0.1 N hydrochloric acid and diluted to volume with methanol.

2.8.3 Oxidative degradation: An aliquot (1.0 mL) of the mixed standard stock solution was treated with 1.0 mL of 3% (v/v) hydrogen peroxide and allowed to stand at room temperature for 3 h. The solution was subsequently diluted to volume with methanol.

2.8.4 Photolytic degradation: Accurately weighed quantities of DAPA (10 mg) and SITA (100 mg) were placed in a Petri dish and exposed to ultraviolet radiation for 24 h. The exposed samples were transferred to a 10 mL volumetric flask, dissolved in methanol, and diluted to

volume to obtain the stock solution. A 1.0 mL aliquot of the stock solution was further diluted to 10 mL with methanol to obtain the working solution.

2.8.5 Thermal degradation: Accurately weighed quantities of DAPA (10 mg) and SITA (100 mg) were exposed to dry heat in a hot-air oven at 70 °C for 6 h. The degraded samples were dissolved in methanol and diluted to volume to obtain the stock solution. Subsequently, 1.0 mL of the stock solution was diluted to 10 mL with methanol to prepare the working solution.

Following each stress treatment, 6 µL of the resulting solution was applied to the HPTLC plate and analysed under the optimized chromatographic conditions. The extent of degradation and the separation of degradation products from the parent analytes were evaluated to confirm the stability-indicating nature of the developed method.

2.9 Assay of Marketed Formulation

The proposed method was applied for the quantitative determination of DAPA and SITA in a commercially available tablet formulation, **SITASON-D** (Unison Pharmaceuticals Pvt. Ltd., Ahmedabad, India), labeled to contain 10 mg of DAPA and 100 mg of SITA per tablet. Twenty tablets were accurately weighed, and the average tablet weight was determined before being finely powdered. An accurately weighed quantity of tablet powder equivalent to 10 mg of DAPA and 100 mg of SITA (245 mg) was transferred to a 10 mL volumetric flask. Approximately 5 mL of methanol was added, and the mixture was sonicated for 30 min to ensure complete extraction of the analytes. The solution was diluted to volume with methanol and filtered through a 0.45 µm membrane syringe filter.

A 1.0 mL aliquot of the filtrate was transferred to a 10 mL volumetric flask and diluted to volume with methanol to obtain working concentrations of 100 µg mL⁻¹ of DAPA and 1000 µg mL⁻¹ of SITA. A 6 µL aliquot of both the standard and sample solutions was applied to the HPTLC plate, and chromatographic development was carried out under the optimized conditions. Quantification was performed by comparing the peak areas of the sample with those of the corresponding reference standards, and the assay results were expressed as the percentage of the labelled claim.

2.10 Green Analytical Assessment

The environmental performance of the proposed AQbD-assisted HPTLC method was evaluated and compared with the reported RP-HPLC method for simultaneous determination of dapagliflozin (DAPA) and sitagliptin (SITA). Four complementary green analytical chemistry tools, namely the Analytical Eco-Scale, AGREE (Analytical GREENness), GAPI (Green Analytical Procedure Index), and AGREEprep, were selected for the assessment.

The Analytical Eco-Scale was calculated from the penalty points associated with reagent hazards, solvent consumption, energy requirements, and waste generation. AGREE was applied according to the twelve principles of Green Analytical Chemistry and expressed as a score between 0 and 1. GAPI was used to provide a pictorial assessment of the environmental impact of the complete analytical workflow. AGREEprep was used to assess the sustainability of sample preparation based on solvent consumption, sample amount, number of preparation steps, waste, energy requirements, and related factors.

For comparative assessment, the reported RP-HPLC procedure and the proposed AQbD-assisted HPTLC procedure were evaluated using their reported chromatographic conditions, solvent systems, analytical formats, and available sample-preparation information. The green metrics were calculated using the corresponding reported experimental parameters. Where complete information for the literature method was unavailable, the resulting assessment was considered provisional and was not interpreted as an experimentally verified score.

3. RESULTS AND DISCUSSION:

3.1 Selection of Detection Wavelength:

The UV absorption spectra of DAPA and SITA were recorded over 200–400 nm, and 210 nm was selected as the analytical wavelength based on the adequate absorbance of both analytes at this wavelength (Figure 3)

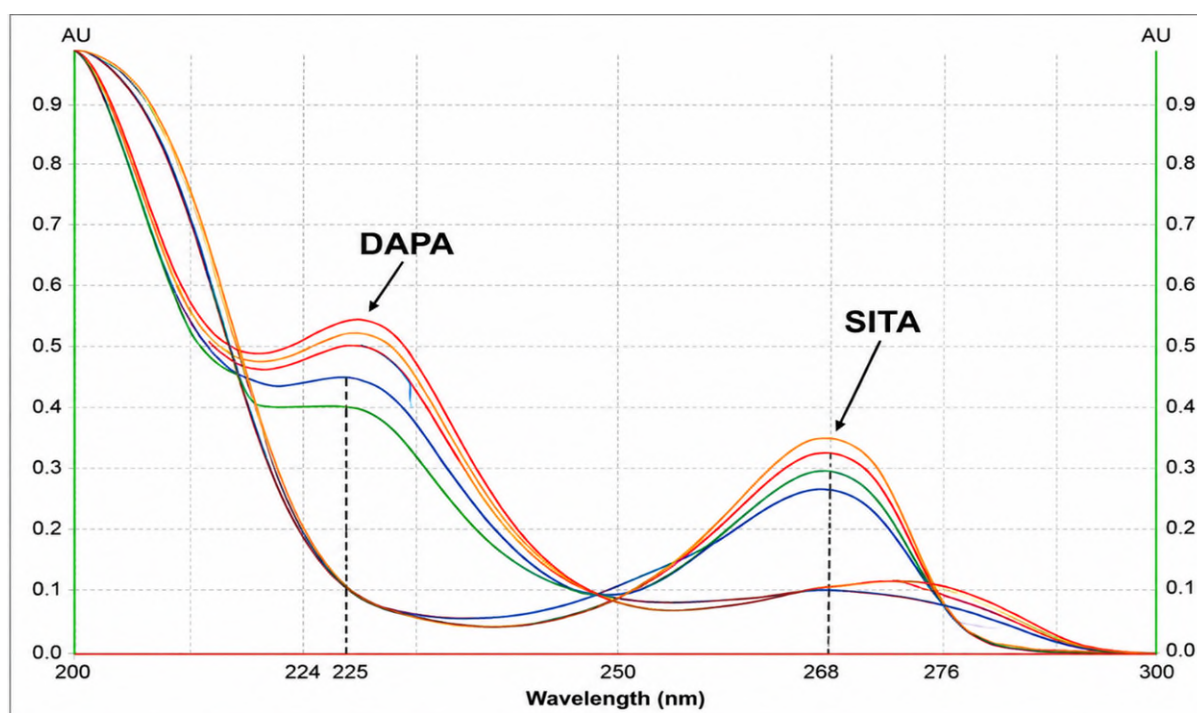


Fig: 3 UV absorption spectra of dapagliflozin propanediol monohydrate (DAPA) and sitagliptin phosphate monohydrate (SITA) recorded over 200–400 nm, with 210 nm selected as the analytical wavelength for simultaneous densitometric determination.

3.2 Preliminary Mobile-Phase Screening

Several mobile-phase compositions containing ethyl acetate, methanol, chloroform, acetonitrile, toluene, hexane, ammonia, and formic acid were initially screened. Several solvent systems resulted in poor migration, band broadening, tailing, or inadequate separation of DAPA and SITA. Among the tested systems, toluene: ethyl acetate: methanol (5:2.5:2.5:0.5, v/v/v/v) and ethyl acetate: methanol: formic acid (8.5:1:0.5, v/v/v) provided comparatively better band characteristics. However, further optimization was required to achieve consistent and simultaneous separation of both analytes. Therefore, an AQbD-based Design of Experiments

(DoE) approach was employed to systematically optimize the mobile-phase composition and establish the optimum chromatographic conditions.

3.3 AQbD-Based Optimization of Mobile-Phase Composition

3.3.1 Optimization of Mobile-Phase Composition Using Central Composite Design

The effects of ethyl acetate and methanol on the chromatographic migration of DAPA and SITA were investigated using a two-factor CCD. Across the 13 experimental runs, the observed *R_f* values ranged from 0.661 to 0.829 for DAPA and from 0.240 to 0.390 for SITA. The centre-point experiments performed at 7.5 mL ethyl acetate and 2.0 mL methanol yielded highly consistent responses, with DAPA *R_f* values ranging from 0.739 to 0.742 and SITA values of 0.309–0.312. This low variability at the centre point supported the repeatability of the chromatographic response within the investigated design region.

Table 4. Effect of mobile-phase composition on the *R_f* values of DAPA and SITA

Run	Design point	Ethyl acetate (mL)	Methanol (mL)	DAPA <i>R_f</i>	SITA <i>R_f</i>
1	Axial	7.50	0.59	0.661	0.24
2	Factorial	7.00	1.00	0.691	0.26
3	Axial	6.79	2.00	0.709	0.28
4	Factorial	8.00	1.00	0.721	0.30
5	Centre	7.50	2.00	0.739	0.31
6	Centre	7.50	2.00	0.740	0.31
7	Centre	7.50	2.00	0.740	0.31
8	Centre	7.50	2.00	0.740	0.31
9	Centre	7.50	2.00	0.741	0.31
10	Factorial	7.00	3.00	0.759	0.33
11	Axial	8.21	2.00	0.781	0.35
12	Axial	7.50	3.41	0.799	0.37
13	Factorial	8.00	3.00	0.829	0.39

Note: Formic acid was maintained at a constant volume of 0.5 mL throughout the experimental design. This concentration was selected during preliminary chromatographic screening because it provided acceptable band compactness and migration characteristics for both analytes. Accordingly, ethyl acetate and methanol were considered the principal critical method parameters and were subjected to systematic optimization using CCD.

3.3.2 Model Fitting and Statistical Evaluation

The experimental CCD data were fitted to quadratic polynomial models to investigate the effects of ethyl acetate (*X*₁) and methanol (*X*₂) on the *R_f* values of DAPA and SITA. The

adequacy and predictive performance of the models were assessed using the coefficient of determination (R^2), adjusted R^2 , coefficient of variation (CV), adequate precision, and ANOVA.

For DAPA, the quadratic model showed a high R^2 of 0.9886 and adjusted R^2 of 0.9804, indicating that the model explained 98.86% of the variability in the observed R_f response. The low CV (0.83%) demonstrated good experimental precision, while the adequate precision value of 38.80 indicated a satisfactory signal-to-noise ratio. The close agreement between R^2 and adjusted R^2 further supported the adequacy of the fitted model.

For SITA, the quadratic model exhibited an R^2 of 0.9452 and adjusted R^2 of 0.9060, indicating that 94.52% of the variability in the response was explained by the model. The CV of 4.07% and adequate precision of 16.92 indicated acceptable model precision and an adequate signal-to-noise ratio. ANOVA demonstrated that the SITA quadratic model was statistically significant ($F = 24.14$, $p = 0.0003$), confirming the significance of the fitted model within the investigated experimental region.

Overall, the high R^2 and adjusted R^2 values, together with the low CV and adequate precision values, demonstrated that the quadratic models adequately described the influence of mobile-phase composition on the chromatographic migration of DAPA and SITA. Thus, the models were considered suitable for subsequent response-surface analysis and optimization of the mobile-phase composition.

Table 5. Statistical evaluation of the quadratic CCD models for DAPA and SITA R_f responses

Response	R^2	Adjusted R^2	CV (%)	Adequate precision	Model F-value	p -value
DAPA R_f	0.9886	0.9804	0.83	38.80	Not available	Not available
SITA R_f	0.9452	0.9060	4.07	16.92	24.14	0.0003

Note: DAPA model F-value and corresponding p -value were not reported because the original ANOVA output was unavailable and the values could not be reliably reconstructed from the rounded ANOVA data.

3.3.3 Regression Model Equations

The experimental R_f responses of DAPA and SITA were fitted to second-order polynomial models using the coded levels of ethyl acetate and methanol as the independent variables. The developed quadratic models described the relationship between mobile-phase composition and chromatographic migration within the investigated design space. The regression equations obtained from the CCD analysis were:

$$\text{DAPA } R_f (Y_1): [Y_1 = 0.74 + 0.02A + 0.04B + 0.005AB + 0.007A^2 - 0.001B^2]$$

$$\text{SITA } R_f (Y_2): [Y_2 = 0.31 + 0.02A + 0.043B + 0.005AB + 0.0078A^2 - 0.0012B^2]$$

where A represents the coded level of ethyl acetate (X_1) and B represents the coded level of methanol (X_2). The AB term represents the interaction between ethyl acetate and methanol,

whereas A^2 and B^2 represent the quadratic effects of the respective variables. Positive regression coefficients indicate a positive contribution to the response, whereas negative coefficients indicate an inverse contribution within the investigated design space.

3.3.4 Effect of Mobile-Phase Variables on DAPA R_f

The CCD analysis demonstrated that variations in ethyl acetate and methanol concentrations influenced the chromatographic migration of DAPA. The developed quadratic model was statistically significant confirming that the selected mobile-phase variables collectively contributed significantly to the variation in the DAPA R_f response.

The model exhibited a high coefficient of determination ($R^2 = 0.9886$) and adjusted R^2 (0.9804), indicating a strong agreement between the experimental and model-predicted DAPA R_f values. The low coefficient of variation ($CV = 0.83\%$) demonstrated good experimental precision. Moreover, the adequate precision value of 38.80, which was considerably higher than the minimum acceptable value of 4, indicated an adequate signal-to-noise ratio and confirmed the suitability of the model for optimization within the investigated design space.

The experimental DAPA R_f values obtained from the 13 CCD runs ranged from 0.661 to 0.829. In general, an increase in methanol concentration resulted in increased DAPA migration, although the magnitude of the response varied according to the corresponding ethyl acetate concentration. This behaviour indicates that the chromatographic migration of DAPA was governed by the combined influence of the mobile-phase components.

The combined effects of ethyl acetate and methanol on DAPA R_f are illustrated by the contour and three-dimensional response-surface plots (Figures 4 and 5). The response surface demonstrated systematic variation in DAPA R_f across the experimental design space and facilitated identification of the region corresponding to the desired chromatographic migration.

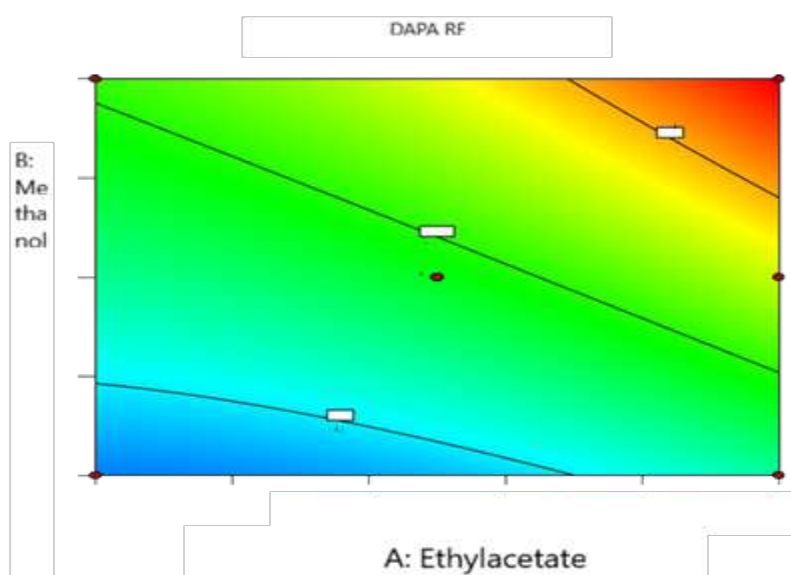


Fig. 4. Contour plot illustrating the combined effect of ethyl acetate and methanol on the R_f value of DAPA.

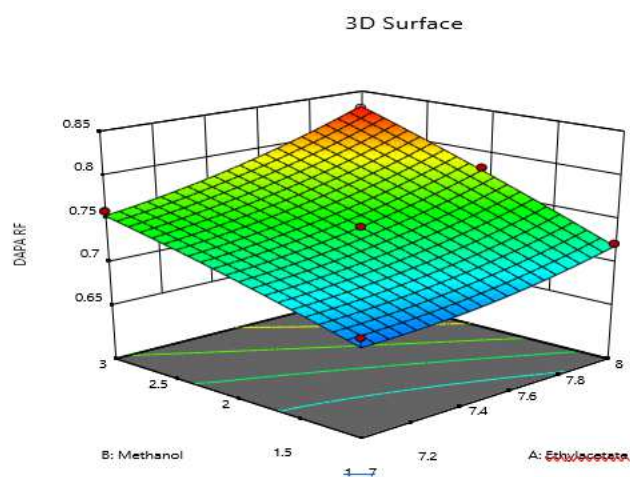


Fig. 5. Three-dimensional response-surface plot illustrating the combined effect of ethyl acetate and methanol on the R_f value of DAPA.

3.3.5 Effect of Mobile-Phase Variables on SITA R_f

The chromatographic migration of SITA was also influenced by the composition of the mobile phase. The developed quadratic model was statistically significant ($F = 24.14$, $p = 0.0003$), demonstrating that the selected mobile-phase variables significantly affected the SITA R_f response.

The model yielded an R^2 of 0.9452 and an adjusted R^2 of 0.9060, indicating satisfactory agreement between the experimental and model-predicted responses. The CV of 4.07% indicated acceptable experimental variability, while the adequate precision value of 16.92 was substantially higher than the recommended value of 4, confirming an adequate signal-to-noise ratio and the suitability of the model for optimization.

Analysis of the individual model terms indicated that the interaction between ethyl acetate and methanol was not statistically significant ($p = 0.2833$). In contrast, the quadratic effect of ethyl acetate was statistically significant ($p = 0.0270$), whereas the quadratic effect of methanol was not statistically significant ($p = 0.7645$). These findings indicate that the two mobile-phase variables contributed differently to the SITA R_f response within the investigated design space.

The experimental SITA R_f values ranged from 0.240 to 0.390 across the 13 CCD runs. An overall increase in SITA migration was observed with increasing methanol concentration, although the magnitude of this effect depended on the corresponding ethyl acetate level. The contour and three-dimensional response-surface plots further illustrate the combined influence of the two mobile-phase variables on SITA migration (Figures 6 and 7).

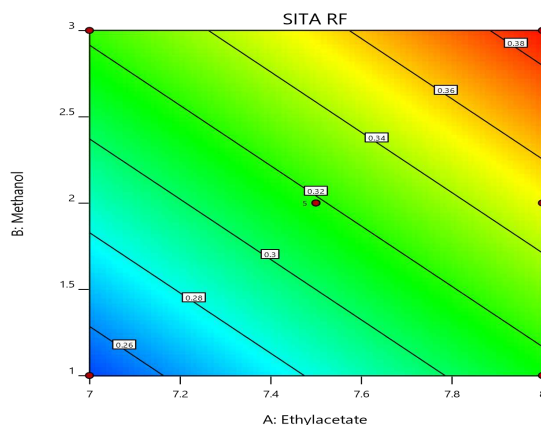


Fig. 6. Contour plot illustrating the combined effect of ethyl acetate and methanol on the *R_f* value of SITA.

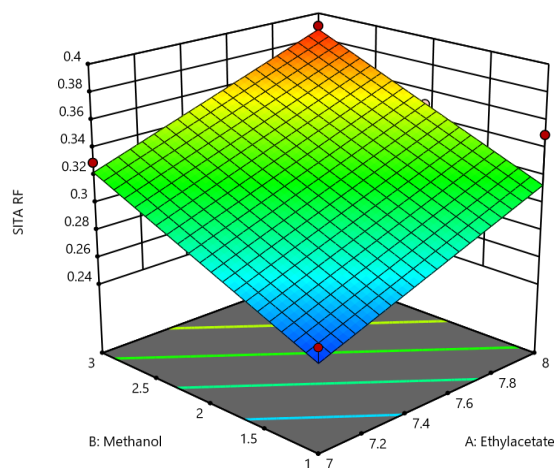


Fig. 7. Three-dimensional response-surface plot illustrating the combined effect of ethyl acetate and methanol on the *R_f* value of SITA.

3.3.6 Response-Surface and Contour-Plot Analysis

Contour and three-dimensional response-surface plots were generated to visualize the individual and combined effects of ethyl acetate and methanol on the *R_f* responses of DAPA and SITA. The graphical analysis demonstrated that changes in methanol concentration produced a pronounced effect on the migration of both analytes, with increasing methanol content generally resulting in higher *R_f* values.

The effect of ethyl acetate varied between the two analytes, reflecting differences in their chromatographic interactions with the stationary phase and mobile phase. The response surfaces further demonstrated that simultaneous control of the two *R_f* responses was feasible within a defined region of the experimental design space.

The overlapping response regions provided the basis for simultaneous optimization of the chromatographic conditions. Accordingly, numerical optimization was subsequently performed using a desirability-function approach to identify the mobile-phase composition capable of producing the predefined target *R_f* values for both analytes.

3.3.7 Numerical Optimization Using the Desirability Function

Numerical optimization was performed using the desirability-function approach to simultaneously achieve the predefined chromatographic targets for DAPA and SITA. The target R_f values were set at approximately 0.74 for DAPA and 0.31 for SITA, which provided suitable migration and effective chromatographic discrimination of the two analytes.

The numerical optimization identified ethyl acetate:methanol:formic acid (7.5:2.0:0.5, v/v/v) as the optimum mobile-phase composition, with an overall desirability of 0.939. The predicted responses under the optimized condition were close to the predefined target values.

The optimized mobile phase produced R_f values of approximately 0.74 for DAPA and 0.31 for SITA, with compact and well-defined chromatographic bands. The optimized composition was therefore selected for experimental verification and subsequent method validation.

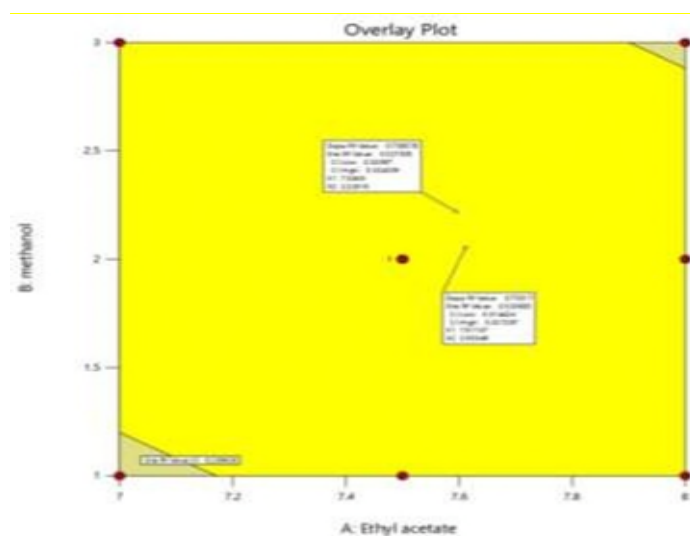


Fig. 8. Overlay plot illustrating the feasible design space for simultaneous optimization of DAPA and SITA R_f responses.

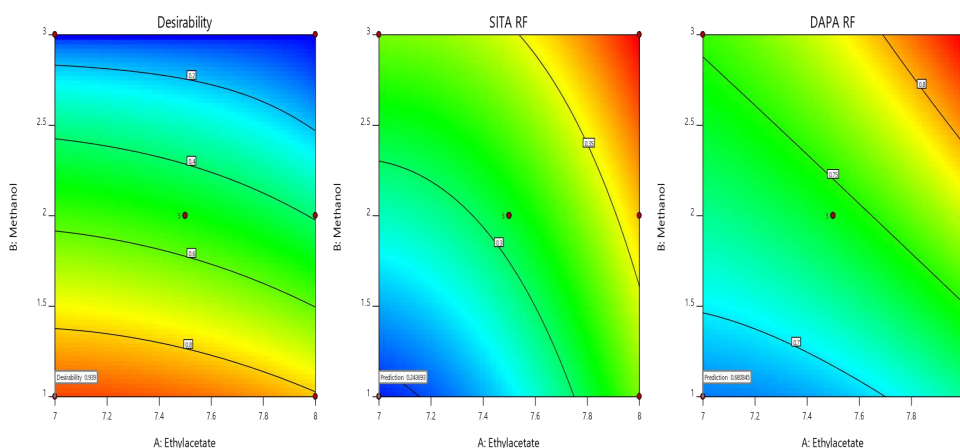


Fig. 9. Desirability plot illustrating the predicted responses and overall desirability of the optimized chromatographic condition.

3.3.8 Experimental Verification of the Optimized Condition

The optimized chromatographic condition was experimentally verified to assess the predictive capability and reliability of the developed CCD models. The optimized mobile-phase

composition, ethyl acetate:methanol:formic acid (7.5:2.0:0.5, v/v/v), was employed under the optimized chromatographic conditions.

The experimentally observed *R_f* values were 0.740 for DAPA and 0.310 for SITA, respectively. These experimental responses were compared with the corresponding model-predicted responses obtained from Design-Expert software.

The prediction error was calculated according to the following equation:

$$\% \text{ Prediction error} = \left| \frac{\text{Predicted} - \text{Experimental}}{\text{Predicted}} \right| \times 100$$

The close agreement between the experimentally observed and model-predicted *R_f* values demonstrated the predictive capability of the developed quadratic models. The low prediction error obtained for the optimized condition further supported the adequacy of the CCD-based optimization approach.

3.3.9 Prediction Error Analysis of the CCD Model

The prediction capability of the developed models was further evaluated using the experimental and predicted *R_f* values obtained for the 13 CCD runs. Prediction error was calculated independently for DAPA and SITA using the corresponding model-predicted values generated by Design-Expert software.

It is important to distinguish model prediction error from deviation from the target response. The percentage values calculated using 0.74 as the DAPA target and 0.31 as the SITA target represent percentage deviations from the predefined target values and should not be reported as model prediction errors.

Therefore, the prediction-error analysis should be presented using the original Design-Expert predicted values, as shown below:

Table 6 Experimental and Design-Expert®-predicted *R_f* values of dapagliflozin propanediol monohydrate (DAPA) and sitagliptin phosphate monohydrate (SITA), with corresponding prediction errors (%) for the 13 CCD experimental runs.

Run	DAPA experimental R _f	DAPA estimated R _f	DAPA prediction error (%)	SITA experimental R _f	SITA estimated R _f	SITA prediction error (%)
1	0.829	0.7713	7.48	0.240	0.2882	16.74
2	0.798	0.7199	10.85	0.260	0.3262	20.29
3	0.661	0.6931	4.63	0.390	0.3576	9.07
4	0.816	0.8277	1.42	0.270	0.2555	5.67
5	0.740	0.7404	0.05	0.310	0.3104	0.13
6	0.741	0.7404	0.08	0.311	0.3104	0.19
7	0.739	0.7404	0.19	0.309	0.3104	0.45
8	0.740	0.7404	0.05	0.310	0.3104	0.13
9	0.742	0.7404	0.22	0.312	0.3104	0.52

10	0.681	0.7675	11.27	0.365	0.2911	25.39
11	0.829	0.7885	5.14	0.250	0.2902	13.84
12	0.690	0.6986	1.23	0.350	0.3458	1.21
13	0.720	0.7574	4.94	0.330	0.3004	9.84

The experimental responses showed good consistency with the response-surface model, particularly around the central and optimized design region. The close agreement between the model-predicted and experimentally observed responses at the optimized condition confirmed that the developed CCD models adequately described the chromatographic behaviour of DAPA and SITA within the investigated design space.

3.3.10 Selection of the Optimized Chromatographic Condition

The optimized chromatographic condition was established using the fitted quadratic models, response-surface analysis, desirability-function optimization, and experimental verification. The selected mobile phase was **ethyl acetate:methanol:formic acid (7.5:2.0:0.5, v/v/v)**. Under these conditions, DAPA and SITA exhibited well-defined and reproducible bands at R_f values of 0.74 ± 0.02 and 0.31 ± 0.02 , respectively, with satisfactory migration and adequate chromatographic separation (Figure 11). The optimized conditions were subsequently employed for method validation and forced-degradation studies.

Optimized chromatographic conditions:

Mobile phase	: Ethyl acetate:methanol acid (7.5:2.0:0.5, v/v/v)
Chamber saturation time	: 30 min
Migration distance	: 70 mm
Detection wavelength	: 210 nm
R_f of DAPA	: 0.74 ± 0.02
R_f of SITA	: 0.31 ± 0.02

3.4 METHOD VALIDATION

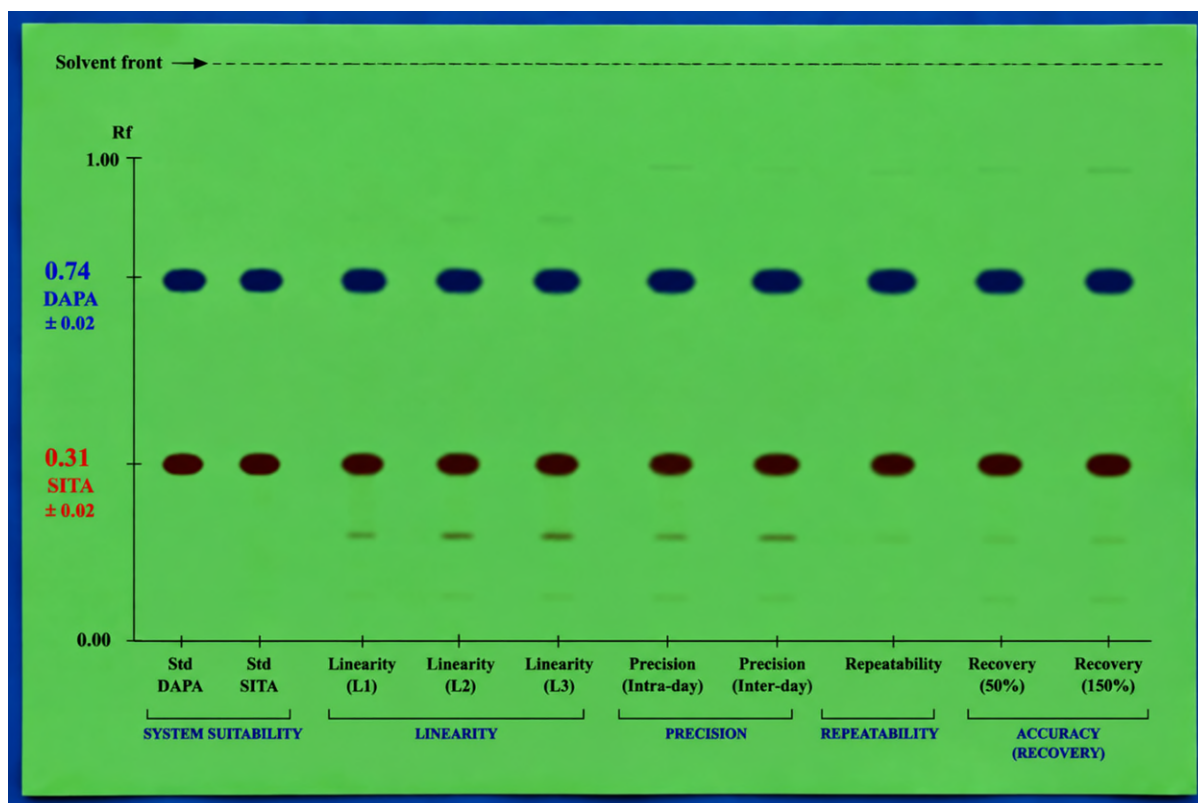


Fig 11. Optimized mobile-phase composition obtained using the desirability function: ethyl acetate: methanol: formic acid. (7.5:2.0:0.5, v/v/v).

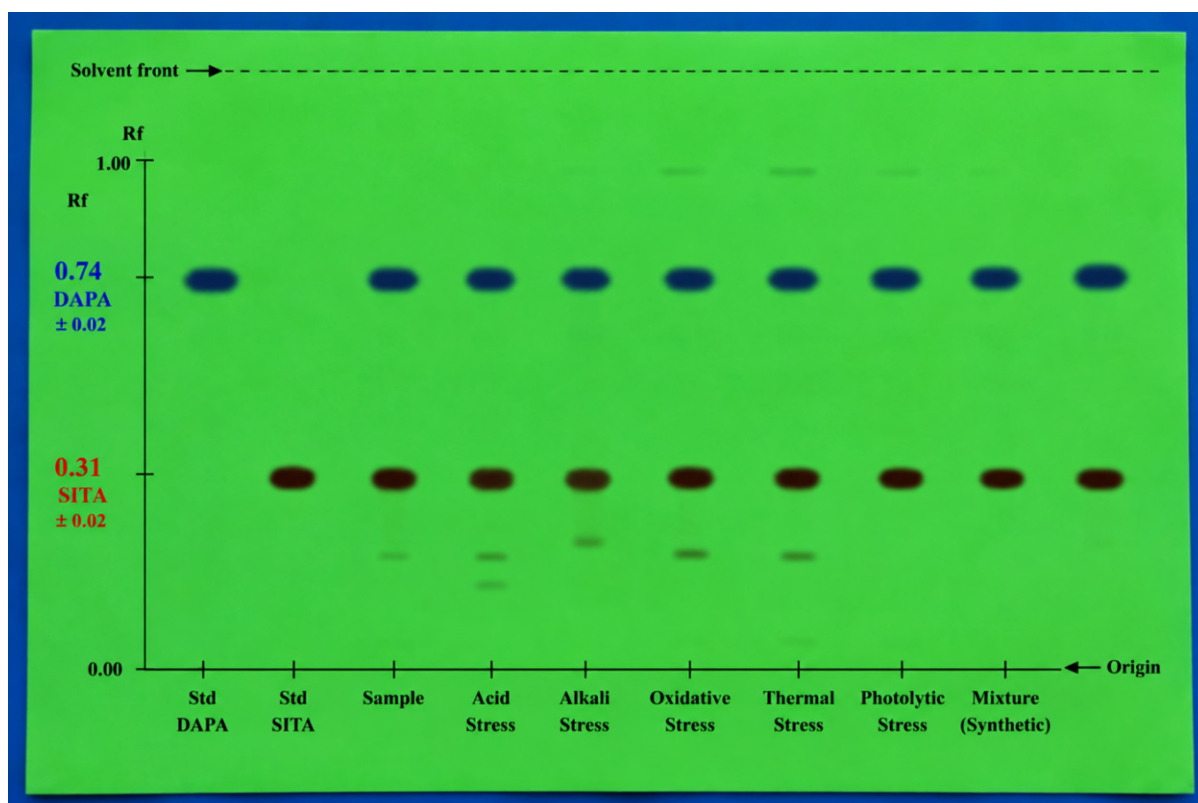


Fig 11. Optimized mobile-phase composition obtained using the desirability function: ethyl acetate: methanol: formic acid. (7.5:2.0:0.5, v/v/v).

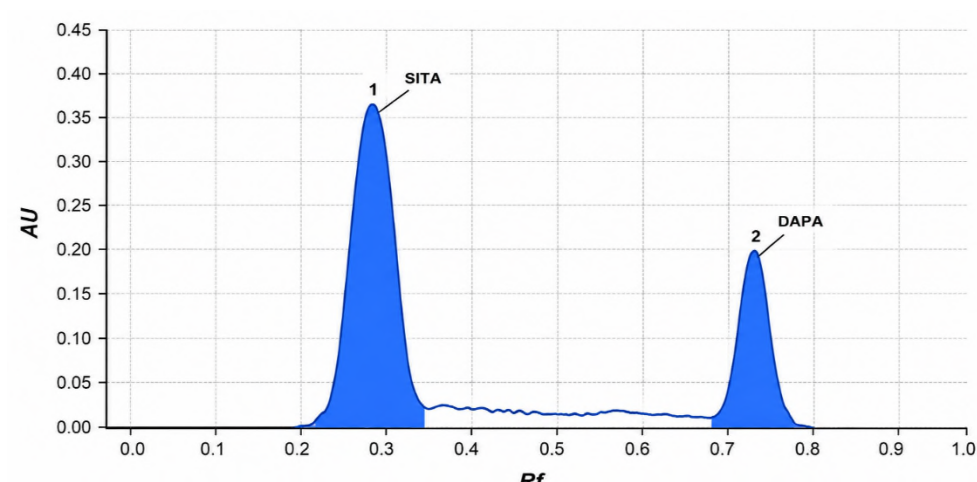


Fig 12 Representative HPTLC densitogram of Dapagliflozin Propanediol Monohydrate (DAPA) and Sitagliptin Phosphate Monohydrate (SITA) obtained using the optimized mobile phase consisting of ethyl acetate:methanol:formic acid (7.5:2.0:0.5, v/v/v).

3.4.1 Specificity and Peak Purity:

The peak purity of DAPA and SITA was confirmed by λ values of **0.9975/0.9997** and **0.9991/0.9999**, respectively, indicating excellent spectral homogeneity and confirming the specificity of the proposed method.

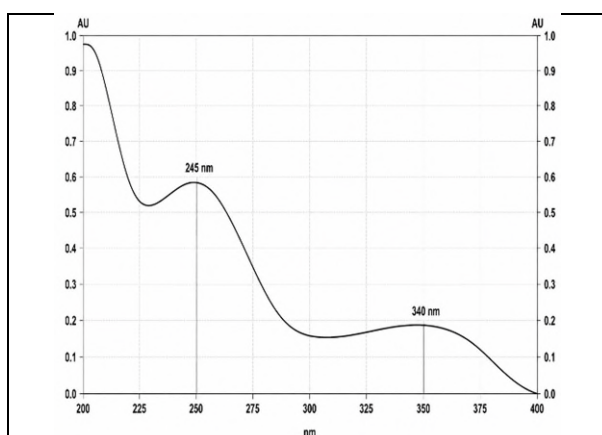


Fig 13: Peak purity of DAPA

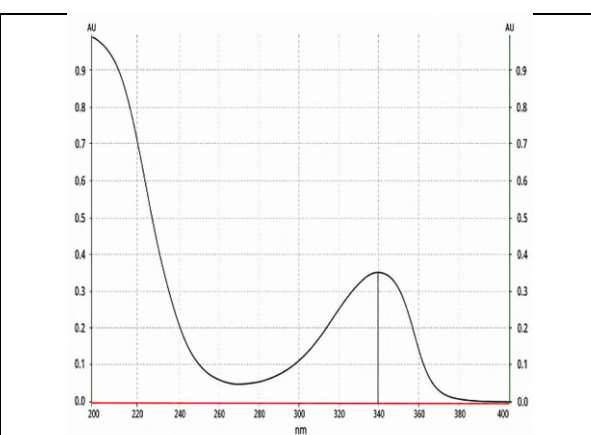


Fig 14: Peak purity of SITA

3.4.2 Linearity

The linearity of the developed HPTLC method was assessed over the concentration ranges of **200–1000 ng/band for DAPA** and **2000–10,000 ng/band for SITA**. Calibration curves were generated by plotting the **mean peak area versus the corresponding analyte concentration**,

and the data were subjected to linear regression analysis. The method exhibited a highly linear densitometric response across the investigated ranges, with coefficients of determination (R^2) of **0.9980 for DAPA** and **0.9991 for SITA**. The corresponding regression equations were $y = 0.791x + 77.46$ for DAPA and $y = 0.1997x + 550.59$ for SITA. The high R^2 values indicate a strong and consistent relationship between analyte concentration and peak response, demonstrating the suitability of the proposed method for reliable simultaneous quantification of DAPA and SITA in pharmaceutical formulations.

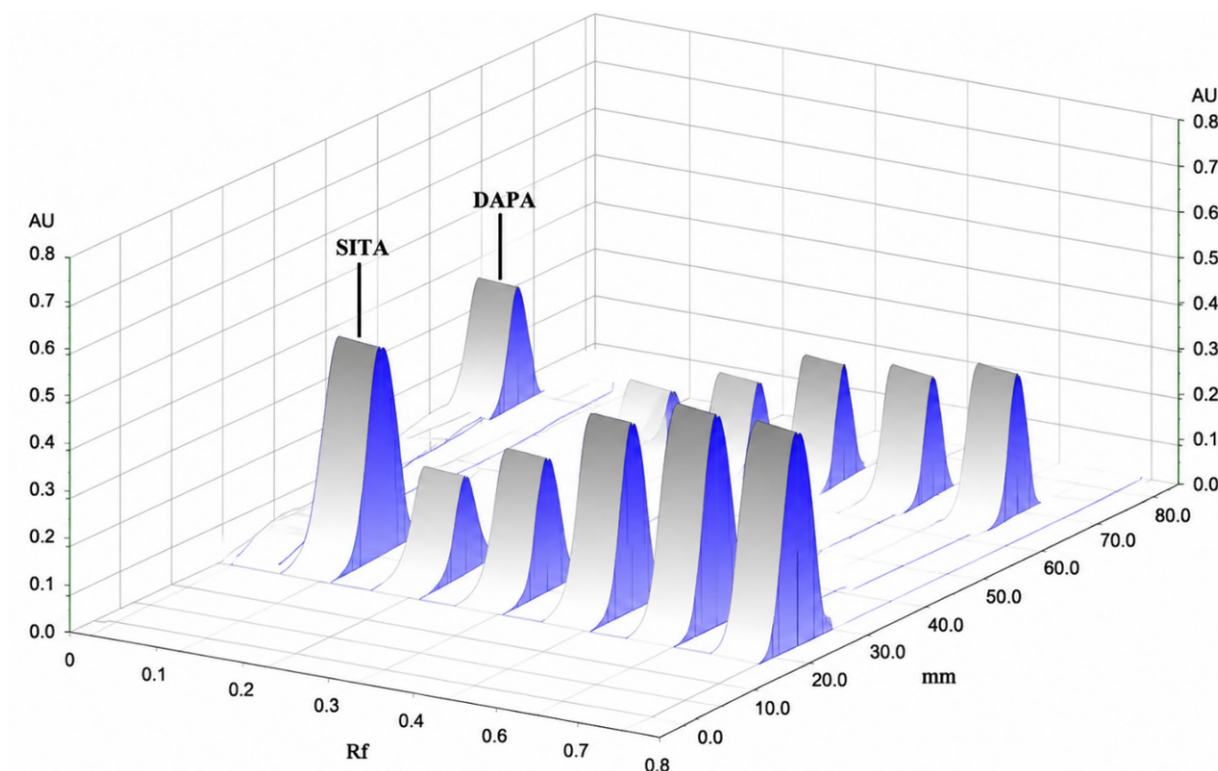


Fig 15 Three-dimensional (3D) TLC/HPTLC densitogram showing the separation of Sitagliptin (**SITA**) and Dapagliflozin (**DAPA**) across multiple tracks. The x-axis represents the retardation factor (R_f), the y-axis indicates the track position in millimeters (mm), and the z-axis denotes absorbance intensity in arbitrary units (AU).

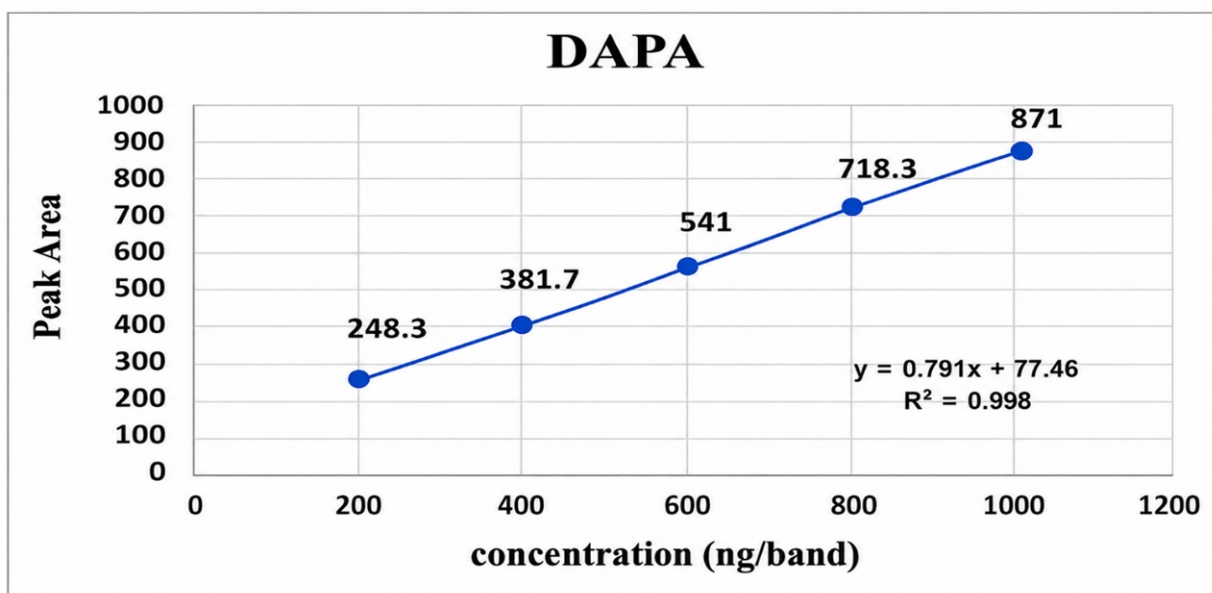


Fig 15 Calibration curves of Dapagliflozin Propanediol Monohydrate (200–1000 ng /band)

Table 7: Calibration data of DAPA

Sr No.	Concentration (ng/band)	Area (AU) $\times 10^{-3} \pm$ SD (n=3)	%RSD
1.	200	248.3 $\pm$ 3.51	1.41
2.	400	381.7 $\pm$ 3.51	0.92
3.	600	541.0 $\pm$ 3.61	0.67
4.	800	718.3 $\pm$ 6.51	0.91
5.	1000	871.0 $\pm$ 3.61	0.41

Table 8: Calibration data of SITA

Sr No.	Concentration (ng/band)	Area (AU) $\times 10^{-3} \pm$ SD (n=3)	%RSD
1.	2000	947.7 $\pm$ 7.51	0.79
2.	4000	1366.0 $\pm$ 11.53	0.84

3.	6000	1742.3 ± 11.59	0.66
4.	8000	2122.3 ± 12.50	0.59
5.	10000	2567.0 ± 12.53	0.49

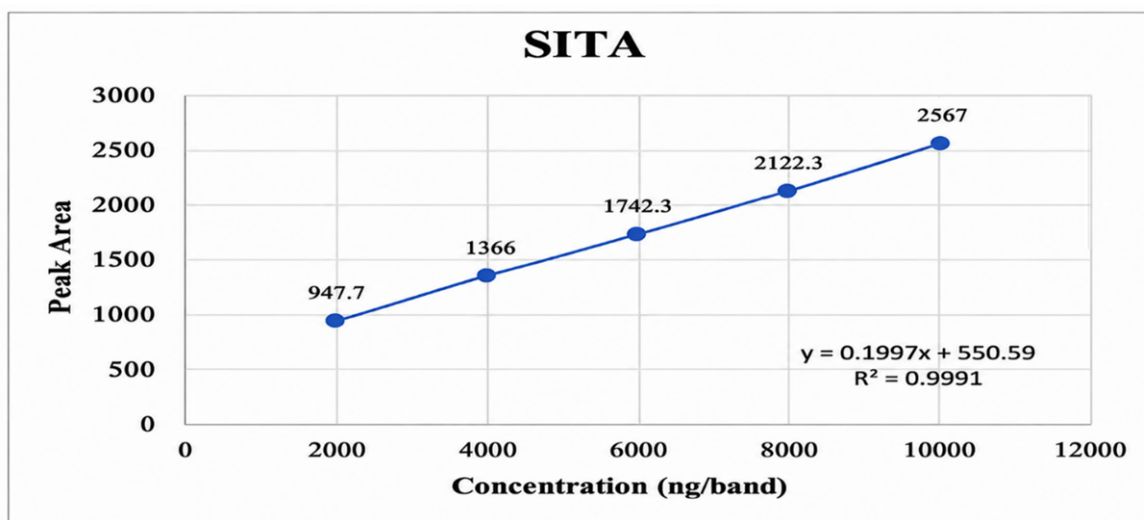


Fig 15 Calibration curves of Sitagliptin Phosphate Monohydrate (2000–10000 ng/band).

Table 9 Regression analysis of DAPA and SITA

Parameters	DAPA	SITA
Range (ng/band)	200-1000	2000-10000
Regression equation ($y = mx + c$)	$y = 0.791x + 77.46$	$y = 0.1997x + 550.59$
Slope (m)	0.791	0.1997
Intercept (c)	77.46	550.59
Correlation coefficient (R^2)	0.998	0.9991

3.4.3 Precision

The precision of the method was evaluated as repeatability, intra-day precision, and inter-day precision. Repeatability was assessed using six replicate applications, whereas intra-day and inter-day precision were evaluated at three concentration levels. The results were expressed

as %RSD. All observed %RSD values were within the predefined acceptance criterion of less than 2%, demonstrating satisfactory precision of the proposed HPTLC method.

3.4.3.1 Repeatability

The repeatability of the scanning device and injector was studied by applying and analysing DAPA (600 ng/band) and SITA (6000 ng/band) samples 6 times. The repeatability studies were given in following Table. The % RSD values obtained were less than 2%. The results indicated that the method is precise in terms of repeatability.

Table 10 Repeatability studies of DAPA (600 ng/band) & SITA (6000 ng/band)

Sr No.	Drugs	Injector (Area) $\times 10^{-3}$		Injector (Area) $\times 10^{-3}$
1.	of DAPA (600 ng/band)	542	SITA (6000 ng/band)	1745
2.		538		1738
3.		545		1752
4.		540		1740
5.		543		1748
6.		539		1739
Mean $\pm$ SD		541.16 $\pm$ 2.63		1743.7 $\pm$ 7.0
% RSD		0.48		0.40

3.4.3.2 Intra-day precision

The intra-day precision studies were performed using standard sample on same day. The intra-day data were given in Table 11. The %RSD for both drugs was less than 2, indicated that the method is precise.

Table 11 Intra-day precision studies of DAPA and SITA

Conc. (ng/band)		Area $\times 10^{-3}$ Mean $\pm$ SD (n=3)		%RSD	
DAPA	SITA	DAPA	SITA	DAPA	SITA
200	2000	248.33 $\pm$ 2.1	948.33 $\pm$ 2.8	0.83	0.30
600	6000	541.66 $\pm$ 3.5	1744 $\pm$ 5.6	0.64	0.31
1000	10000	871.66 $\pm$ 3.5	2568.33 $\pm$ 5.6	0.40	0.22
Mean				0.62	0.28

3.4.3.3 Inter-day precision

The inter-day precision studies were performed using standard sample on different days. The inter-day data were given in Table 12. The %RSD for both drugs were less than 2, indicated that the method is precise.

Table 12 Inter-day precision studies of DAPA and SITA

Conc. (ng/band)		Area $\times 10^{-3}$ Mean $\pm$ SD (n=3)		%RSD	
DAPA	SITA	DAPA	SITA	DAPA	SITA
200	2000	267.33 $\pm$ 2.52	947.7 $\pm$ 1.00	0.94	1.49
600	6000	417.67 $\pm$ 2.08	1742.3 $\pm$ 1.53	0.50	1.07
1000	10000	565 $\pm$ 3.61	2567.0 $\pm$ 1.54	0.64	0.70
Mean				0.69	1.08

3.4.4 Accuracy

Accuracy was evaluated by the standard-addition method at 80%, 100%, and 120% of the target concentration. Mean recoveries ranged from **99.49–100.91 % for DAPA** and **99.96–100.06 % for SITA**, demonstrating satisfactory agreement between the added and recovered amounts.

Table 13 Accuracy studies of DAPA and SITA

Drug	Spiking level	Amount of sample taken (ng/band)	Amount standard drug spiked (ng/band)	Average amount of drug found Mean $\pm$ SD (n=3)	% Recovery Mean $\pm$ SD (n=3)
DAPA	80 %	400	320	720.91 $\pm$ 3.2	100.91 $\pm$ 1
	100 %	400	400	799.72 $\pm$ 2.5	99.97 $\pm$ 0.64
	120 %	400	480	879.99 $\pm$ 2.5	99.99 $\pm$ 0.53
SITA	80 %	4000	3200	7199.84 $\pm$ 18.11	99.96 $\pm$ 0.56
	100 %	4000	4000	8003.04 $\pm$ 15.01	100.04 $\pm$ 0.37
	120 %	4000	4800	8804.39 $\pm$ 15.01	100.06 $\pm$ 0.31

3.4.5 LOD and LOQ

The LOD is the lowest amount of analyte which can be detected but not quantified. LOQ is the lowest amount of analyte which can be quantitatively determined with suitable precision and accuracy. LOD of DAPA and SITA was found to be 17.31 ng/band and 184 ng/band respectively. LOQ of DAPA and SITA was found to be 52.47 ng/band and 557.33 ng/band respectively. LOD and LOQ data of both the drugs was given in Table 14.

Table 14 LOD and LOQ data of DAPA and SITA

Parameter	DAPA	SITA
LOD (ng/band)	17.31	184.00
LOQ (ng/band)	52.47	557.33

3.4.6 Robustness:

Robustness was evaluated by deliberately varying selected chromatographic parameters, including mobile-phase composition, chamber saturation time, and migration distance. Each parameter was varied around the optimized condition while keeping the remaining parameters constant. The effect of each variation was assessed using the *R_f* values and peak areas of DAPA and SITA. The method was considered robust when the resulting variation remained within the predefined acceptance criteria mentioned at **Table 15**.

Table 15 Robustness studies of DAPA and SITA

Parameters	Change in condition	DAPA <i>R_f</i>	DAPA Peak area $\pm$ SD	DAPA %RSD	SITA <i>R_f</i>	SITA Peak area $\pm$ SD	SITA %RSD
Saturation time (30 min)	35 min	0.75	383.20 $\pm$ 2.10	0.55	0.32	1367.40 $\pm$ 3.10	0.23
	25 min	0.73	381.40 $\pm$ 1.90	0.50	0.30	1363.80 $\pm$ 2.90	0.21
Mobile phase (7.5:2:0.5)	7.0:2.5:0.5	0.72	381.60 $\pm$ 2.00	0.52	0.29	1363.50 $\pm$ 3.20	0.23
	8.0:1.5:0.5	0.76	383.10 $\pm$ 2.20	0.57	0.33	1366.90 $\pm$ 3.00	0.22
	65 mm	0.73	381.80 $\pm$ 2.10	0.55	0.30	1364.20 $\pm$ 3.10	0.23

Migration distance (70 mm)	75 mm	0.75	382.90 ± 2.00	0.52	0.32	1366.30 ± 2.80	0.20
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3.5 Force degradation:

Forced-degradation studies were performed under acidic, alkaline, oxidative, thermal, and photolytic conditions to evaluate the stability-indicating capability of the developed HPTLC method. The stressed samples were analysed to determine the extent of degradation and to assess the specificity of the method.

3.5.1 Acid hydrolysis:

Application of 6 μ L of solution provided 600 ng/band of DAPA and 6000 ng/band of SITA. Under acidic conditions, DAPA showed 9.3% degradation at Rf 0.18, while SITA showed 8.05% degradation at Rf 0.64.

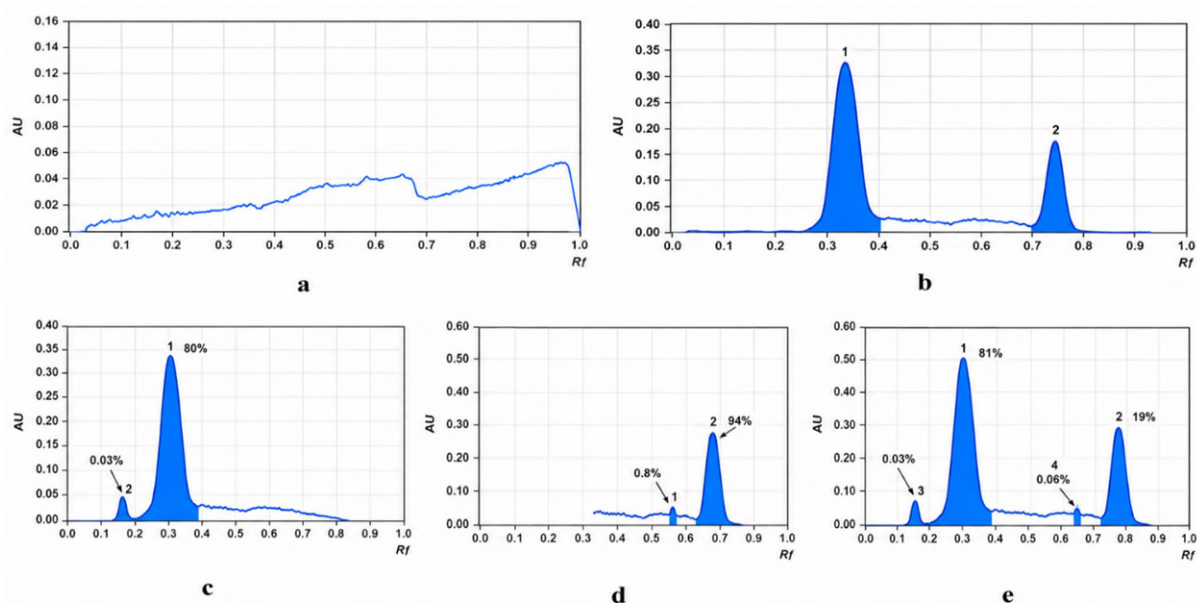


Fig 18 : HPTLC densitograms illustrating acid-induced degradation of DAPA and SITA: (a)blank; (b) control densitogram of DAPA (600 ng/band) and SITA (6000 ng/band) mixture; (c) acid-hydrolyzed DAPA (600 ng/band); (d) acid-hydrolyzed SITA (6000 ng/band); and (e) acid-hydrolyzed mixture of DAPA (600 ng/band) and SITA (6000 ng/band).

3.5.2 Alkaline hydrolysis

Application of 6 μ L of solution provided 600 ng/band of DAPA and 6000 ng/band of SITA. Under alkaline conditions, DAPA showed 12.9% degradation at Rf 0.48, while

SITA showed 11.0% degradation at R_f 0.85.

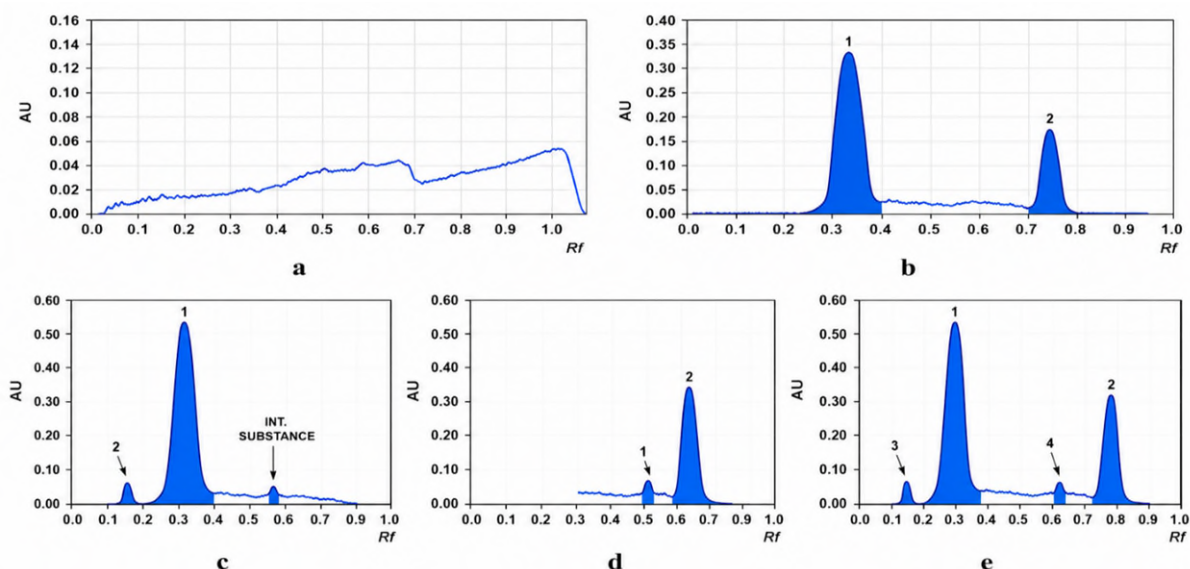


Fig 19 HPTLC densitograms illustrating alkaline-induced degradation of DAPA and SITA: (a) blank; (b) control densitogram of DAPA (600 ng/band) and SITA (6000 ng/band) mixture; (c) alkaline-hydrolyzed DAPA (600 ng/band); (d) alkaline-hydrolyzed SITA (6000 ng/band); and (e) alkaline-hydrolyzed mixture of DAPA (600 ng/band) and SITA (6000 ng/band).

3.5.3 Oxidative degradation

Application of 6 μ L of solution provided 600 ng/band of DAPA and 6000 ng/band of SITA. Under oxidative conditions, DAPA showed 7.8% degradation at R_f 0.17, while SITA showed 4.2% degradation at R_f 0.86.

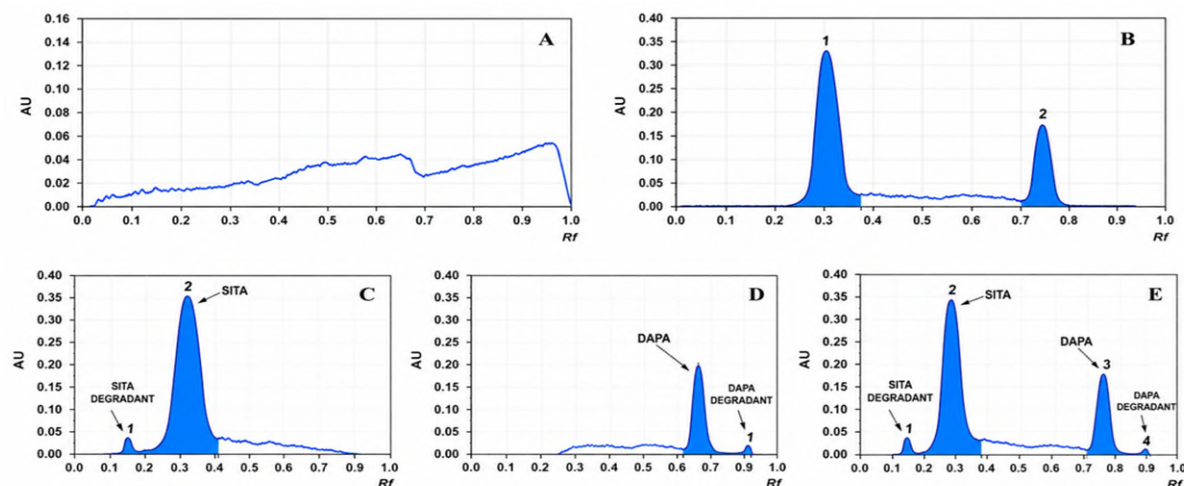


Fig 20: HPTLC densitograms illustrating oxidative degradation of DAPA and SITA: (a) blank; (b) control densitogram of DAPA (600 ng/band) and SITA (6000 ng/band) mixture; (c) oxidative degradation densitogram of DAPA (600 ng/band); (d) oxidative degradation densitogram of SITA (6000 ng/band); and (e) oxidative degradation densitogram of the DAPA (600 ng/band) and SITA (6000 ng/band) mixture.

3.5.4 Photolytic degradation

Application of 6 μ L of solution provided 600 ng/band of DAPA and 6000 ng/band of SITA. Under photolytic conditions, DAPA showed 4.9% degradation at R_f 0.20, while SITA showed 3.33% degradation at R_f 0.83.

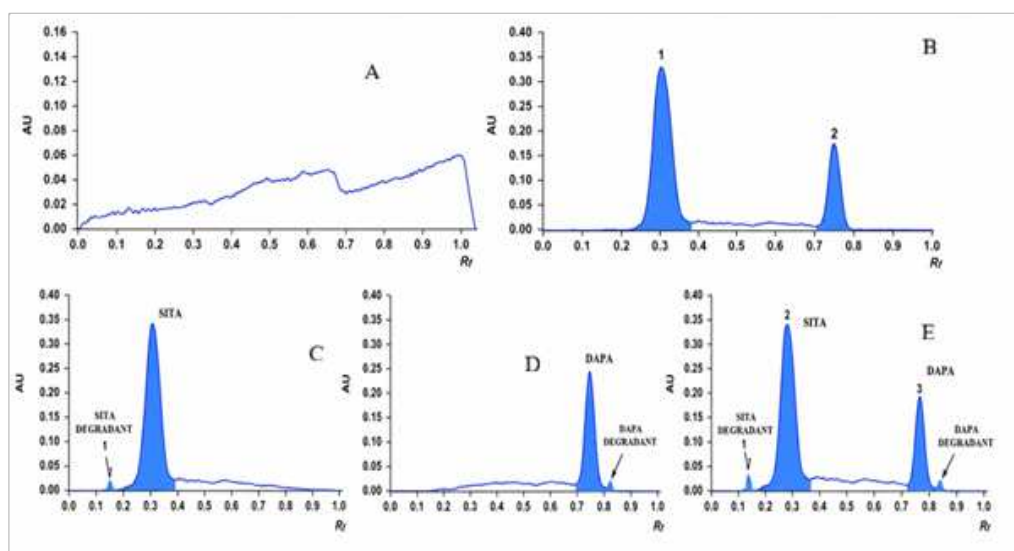


Fig 21: HPTLC densitograms illustrating photolytic degradation of DAPA and SITA: (a) blank; (b) control densitogram of DAPA (600 ng/band) and SITA (6000 ng/band) mixture; (c) photolytic degradation densitogram of DAPA (600 ng/band); (d) photolytic degradation densitogram of SITA (6000 ng/band); and (e) photolytic degradation densitogram of the DAPA (600 ng/band) and SITA (6000 ng/band) mixture.

3.5.5 Thermal degradation:

Application of 6 μ L of solution provided 600 ng/band of DAPA and 6000 ng/band of SITA. Under thermal conditions, DAPA showed 3.14% degradation at R_f 0.18, while SITA showed 2.3% degradation at R_f 0.82

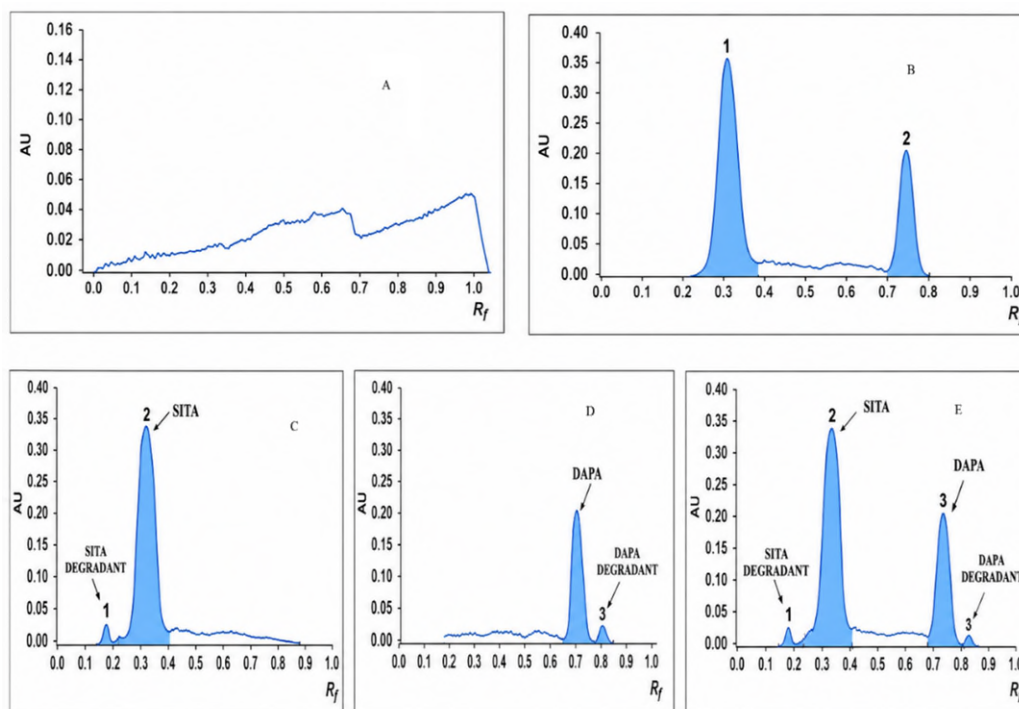


Fig 22: HPTLC densitograms illustrating thermal degradation of DAPA and SITA: (a) blank; (b) control densitogram of DAPA (600 ng/band) and SITA (6000 ng/band) mixture; (c) thermal degradation densitogram of DAPA (600 ng/band); (d) thermal degradation densitogram of SITA (6000 ng/band); and (e) thermal degradation densitogram of the DAPA (600 ng/band) and SITA (6000 ng/band) mixture.

Table 16 Summary of forced degradation.

Stress Condition	Condition Applied	% Degradation (DAPA)	% Degradation (SITA)	Observation
Acid Hydrolysis	0.1 N HCl, 60 °C, 2 h	9.30	8.05	Moderate degradation
Alkaline Hydrolysis	0.1 N NaOH, 60 °C, 1 h	12.90	11.00	Maximum degradation
Oxidative Degradation	3% H ₂ O ₂ , room temperature, 3 h	7.80	4.20	Moderate degradation
Thermal Degradation	70 °C, 6 h	3.49	4.95	Slight degradation
Photolytic Degradation	UV light, 24 h	4.90	3.33	Slight degradation

The stability-indicating capability of the proposed HPTLC method was evaluated under acidic, alkaline, oxidative, thermal, and photolytic stress conditions. DAPA exhibited degradation of

9.30%, 12.90%, 7.80%, 3.49%, and 4.90% under acidic, alkaline, oxidative, thermal, and photolytic conditions, respectively. The corresponding degradation of SITA was 8.05%, 11.00%, 4.20%, 4.95%, and 3.33%.

Among the investigated conditions, alkaline hydrolysis produced the greatest degradation for both analytes, indicating greater susceptibility of DAPA and SITA to alkaline stress under the applied experimental conditions. The degradation products were chromatographically separated from the corresponding parent analyte bands, supporting the stability-indicating capability of the developed method.

3.6 Assay of Marketed Formulation:

The developed method was applied to the simultaneous determination of DAPA and SITA in SITASON-D tablets labelled to contain 10 mg of DAPA and 100 mg of SITA per tablet. The mean assay results were $98.79 \pm 0.7\%$ **for DAPA** and $100.01 \pm 0.6\%$ **for SITA**. These results were within the acceptable range of the labelled content and demonstrated the applicability of the developed HPTLC method for routine assay of the combined pharmaceutical formulation.

Table: 17 Analysis of Marketed Formulation DAPA and SITA

Formulation		Drug	Amount of drug taken (ng/band)	Average amount of drug found (ng/band) (n=3)	% Average amount of drug found $\pm$ SD (n=3)
SITASON-D	Unison Pharmaceuticals Pvt. Ltd., Ahmedabad, India	DAPA	600	592.76	98.79 ± 0.7
		SITA	6000	5994.52	100.01 ± 0.6

3.7 Green Analytical Assessment

The comparative green analytical assessment is presented in Table 18 and Fig.23. The proposed AQbD-assisted HPTLC method exhibited a more favorable environmental profile than the reported RP-HPLC procedure across the evaluated green analytical metrics.

The Analytical Eco-Scale scores were **82 for the RP-HPLC method and 85 for the proposed HPTLC method**, indicating a modest improvement in environmental performance for the HPTLC procedure. The higher score was mainly associated with its low-volume planar development and the absence of acetonitrile.

The AGREE scores were **0.52 and 0.73 for the RP-HPLC and HPTLC methods, respectively**. The higher AGREE score for HPTLC was attributed mainly to lower solvent consumption, reduced waste generation, elimination of acetonitrile, miniaturized chromatographic operation, and the possibility of simultaneous analysis of multiple samples on a single plate.

The provisional AGREEprep scores were **0.55 for RP-HPLC** and **0.75 for HPTLC**, suggesting a more favorable sample-preparation profile for the proposed procedure. However, these values should be interpreted cautiously because complete sample-preparation information for the reported literature method was unavailable.

The GAPI assessment further supported the favorable environmental profile of the HPTLC procedure, particularly with respect to solvent consumption, waste generation, and chromatographic operation. Overall, the combined assessment indicates that the proposed AQbD-assisted HPTLC method provides a **greener and more resource-efficient alternative** to the reported RP-HPLC method while maintaining satisfactory analytical performance.

It should be emphasized that the proposed procedure is described as a **greener analytical method rather than a completely green method**, because methanol and other chemical reagents remain part of the analytical workflow.

Table 18 Comparative green analytical assessment of the reported RP-HPLC and proposed AQbD-assisted HPTLC methods

Metric	RP-HPLC	AQbD-HPTLC	Difference
Analytical Eco-Scale	82	85	+3
AGREE	0.52	0.73	+0.21
AGREEprep*	0.55	0.75	+0.20
GAPI	Less favorable	More favorable	---

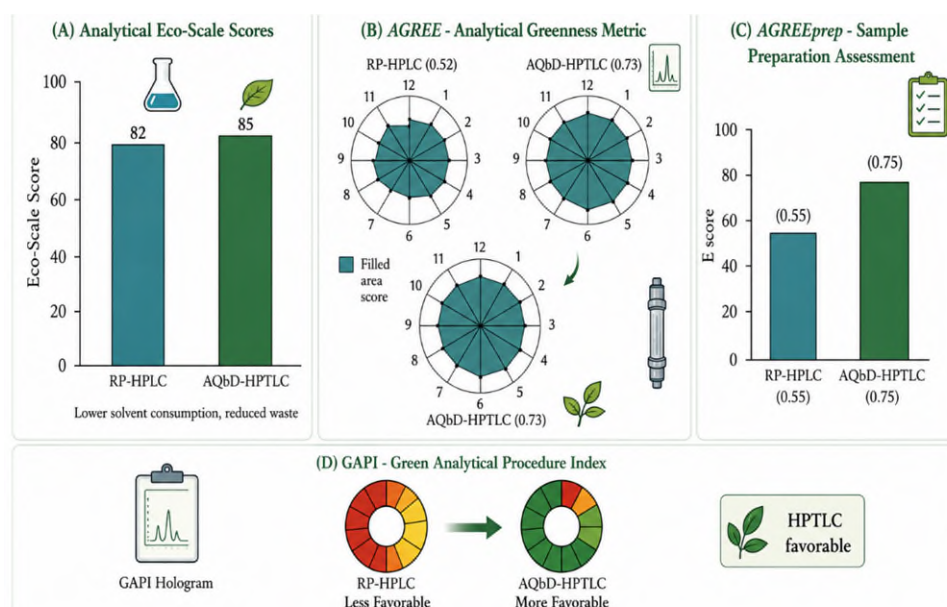


Fig 23 Comparative green analytical assessment of the reported RP-HPLC and proposed AQbD-assisted HPTLC methods: (A) Analytical Eco-Scale, (B) AGREE, (C) AGREEprep, and (D) GAPI pictograms.

4 CONCLUSION

An AQbD-assisted HPTLC-densitometric method was successfully developed and optimized for the simultaneous determination of DAPA and SITA in pharmaceutical formulations. A two-factor central composite design enabled systematic evaluation of ethyl acetate and methanol as critical method parameters, and the resulting quadratic models provided an experimentally verifiable basis for optimization of the chromatographic conditions. The optimized mobile phase, ethyl acetate:methanol:formic acid (7.5:2.0:0.5, v/v/v), produced reproducible R_f values of approximately 0.74 for DAPA and 0.31 for SITA.

The method demonstrated satisfactory specificity, linearity, precision, accuracy, sensitivity, and robustness in accordance with the requirements of ICH Q2(R2). Forced-degradation studies under acidic, alkaline, oxidative, thermal, and photolytic conditions generated degradation products that were chromatographically separated from the parent analyte bands, while peak-purity assessment supported the spectral homogeneity of the analyte bands. Application to the marketed formulation provided assay values of $98.79 \pm 0.70\%$ for DAPA and $100.01 \pm 0.60\%$ for SITA.

Comparative assessment using the Analytical Eco-Scale, AGREE, GAPI, and AGREEprep indicated that the proposed HPTLC procedure has a more favorable environmental profile than the compared RP-HPLC procedure. Overall, the developed method combines systematic AQbD-based optimization, stability-indicating capability, satisfactory analytical performance, and comparatively improved environmental characteristics, supporting its potential application in routine quality control of DAPA–SITA pharmaceutical formulations.

Ethics approval and consent to participate:

Not applicable

Consent for publication:

Not applicable

Data availability

The data supporting the finding of this study are available from corresponding author upon reasonable request.

Competing interests:

The authors declare that they have no competing interests.

Authors' contributions:

Priyanka Malani: Conceptualization, methodology, investigation, data analysis, writing—original draft, and supervision. Zeel Shah: Investigation and experimental work. Lakshmi Radhika G, Salaj Khare, Kautuk P. Shah, Archana Barot, Imtiyaz Bagban, Bansari Patel, Ashim Kumar Sen, and Hamza Shaikh: Review, scientific input, and manuscript editing. All authors reviewed and approved the final manuscript.

I confirm that all authors listed on the title page have contributed significantly to the work, have read the manuscript, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission

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Abbreviations List

- **ADC 2:** Automatic Developing Chamber 2
- **AGREE:** Analytical GREEnness Metric Approach
- **AGREEprep:** Analytical GREEnness Metric for Sample Preparation
- **ANOVA:** Analysis of Variance
- **AQbD:** Analytical Quality by Design
- **ATP:** Analytical Target Profile
- **CAA:** Critical Analytical Attribute
- **CCD:** Central Composite Design
- **CMP:** Critical Method Parameter
- **CV:** Coefficient of Variation
- **DAPA:** Dapagliflozin Propanediol Monohydrate
- **DM:** Diabetes Mellitus
- **DoE:** Design of Experiments
- **DPP-4:** Dipeptidyl Peptidase-4
- **GAC:** Green Analytical Chemistry
- **GAPI:** Green Analytical Procedure Index
- **HPTLC:** High-Performance Thin-Layer Chromatography
- **ICH:** International Council for Harmonisation
- **LC–MS:** Liquid Chromatography–Mass Spectrometry
- **LOD:** Limit of Detection
- **LOQ:** Limit of Quantification
- **R²:** Coefficient of Determination

- **R_f**: Retardation Factor
- **RP-HPLC**: Reversed-Phase High-Performance Liquid Chromatography
- **%RSD**: Percentage Relative Standard Deviation
- **SD**: Standard Deviation
- **SGLT2**: Sodium–Glucose Cotransporter-2
- **SITA**: Sitagliptin Phosphate Monohydrate
- **T2DM**: Type 2 Diabetes Mellitus
- **TLC**: Thin-Layer Chromatography
- **UV**: Ultraviolet
- **v/v**: Volume by Volume
- **v/v/v**: Volume by Volume by Volume

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