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Development and Validation of a High-Performance Thin-Layer Chromatography (HPTLC) Method for Accurate Quantification of Cyclosporine in Pharmaceutical Formulations

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ABSTRACT: This study presents the development of a new HPTLC method for the quantification of cyclosporine in bulk drug substances and pharmaceutical formulations, adhering to ICH guidelines. The technique employs a mobile phase consisting of toluene, ethyl acetate, and acetic acid (3:7). Cyclosporine is detected using densitometric scanning at 202 nm, with separation performed on silica gel 60 F254 TLC plates (0.2 mm). The method demonstrates excellent linearity ($R^2 > 0.999$) over the concentration range of 50–150%. Accuracy studies show recoveries within 98–102%, while precision analysis reveals %RSD values below 2.0%. Specificity testing confirms that cyclosporine is well resolved from excipients and impurities, ensuring accurate quantification. The limit of detection (LOD) is determined to be 44.02 ng/band, and the limit of quantification (LOQ) is 124.3 ng/band, highlighting the method's high sensitivity. A robustness assessment indicates that variations in chamber saturation time and mobile phase composition have minimal impact on the method's performance. Due to its simplicity, low cost, and reliable performance, the developed HPTLC method is ideal for routine quality control of cyclosporine in pharmaceutical formulations. This work provides a foundation for using this method in stability testing, impurity identification, and the determination of therapeutic drug levels in body fluids.

KEYWORDS: HPTLC, Cyclosporine, Method Validation, ICH Guidelines, Pharmaceutical Analysis, Robustness.

I. INTRODUCTION

Cyclosporine is a widely used immunosuppressant essential for preventing transplant rejection and managing autoimmune diseases. It works by inhibiting T-cell activation, a crucial process in regulating immune responses. However, due to its narrow therapeutic window, even small variations in dosage can lead to toxicity or reduced effectiveness. This necessitates accurate monitoring, highlighting the need for reliable and precise analytical techniques to ensure its safe and effective use in pharmaceutical formulations [1,2].

Thin-layer chromatography (TLC) has evolved significantly, leading to the advancement of high-performance thin-layer chromatography (HPTLC), which offers enhanced resolution, sensitivity, and reproducibility. HPTLC has become a valuable tool in pharmaceutical analysis, providing a rapid and efficient method for drug identification, quantification, and quality control. This method has several advantages, such as ease of use, cost-effectiveness, and low solvent consumption. Additionally, multiple samples can be analyzed simultaneously within a short time. These factors make HPTLC a more attractive choice compared to sophisticated techniques such as HPLC and LC-MS, which require specialized equipment and longer sample preparation times [3–5].

Despite the general acceptance of HPTLC in pharmaceutical method development and the availability of methodologies for cyclosporine determination, its HPTLC analysis remains under-researched. Many studies have described the use of HPLC or LC-MS, which, while ultra- sensitive, may not be suitable for routine quality control analysis due to their operational complexity and cost [6,7]. Furthermore, to the best of the authors' knowledge, no reports have confirmed HPTLC methods for cyclosporine analysis in compliance with ICH guidelines, which ensure accuracy, precision, and reliability [8,9].

This study aims to develop and validate a novel HPTLC method for the quantification of cyclosporine. The proposed technique is simple, efficient, and adheres to ICH guidelines, ensuring its suitability for pharmaceutical quality control [10,11].

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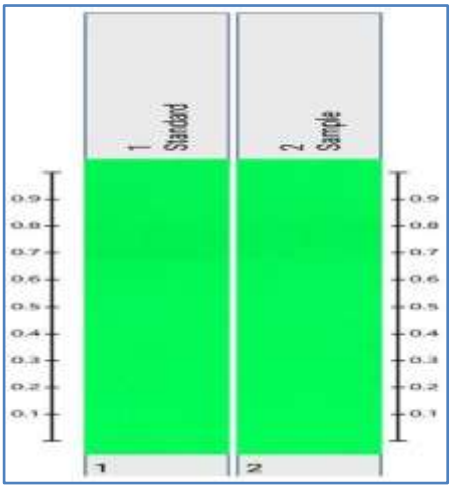

Figure 1: HPTLC Plate Visualization at UV-254nm showing distinct Cyclosporine bands separated using the optimized mobile phase.

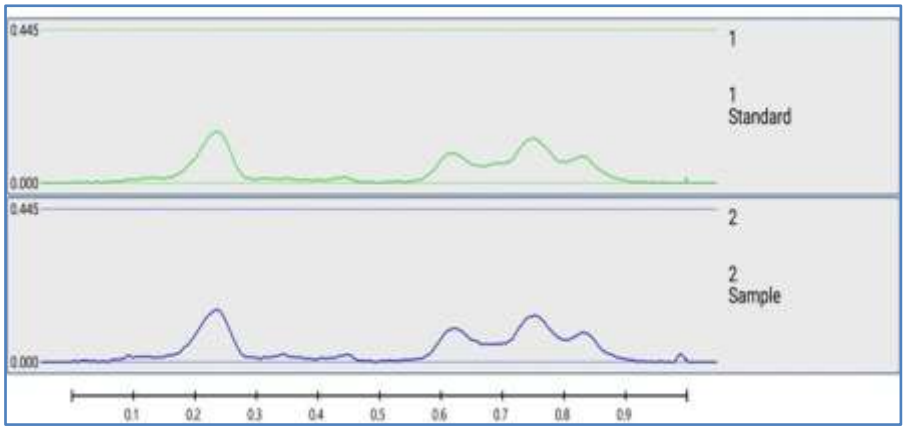

Figure 2: Densitogram of Cyclosporine Standard and Test Solutions scanned at 202nm, illustrating sharp and well-defined peaks.

II. MATERIALS AND METHODS

Materials

The materials used in this study included a cyclosporine standard (≥99% purity) purchased from Steris Healthcare Private Limited. Cyclosporine Eye Drops BP (0.5 mg/mL) were obtained from a reputable supplier. HPLC-grade methanol was sourced from leading manufacturers such as Merck or Sigma-Aldrich. Additional chemicals, including toluene and ethyl acetate of analytical reagent grade, as well as glacial acetic acid, were procured fromrecognized chemical vendors. TLC plates (silica gel 60 F254, 0.2 mm thickness) were supplied by Merck. A 0.22 µm syringe filter, obtained from a trusted laboratory supplier, was used for solution filtration.

Instrumentation

The study utilized a CAMAG High-Performance Thin-Layer Chromatography (HPTLC) system equipped with VisionCATS software (version 3.2 SP2) for method development and analysis. TLC plate development and densitometric scanning were performed using a CAMAG densitometer. Fluorescence visualization was conducted under UV light at 254 nm, while densitometric scans were recorded at 202 nm for optimal detection. This system enabled high-precision, accurate, and efficient analysis of cyclosporine [12,13].

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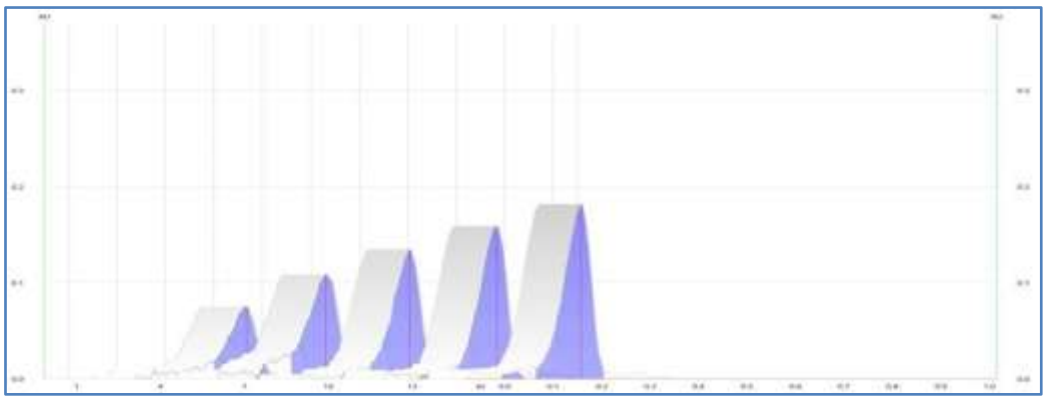

Figure 3: 3D Linearity Image of Cyclosporine Standard (Scan at 202 nm) demonstrating consistent analytical response across the tested concentration range.

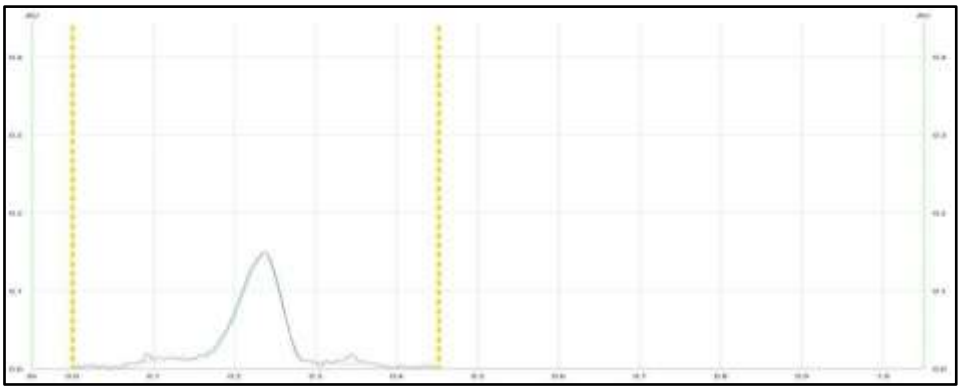

Figure 4: Overlay of Standard and Test Solution Densitograms showcasing comparable retention factors and peak characteristics.

Table 1: Linearity data for Cyclosporine showing the standard and mean areas at different concentration levels.

Level	StandardAreaof Cyclosporine	MeanStandardAreaof Cyclosporine
50%level	0.00518	0.00509
	0.00492	
	0.00518	
75%level	0.00715	0.00713
	0.00709	
	0.00714	
100%level	0.00880	0.00879
	0.00879	
	0.00879	

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125%level	0.01057	0.01061
	0.01059	
	0.01066	
150%level	0.01231	0.01243
	0.01242	
	0.01255	

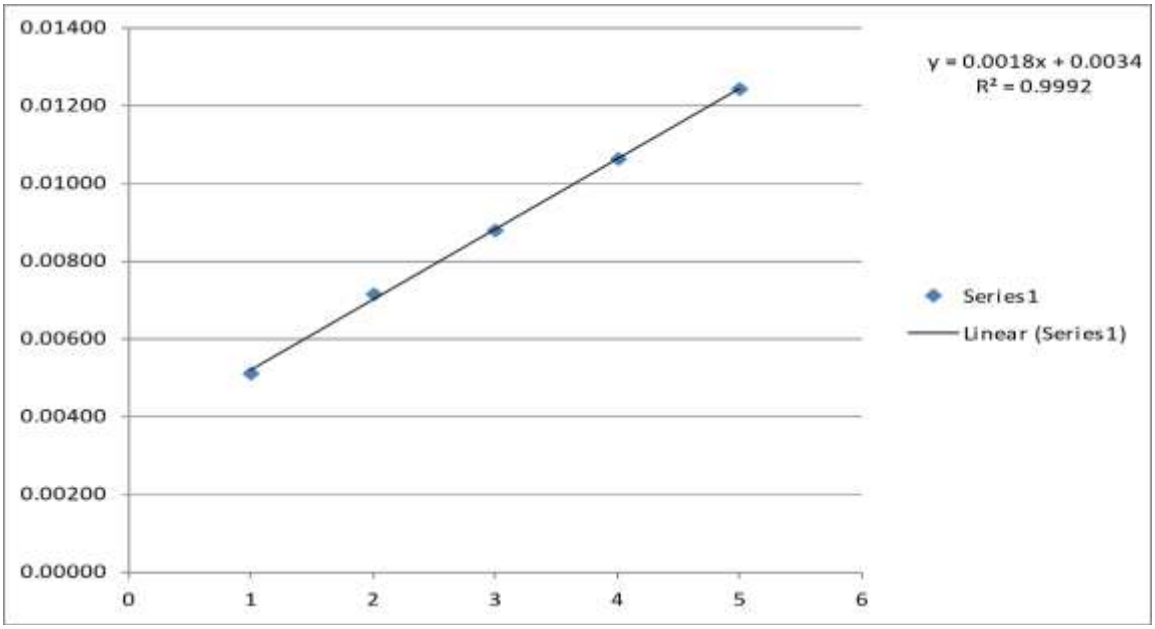

Figure 5: Calibration curve for Cyclosporine demonstrating the linear relationship between concentration and standard area.

Table 2: Accuracy/Recovery data for Cyclosporine showing the percentage recovery and %RSD at different spiking levels.

Sr. No.	Spike level	Amount added (mg) Cyclosporine STD in Sample	Area of Cyclosporine STD in Sample	% Recovery of Cyclosporine STD in Sample	Amount Found (mg) of Cyclosporine STD in Sample	Mean % Recovery of Cyclosporine STD in Sample	RSD %

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1	100% Base Level	0	0.00848	99.71	**0**	99.47	0.24
2	100% Base Level		0.00844	99.24			
3	100% Base Level		0.00846	99.47			
4	10% Spike	1	0.00941	100.58	1.00	100.23	0.44
5	10% Spike		0.00939	100.37			
6	10% Spike		0.00933	99.73			
7	20% Spike	2	0.0102	99.94	1.990	99.48	0.63
8	20% Spike		0.01018	99.74			
9	20% Spike		0.01008	98.76			
10	30% Spike	3	0.01112	100.57	3.022	100.72	0.34
11	30% Spike		0.01118	101.12			

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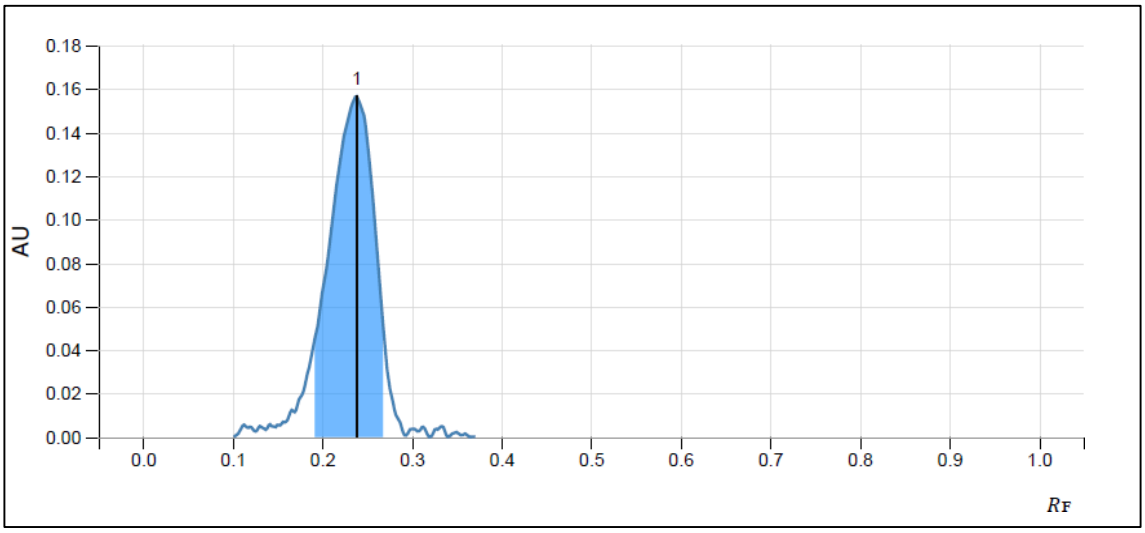

Figure 6: Densitogram of Cyclosporine Standard Area demonstrating %RSD within acceptable limits.

Table 3: Method precision data for Cyclosporine showing the standardand sample area with %RSD and percentage assay within the acceptable range.

SrNo.	**Sample Area of Cyclosporine**	**%ofCyclosporineSTD inSample**
1	0.00871	99.37
2	0.00873	99.59
3	0.00867	98.91
4	0.00871	99.37
5	0.00874	99.71
6	0.00869	99.14
Mean	0.00871	99.3
SD	0.00003	0.29234
%RSD	**0.29**	**0.29**

Figure 7: Densitogram of Cyclosporine standard areas illustrating method precision with %RSD within acceptable limits.

Preparation of Standard Solution

A cyclosporine standard solution was prepared from a stock solution at a concentration of 100 µg/mL. Accurately, 1 mg of cyclosporine was weighed and dissolved in a 10 mL volumetric flask. Initially, 5 mL of methanol was added, and the solution was sonicated at 25 °C for 15 minutes to ensure complete dissolution [14]. After sonication, the volume was adjusted to 10 mL with methanol, resulting in a final working concentration of 0.1 mg/mL (100 ppm). The solution was then centrifuged at 10,000 rpm for 10 minutes, and the obtained supernatant was filtered through a 0.22 µm syringe filter before analysis [15,16].

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Preparation of Sample Solution

To prepare the sample solution, 2 mL of Cyclosporine Eye Drops BP (0.5 mg/mL) was accurately pipetted into a 10 mL volumetric flask. Methanol (5 mL) was added, and the solution was subjected to ultrasonic treatment at 25 °C for 15 minutes to ensure complete dissolution. The volume was then adjusted with methanol to achieve a final working concentration of 0.1 mg/mL (100 ppm). The solution was subsequently centrifuged at 10,000 rpm for 10 minutes, and the resulting supernatant was filtered through a 0.22 µm syringe filter before application onto the HPTLC plate [17].

Chromatographic Conditions

Separation of the components was achieved using pre-coated silica gel 60 F254 plates with a thickness of 0.25 mm (Merck) as the stationary phase. The mobile phase consisted of a mixture of toluene, ethyl acetate, and acetic acid in the ratio of 3.0:7.0:0.1 (v/v/v). Before sampleapplication, the TLC chamber was preconditioned with the mobile phase for 20 minutes to ensure optimal separation of the compounds. In this study, both samples and standards were applied as 5 µL bands onto the plate using the CAMAG Linomat applicator. The plates were then developed in the mobile phase up to a distance of 70 mm and air-dried.

Visualization and Detection

The developed TLC plates were first examined under UV light at 254 nm for the preliminary analysis of the separated bands. Densitometric scanning was then performed at 202 nm using a CAMAG TLC Scanner to accurately quantify the peaks. The resulting densitograms were analyzed quantitatively, with peak identification performed using the standard solution. A 3D densitometric image of the standard glycan was generated, along with an overlay of the standard and sample peaks for comparison and further analysis [18,19].

Validation Parameters

The validation of the HPTLC method was carried out in accordance with the guidelines set by the International Council for Harmonisation (ICH). Each validation parameter was thoroughly evaluated as outlined below:

Linearity

Chromatographic linearity was assessed using cyclosporine solutions prepared at concentrations ranging from 20 ppm to 120 ppm as stock solutions, which were applied to the TLC plate. The densitograms were recorded at 202 nm, and the peak areas were plotted against their respective concentrations. Linear regression analysis was performed to derive the regression equation, and the coefficient of determination (r^2) was calculated to confirm linearity within the specified concentration range [20].

Accuracy (Recovery Studies)

The validity of the method was further confirmed by preparing a known amount of cyclosporine standard at three concentration levels (80%, 100%, and 120%) within the sample matrix. The spiked samples were analyzed, and the percentage recovery of the actual spiked concentration was determined based on the obtained results [21].

Precision

The precision of the method was assessed by evaluating both repeatability and intermediate precision. Repeatability was determined by analyzing six replicates of the standard solution at 100 ppm on the same day under identical conditions. Intermediate precision was assessed by repeating the analysis on different days and involving a second analyst to ensure the method's robustness and reliability [22].

Specificity

The selectivity of the method was evaluated using both standard and sample solutions to assess its ability to separate cyclosporine from potential excipients or degradation products. The densitograms were carefully examined for any interference at the retention factor (Rf) of cyclosporine to ensure that the method specifically detected the drug without cross- contamination from other components [23].

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) were determined according to ICH guidelines using the formulas:

- LOD = 3.3 × (SD/Slope)
- LOQ = 10 × (SD/Slope)

The method was considered sensitive if the LOD and LOQ values were lower than the lowest standard concentration used in the study.

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Table 4: Specificity data showing distinct separation of Cyclosporine from potential excipients and impurities.

Sample	RetentionFactor (Rf)	Observation
Standard Cyclosporine	0.5	Sharppeak observed
CyclosporineinSample	0.5	Sharppeak observed
ExcipientBlank	Nopeakdetected	No interference

Table 5: Robustness Data for Chamber Saturation Time

SrNo.	Standard Area of Cyclosporine STD(15min)	Rt of Cyclosporine STD(15min)	Standard Area of Cyclosporine STD(25min)	Rt of Cyclosporine STD(25min)
1	0.00885	2.433	0.00917	2.433
2	0.00885	2.433	0.00913	2.422
3	0.00888	2.433	0.00916	2.442
4	0.00886	2.433	0.00993	2.442
5	0.00885	2.442	0.00915	2.442

Table 6: Robustness Data for Mobile Phase Composition

SrNo.	Standard Area of Cyclosporine STD(-10%)	Rt of Cyclosporine STD (-10%)	Standard Area of Cyclosporine STD (+10%)	Rt of Cyclosporine STD (+10%)
1	0.00939	2.433	0.0094	2.433
2	0.00935	2.442	0.0094	2.433
3	0.00929	2.433	0.00939	2.433
4	0.00932	2.442	0.00944	2.433
5	0.00938	2.442	0.00952	2.433

Robustness

The robustness of the method was evaluated by deliberately making small changes to one or more method parameters, such as the composition of the mobile phase, saturation time in the evaporation chamber, and detection wavelength. The effect of these changes on the number of theoretical plates, calculated from the Rf value and peak area, was further analyzed to assess the method's robustness under varying conditions [24].

Ruggedness

The reliability of the method was assessed by testing the same samples in different laboratories using different instruments and analysts. These conditions were tested to validate the method's applicability, and the consistent results obtained supported its robustness [25,26]

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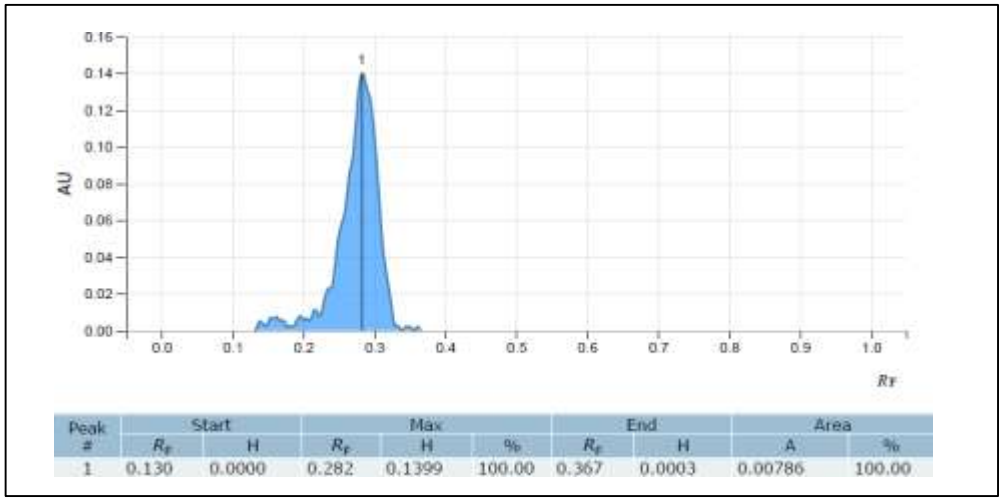

Peak #	Start		Max			End		Area	
	R_f	H	R_f	H	%	R_f	H	A	%
1	0.130	0.0000	0.282	0.1399	100.00	0.367	0.0003	0.00786	100.00

Figure 8: Densitogram showing the robustness of the HPTLC method with consistent retention factor (Rf) and peak area under varied conditions.

III. RESULTS AND DISCUSSION

3.1 HPTLC Method Development

The optimization of the HPTLC method for the determination of cyclosporine was carried out and is presented here. The selection of the mobile phase, comprising toluene, ethyl acetate, and acetic acid in a ratio of 3.0:7.0:0.1 (v/v/v), resulted in excellent baseline separation of cyclosporine with a well-defined, sharp peak. The mobile phase, dichloromethane: methanol (9:1 v/v), and the pre-coated silica gel 60 F254 TLC plates showed no interference from excipients or impurities. A chamber saturation time of 20 minutes ensured an even solvent front, while a solvent migration distance of 70 mm resulted in consistent and reproducible results. Visualization under UV light at 254 nm produced distinct bands, and a densitometric scan at 202 nm was found to be suitable for qualitative analysis.

3.2 Visualization and Photo-Documentation

The HPTLC method for cyclosporine analysis was successfully developed and optimized, yielding accurate and repeatable results. The mobile phase, consisting of toluene, ethyl acetate, and acetic acid in a 3.0:7.0:0.1 (v/v/v) ratio, provided excellent resolution, producing sharp and symmetrical peaks for cyclosporine. Pre-coated silica gel 60 F254 TLC plates (Whatman) were utilized, ensuring high resolution and effective separation without interference from excipients or impurities. A chamber saturation time of 20 minutes was employed to achieve a uniform solvent front, with the solvent migrating 70 mm on the plate.

Cyclosporine bands were visualized under UV light at 254 nm, revealing compact and distinct bands, confirming effective separation (Figure 1: HPTLC Plate Visualization at UV-254 nm). Densitometric scanning at 202 nm further verified the separation and quantification, with the resulting densitogram (Figure 2: Densitogram of Cyclosporine Standard and Test Solutions) displaying well-resolved peaks for both the standard and sample, demonstrating the method's accuracy. The method exhibited excellent linearity across the tested concentration range, as shown in the 3D densitogram (Figure 3: Cyclosporine Standard: 3D Linearity Image at 202 nm). The overlay of the standard and test solution densitograms (Figure 4: Standard and Test Solution Densitograms Superimposed) showed consistent retention factors and peak profiles, further confirming the accuracy and specificity of the developed method.

Validation Parameters

Linearity

The linearity of the developed HPTLC method was assessed across five concentration levels, with the incubation terminated at 100% of the test concentration, as well as at 50%, 75%, 125%, and 150% thereof. The mean standard areas of Cyclosporine at each concentration level increased proportionally with concentration, confirming the linearity of the method. The linearity was found to be satisfactory, with the coefficient range not being less than 0.999 (Table

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1, Figure 5). This strong linear relationship further confirms the applicability of the method for the quantitative determination of Cyclosporine across a broad concentration range, making it more reliable for routine quality control.

Accuracy (Recovery Studies)

The accuracy of the developed HPTLC method was assessed through recovery studies by spiking known amounts of Cyclosporine standard into the sample matrix at different levels: no stress, 100% base level, 10% stress, 20% stress, and 30% stress. The percentage recovery and relative standard deviation (%RSD) were calculated to evaluate the method's accuracy.

At the 100% base level, the mean recovery was 99.47%, with a %RSD of 0.24%. For the spiked levels, recovery percentages ranged from 98.76% to 101.12%, with %RSD values below 2.0%, indicating that the method is both precise and accurate. These findings are detailed in Table 2 and Figure 6, which present the accuracy/recovery data for Cyclosporine.

The results confirm the efficiency of the developed method for the quantitative determination of Cyclosporine in drug products. The acceptable %RSD values and near-quantitative recoveries at all levels demonstrate the reliability and versatility of the method for routine quality control analysis.

Precision

The precision of the developed HPTLC method was evaluated by analyzing six replicates of both the Cyclosporine standard and sample. The standard area for Cyclosporine showed a mean value of 0.00868, with a %RSD of 0.39%, which is well within the acceptance limit of 2% RSD. Similarly, the mean value for the sample area of Cyclosporine was 0.00871, with a %RSD of 0.29%.

The % assay of Cyclosporine in the analyzed samples ranged from 98.91% to 99.71%, with an average of 99.3%. These results confirm that the method is reliable, demonstrating good recovery and results well within the acceptable range of 98%–102%. This further supports the accuracy of the developed method for determining Cyclosporine content in pharmaceutical formulations, as shown in Table 3 and Figure 7.

Specificity

The specificity of the developed HPTLC method was assessed by analyzing Cyclosporine in the presence of excipients and impurities. The densitogram showed sharp peaks at the same Rf for both the standard and test samples, with no interference from excipients or degradation products. The method effectively differentiated Cyclosporine without any overlapping,ensuring accurate quantification. These results confirm that the method is fully specific for the determination of Cyclosporine in pharmaceutical formulations, as shown in Table 4.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) for the developed HPTLC method were determined based on the standard deviation of the response and the slope of the calibration curve. The LOD, representing the lowest concentration of Cyclosporine that can be detected, was found to be 44.02 ng/band. The LOQ, or the smallest concentration that can be reliably quantified, was determined to be 124.3 ng/band.

These results demonstrate that the method is highly sensitive, making it suitable for accurately determining Cyclosporine concentrations. This level of sensitivity is particularly important in pharmaceutical quality control, where precise measurements at trace levels are often required.

Robustness

The developed HPTLC method was deliberately challenged by altering the chamber saturation time and the composition of the mobile phase to assess its robustness. To confirm the reliability of the method under these conditions, the %RSD of the standard area and the Rf of Cyclosporine were determined.

1. Change in Chamber Saturation Time ± 5 Minutes (20 Minutes)

1.1 Change in Chamber Saturation Time - 5 Minutes (15 Minutes)

When the chamber saturation time was adjusted to (75 ± 5) minutes, the %RSD obtained for the standard area and retention time was 0.15% and 0.17%, respectively, both well below theacceptable limit of 2.0%. This indicates that the method is relatively unaffected by minor variations in chamber saturation time.

1.2 Change in Chamber Saturation Time + 5 Minutes (25 Minutes)

Extending the chamber saturation time by 5 minutes resulted in a %RSD of 0.19% for the standard area and 0.36% for the retention time. These results confirm that slight variations in

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saturation time do not significantly impact the integrity of the method (Table 5).

2. Change in Mobile Phase Composition ± 10%

To evaluate the stability of the mobile phase, modifications of ±10% were applied to the original composition of 40% ethyl acetate, 60% toluene, and 0.5% acetic acid. The %RSD values for both the standard area and retention time remained within the acceptable limit of 2.0%, indicating the robustness of the method under these conditions.

2.1 Change in Mobile Phase Composition -10% (7.3:2.7:0.1 v/v/v)

With a 10% increase in ethyl acetate, the %RSD for the standard area and retention time was 0.45% and 0.20%, respectively. These results indicate that the method remains stable despite slight modifications in the mobile phase composition.

2.2 Change in Mobile Phase Composition +10% (6.7:3.3:0.1 v/v/v)

When the ethyl acetate ratio was reduced by 10%, the %RSD for the standard area was 0.57%, while the retention time showed no variation (%RSD = 0.00%). These findings confirm that the method remains applicable and reliable even with slight changes in mobile phase composition (Table 6, Figure 8).

IV. DISCUSSION

The validated HPTLC method for Cyclosporine demonstrated excellent reproducibility and was found to be fit for purpose according to ICH guidelines for pharmaceutical analysis. The linearity ($R^2 \geq 0.9994$) was confirmed over a broad concentration range, and the method exhibited a recovery rate between 98%–102%, confirming its accuracy. Precision analyses resulted in %RSD values lower than 2.0%, ensuring the method's reliability andreproducibility. Specificity studies showed good resolution ofCyclosporine from excipients and other related substances, proving the method's ability to differentiate and quantify Cyclosporine accurately.The LOD of 44.02 ng/band and LOQ of 124.3 ng/band demonstrated the high sensitivity of the method, enabling the detection of trace amounts of Cyclosporine. Sensitivity was further evaluated by assessing changes in chamber saturation time and mobile phase composition, with results indicating minimal effects on the retention factor and peak area. This study highlights the simplicity, low cost, and versatility of the developed method, making it a valuable alternative to more complex and expensive chromatographictechniques. The method's effectiveness in optimizing the quality control of Cyclosporine formulations further solidifies its role in pharmaceutical analysis.

V. CONCLUSION

In conclusion, the developed HPTLC method for the estimation of Cyclosporine in dosage forms has been successfully validated according to ICH guidelines. The method demonstrated excellent linearity (R^2>0.999) and accuracy, with percentage recoveries between 98%–102%, and precision with %RSD values below 2.0%. It effectively isolated Cyclosporine from excipients and impurities, with a low LOD of 44.02 ng/band and LOQ of 124.3 ng/band, confirming its high sensitivity. The method was found to be independent of changes in chamber saturation time and mobile phase composition, as minor alterations had minimal impact on the retention factor and peak area. These results affirm the suitability and repeatability of the method for reliable and accurate Cyclosporine analysis in pharmaceutical formulations.

FUTURE PERSPECTIVE

Due to its reliability and cost-effectiveness, the validated HPTLC method for Cyclosporine can serve as an efficient tool in pharmaceutical quality control. Further developments could involve expanding the method's application to biological fluids, including plasma or serum, for therapeutic drug monitoring and pharmacokinetic-related research. Additionally, the method could be adapted for stability studies and impurity analysis, providing insights into the degradation characteristics of Cyclosporine under different storage conditions.

Integrating this HPTLC method with densitometric scanning and computer-based data analysis can enhance throughput and accuracy, making it suitable for large-scale manufacturing. Furthermore, its ease of use and low operational costs make it ideal for laboratories that require affordable yet reliable pharmaceutical testing and analysis, ultimately contributing to the increased availability of quality pharmaceutical products in the market.

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