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Original Article

Development and Validation of High-Performance Thin Layer Chromatographic Method for Estimation of Acyclovir

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ABSTRACT

Background: The antiviral medication genre comprises the synthetic purine nucleoside analogue acyclovir, which particularly is used to treat varicella-zoster and herpes simplex virus infections. By incorporating into viral DNA to stop further synthesis, acyclovir limits DNA synthesis and viral multiplication. The investigation of an economical alternative was spurred by the existence of the currently used HPLC and UV techniques for acyclovir analysis, which are recognized for their solvent-intensive character. The innovation offers a more cost-effective method of pharmaceutical analysis along with improving sustainability. **Materials and Methods:** Using a CAMAG Linomat 5 sampler, the samples were spotted in the form of bands on a precoated silica gel plate using a CAMAG microliter syringe. The application rate was kept constant at 100 nL/sec and the spacing between the tracks was set. Chloroform, ethanol, isopropyl alcohol and strong ammonia (2:4:3:1 v/v/v/v) constitute the mobile phase. The development process was linear ascending in a twin trough glass chamber that was saturated with a mobile phase. To estimate acyclovir, densitometric scanning was carried out in the absorbance mode at 254 nm. **Results:** With r^2=0.9974, the calibration plots' linear regression analysis results demonstrated a strong linear association. The limit of quantification was 11.7726 µg/mL, whereas the limit of detection was 3.884 µg/mL. **Conclusion:** According to statistical analysis of the data, the approach was found to be precise, accurate, repeatable, sensitive and selective for the analysis of acyclovir. The technique will work well for routine quality control when estimating acyclovir as a bulk medication.

Keywords: HPTLC, ICH guidelines, Method development, Acyclovir.

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INTRODUCTION

Acyclovir is a synthetic purine nucleoside analogue (Figure 1) that belongs to the group of antiviral drugs and is prescribed to treat infections caused by the Herpes Simplex Virus (HSV) and the varicella-zoster virus.[1,2] Acyclovir works by inhibiting DNA synthesis and viral replication by integrating itself into viral DNA to prevent further synthesis. It has received FDA approval for the treatment of genital herpes and HSV encephalitis.[2,3] To ensure the accuracy and precision of a specific analyte, an analytical method must be developed and validated. However, the available analytical methods for estimating acyclovir are not entirely satisfactory and many suffer from low sensitivity, simplicity issues, or a lack of selectivity.[4] To address this significant analytical gap, we have developed a specific protocol for detecting and quantifying acyclovir using High Performance Thin Layer Chromatography (HPTLC). The HPTLC method offers a versatile, cost-effective tool for pharmaceutical research and quality control purposes and has the potential to overcome the limitations of the current methods, which often require a high amount of solvents, HPTLC can be considered a more economical approach. Our objective is to enhance sensitivity, improve efficiency, contribute to resource conservation and overcome the limitations of the existing analytical methods. Our study aims to fill the gap in available analytical methods and provide a more cost-effective alternative for the detection and quantification of acyclovir. By developing and validating an HPTLC method, we hope to contribute to the advancement of pharmaceutical research and quality control; which is the purpose of the study.

MATERIALS AND METHODS

Chemicals and reagents

The pharmaceutical-grade working standards of Acyclovir were obtained. Acyclovir formulation (400 mg) was used from the market. The High-Performance Liquid Chromatography (HPLC)-grade Methanol, Ethanol, Chloroform, Propan-2-ol

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and strong ammonia solution were procured from the certified vendor. Distilled water was used for analysis.

Methodology

Preparation of standard stock solution

By dissolving 2 mg of the standard Active Pharmaceutical Ingredient (API) in 10 mL of the volumetric flask with methanol, a stock solution of Acyclovir standard API was prepared. For a period of 10 min, the drug solution was sonicated. A final concentration of 200 µg/mL was achieved as an end result.[5]

Preparation of stock solution of marketed formulation

A stock solution of the Acyclovir marketed formulation was prepared by dissolving 300 mg of powdered tablet in 250 volumetric flasks with methanol. The drug solution was sonicated for 30-35 min. The solution was then filtered twice using a filter paper to obtain a clear solution of Acyclovir.

Optimization of mobile phase

Acyclovir 200 µg/mL standard solution was applied to silica gel $60F_{254}$ Thin Layer Chromatographic (TLC) plates. To get an optimal movement of the drug with a sharp, symmetrical peak, several pure solvents with different polarities and their combinations were tested. The solvent system combining chloroform: ethanol: propane-2-ol: and strong ammonia (2:4:3:1) was determined to be the most suitable after attempting several permutations and combinations. This system exhibited well-resolved symmetrical peaks for the drug.[5]

Mobile phase preparation

Here, 3 mL of HPLC grade Propan-2-ol were added to 4 mL of ethanol accurately and were mixed. Then 2 mL of Chloroform were added to make the total volume of 10 mL of Mobile phase. To regulate the amount of ionization of each molecule and to alter the pH of the mobile phase, one milliliter of strong ammonia was introduced.

HPTLC instrumentation

Using a CAMAG Linomat 5 automated TLC sampler, the samples were spotted as a series of bands of specified width on a precoated silica gel plate $60F_{254}$ (20x10 cm) using a CAMAG microliter syringe. Following a methanol wash, the TLC plates were activated for 20 min at 120°C. The application rate was held constant at 100 nL/sec and the spacing between tracks was set. Along with the slit dimension, a scanning speed was used. Chloroform, ethanol, isopropyl alcohol and strong ammonia (2, 4, 3 and 1 v/v/v/v) constitute the mobile phase. The development process was linear ascending in a twin trough glass chamber that was saturated with a mobile phase. For the mobile phase, the ideal chamber saturation duration was 45 min at room temperature. The chromatogram run extended around 80 mm in length. Using an air drier, TLC plates were dried in an air current following development. For the purpose of estimating acyclovir, densitometric scanning was carried out using CAMAG TLC scanner3 using winCATS software in the absorbance mode at 254 nm. The radiation source that was used was a deuterium lamp.[5]

Calibration of the instrument

Working solutions in various concentrations of 0.2, 0.4, 0.8, 1.2 and 1.6 µL/spot were prepared. Three duplicates of the solutions were spotted on the TLC plate. To estimate the amount of acyclovir, densitometric scanning was carried out in the absorbance mode at 254 nm. The linear regression mode was used for the peak area against drug concentration data.

Solubility data

The solubility data[6-8] is shown in the Table 1.

Optimization of wavelength for densitometric evaluation

Acyclovir was diluted appropriately to make an 11 µg/mL solution, which was then scanned in the spectrum mode between 400 and 200 nm to determine the optimal wavelength. The drug's Ultra-violet (UV) spectrum was used to determine Acyclovir's λ_{max} of 254 nm for the study.

Method Validation

Range

The interval between the greatest and lowest concentrations of the analyte that have been determined with adequate precision, accuracy and linearity which is known as the range.[6,9] It is often stated in the same units as the test findings and is based on either a linear or nonlinear response curve. 50% to 150% of the working concentration should be the range.

Linearity

The recommended HPTLC approach for assessing Acyclovir's linearity has been evaluated through the examination of many reference drug concentrations. In order to determine the concentrations of 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2 and 3.6 µg/spot, various volumes of standard solutions 0.5, 1, 2, 4, 6, 8, 10, 11, 16 and 18 mL were spotted on the TLC plate.[10]

Limit of detection and quantification

The Limits of Quantitation (LOQ) and Detection (LOD) were estimated by spotting the drug solution at a given concentration and the lowest detectable peak area was used to investigate LOD and LOQ. The outcomes were multiplied threefold for LOD and tenfold for LOQ in order to comply with the recommendations set out by the International Conference on Harmonisation.[9]

Accuracy

The extent of agreement between the value derived and the value acknowledged as a conventional true value essentially determines the analytical accuracy of the procedure.[6,9] The commercial formulation containing 200 µg/mL was spiked with the standard of Acyclovir, which is equivalent to 50, 100 and 150% in the original solution. These solutions were subsequently analyzed to establish accuracy. At all phases, the mean and individual recoveries should range from 98.0% to 101.0%.

Precision

Several homogenous analyses of samples were conducted to evaluate the analytical precision of the method.

Precision measurements of the peak area for acyclovir were made both within and between days, demonstrating the repeatability of the method.[9] For a minimum of three times, a total of three different concentrations (0.4, 0.8 and 1.2 µg/spot) were applied to perform the intraday (within-day in three repetitions) and inter-day precision (for three days).

Table 1: Solubility data.

Sl. No.	Solvent	Solubility (2 mg in 10 mL)
1	Water	Soluble
2	Acetonitrile	Insoluble
3	Methanol	Soluble
4	Acetone	Insoluble
5	Dimethyl sulphoxide	Soluble
6	Toluene	Insoluble
7	Ethanol	Soluble

Table 2: Linearity studies.

Sl. No.	Acyclovir	
	Concentration (µg/spot)	Peak Area
1.	0.1	1878.6
2.	0.2	3015.2
3.	0.4	4703.6
4.	0.8	8222.0
5.	1.2	11660.7
6.	1.6	14911.6
7.	2.0	18019.2
8.	2.4	20942.5
9.	2.8	23739.9
10.	3.2	26439.6
11.	3.6	29210.9
Regression Coefficient		0.9974
Slope		7819.1
Intercept		1786.8

Robustness

By slightly altering the optimized mobile phase composition and adjusting the saturation time at concentration ranges of 4, 8 and 12 µL (0.8, 1.6 and 2.4 µg/spot) five times, the robustness parameter was evaluated. The percentage Relative Standard Deviation (%RSD) of the peak regions was also computed.

Ruggedness

The degree to which test findings may be reproducible when analyzing the sample using any number of standard test circumstances, including multiple analysts, tools, days, reagents and TLC plates.[6]

RESULTS

The absorption maximum of acyclovir in methanol was found to be 254 nm and hence all measurements were done at this wavelength. Table 2 presents data on the variance in the peak response, which was repeated three times for each concentration. With a regression coefficient (r^2) value of 0.9974, the calibration plots' linear regression analysis results demonstrated a strong linear association (y=7819.1x+1786.8). Figure 2 illustrate the area visually displayed as a function of analyte concentration. The high values of the regression coefficient, slope and intercept value, which are displayed in Figure 3 and Table 2, support the linearity of the calibration graph (Figure 4).[9]

LOD and LOQ were exhibited at concentrations of 0.1 and 0.2 µg/spot, respectively. It was determined that the limit of quantification was 11.7726 µg/mL, whereas the limit of detection was 3.884 µg/mL (Table 3).

By introducing Acyclovir standard to the commercial formulation containing 200 µg/mL, accuracy was tested (Figure 5). Acyclovir recovery percentage using this approach ranged from 99.9 to

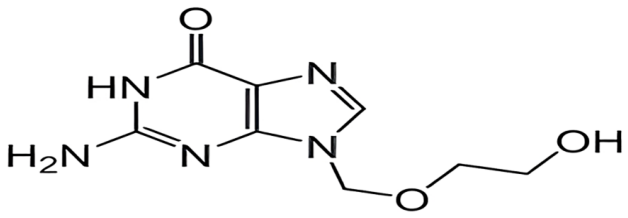

Figure 1: Structure of acyclovir.

Table 3: LOD and LOQ.

Results observed	Acyclovir
Standard Deviation	9205.179
LOD (µg/mL)	3.884
LOQ (µg/mL)	11.7726

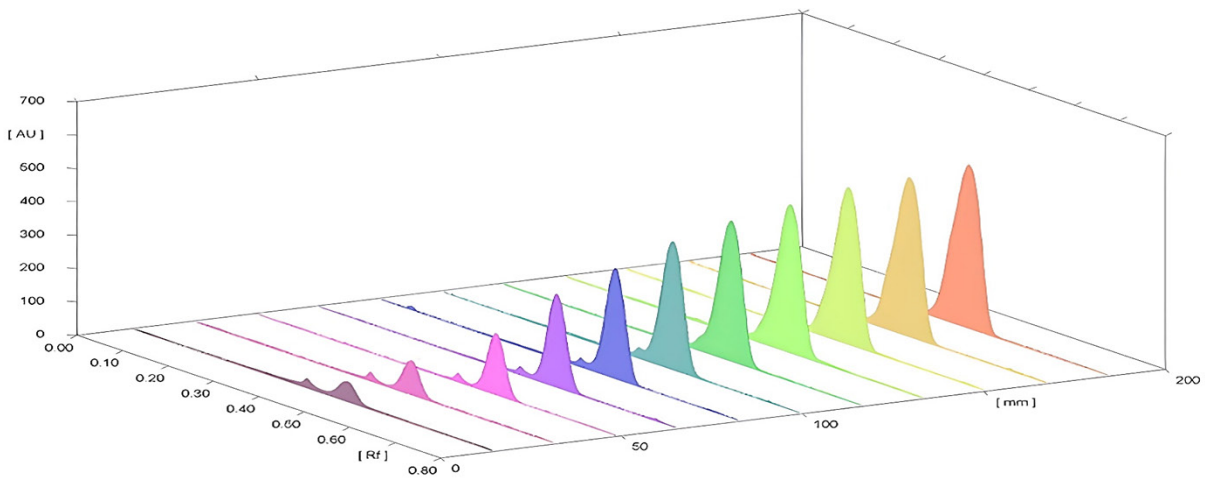

Figure 2: HPTLC densitogram for linearity studies of acyclovir API.

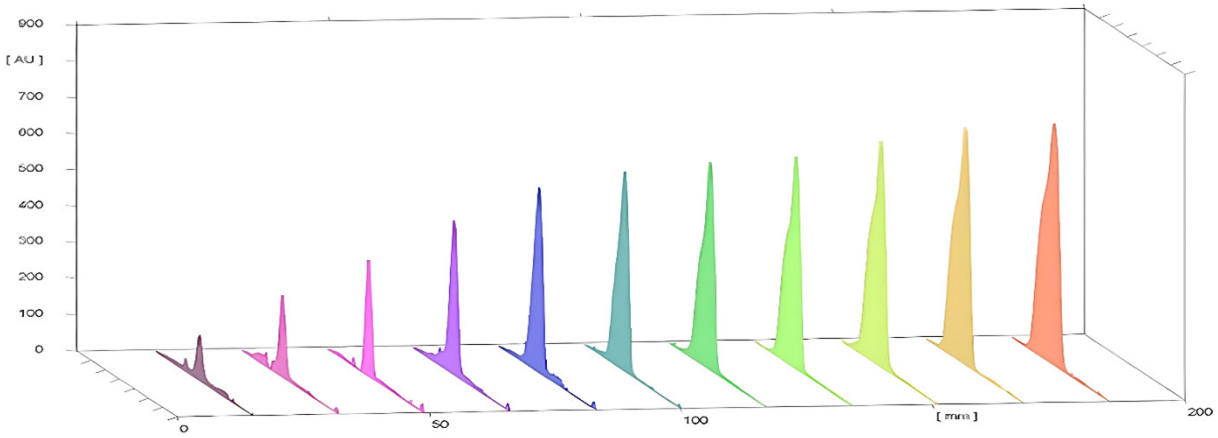

Figure 3: HPTLC densitogram for linearity studies of acyclovir tablets.

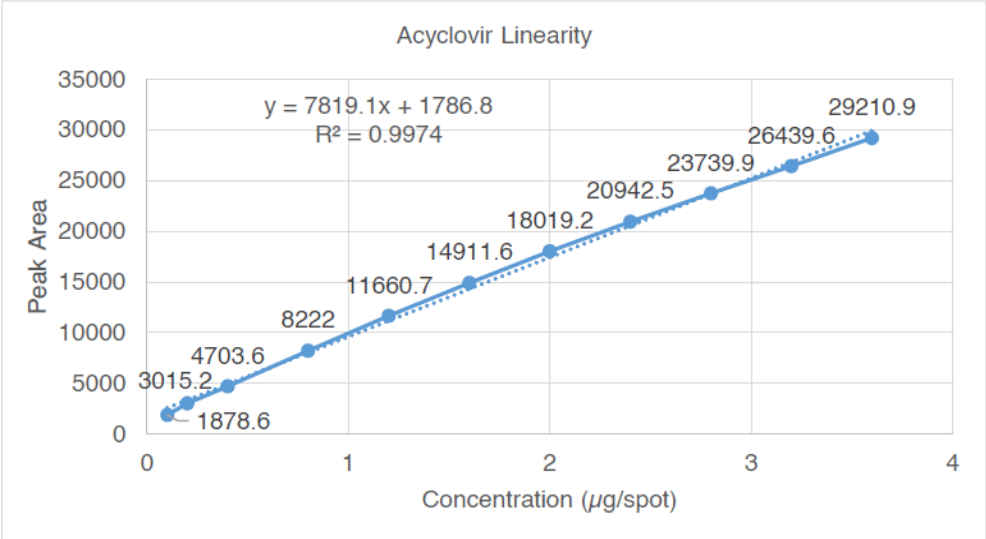

Figure 4: Linearity curve for acyclovir.

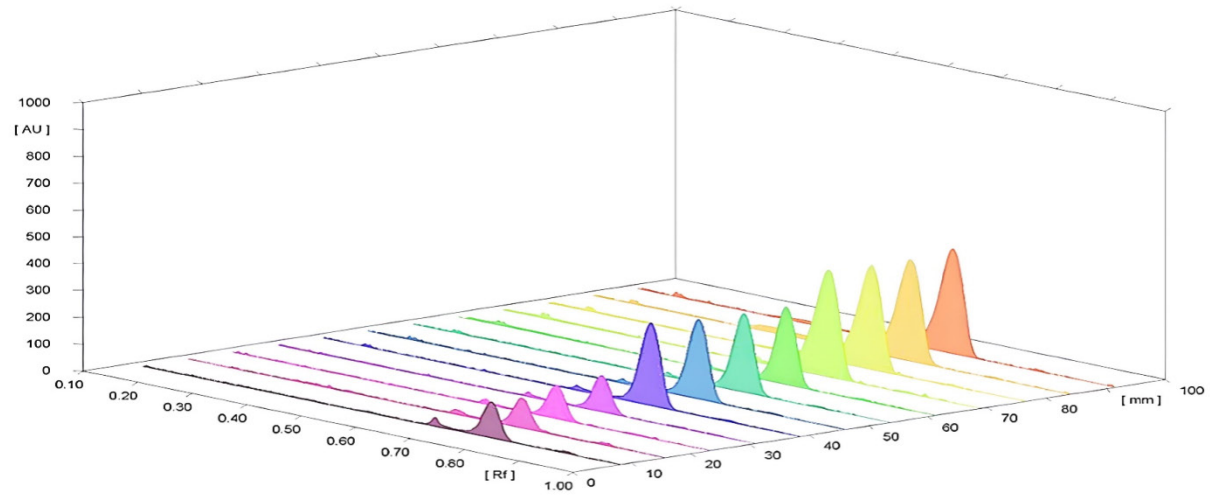

Figure 5: HPTLC densitogram of accuracy studies for acyclovir.

Table 4: Tabulated results for the accuracy study of acyclovir.

Level of Recovery (%)	Volume of injection (µL)	Concentration Of Acyclovir Taken (µg/µL)	Amount of Acyclovir Standard Added (µg/µL)	Total Amount Recovered	% Recovery of Acyclovir	Mean %Recovery of Acyclovir
50	2	0.02	0.02	3606.7	100	100
50	2	0.02	0.02	3433.7	100	
50	2	0.02	0.02	3443.1	100	
50	2	0.02	0.02	3714.8	100	
100	4	0.02	0.06	9573.7	100	100
100	4	0.02	0.06	9299.6	100	
100	4	0.02	0.06	9210.9	100	
100	4	0.02	0.06	9595.8	100	
150	6	0.02	0.10	14548.5	100	100
150	6	0.02	0.10	14508.2	100	
150	6	0.02	0.10	14423.5	100	
150	6	0.02	0.10	14428.9	100	
Across all Levels % Recovery						100

100% (Table 4). The development method's accuracy was proven by its outcomes. The resulting solutions were examined and 100% recovery was achieved.

Peak area measurements for Acyclovir at three different concentrations (0.4, 0.8 and 1.2 µg/spot) were carried out to demonstrate the method's reproducibility (Figure 6). The results demonstrated that the intraday precision was 0.0182% RSD for API and 0.0122% RSD for tablets and the inter-day precision was 0.0216% RSD for API and 0.0104% RSD for tablets. The %RSD obtained within and between days indicates that the suggested approach has excellent precision; it falls within an acceptable range. The precision of the acyclovir API and acyclovir in commercial formulation is displayed in Table 5.

The saturation time and mobile phase composition were slightly adjusted on purpose and the results showed that the % RSD was 0.0032% for 45 min, 0.0011% for 30 min and when the mobile phase composition was altered it resulted in the % RSD of 0.0032% and 0.0096% which were within the accepted limits. The

Table 5: Precision studies.

Concentration (µg/spot)	API				Tablets			
	Intra day		Inter day		Intra day		Inter day	
	Mean area	%RSD	Mean area	%RSD	Mean area	%RSD	Mean area	%RSD
0.8	14225.28	0.0255	12091.58	0.0306	20875.13	0.0209	21340.67	0.0143
1.6	23866.02	0.01679	21084.4	0.0181	40378.8	0.0094	41296.00	0.0108
2.4	32389.02	0.01288	25986.6	0.0162	57622.53	0.0064	59211.53	0.0062
Mean %RSD	0.01839		0.0216		0.0122		0.0104	

Table 6: Results of robustness studies for acyclovir.

Robustness		Saturation Time (In min)		Mobile phase with and without modifier (CHCl3: Ethanol: IPA: Strong ammonia)	
		45	30	1.5:4:3:1.5	1:4:3:2
Acyclovir	Rf	0.490	0.487	0.49	0.53
	Area	17908	17833	17908	10384
	%RSD	0.0032	0.011	0.0032	0.0096

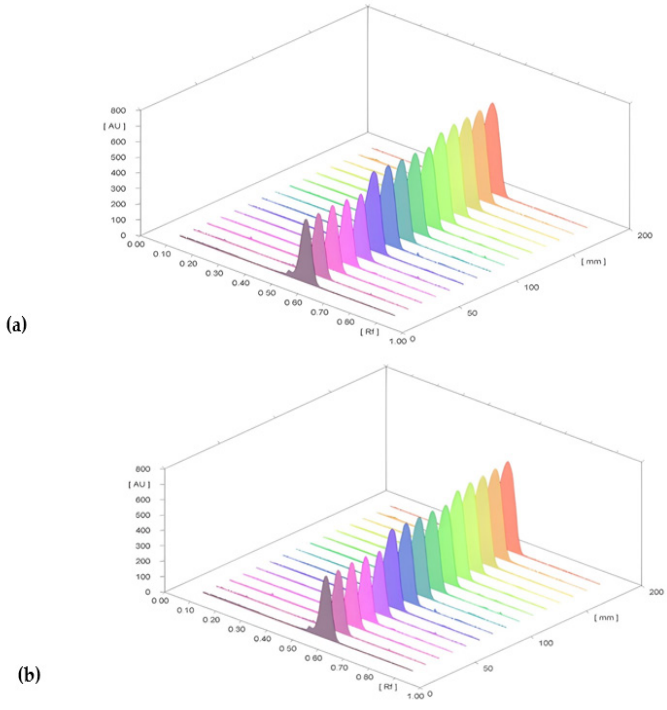

Figure 6: Precision studies for API (a)Intra-day (b) Inter-day.

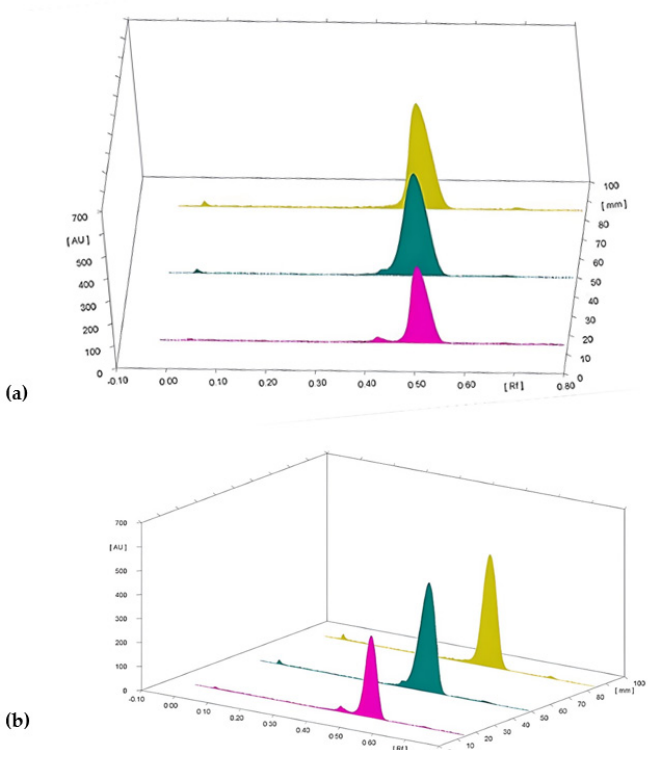

Figure 7: Saturation time of (a) 45 min (b) 30 min.

robustness of the established HPTLC technique was evidenced by the low values of % RSD values that were achieved after making tiny, deliberate adjustments, as listed in Table 6 and Figure 7.

DISCUSSION

Studies undertaken in the current project attempt to create a novel HPTLC analytical technique for the determination of antiviral drug acyclovir. It was discovered that the drug Acyclovir had a linear response within the concentration ranging from 0.2-3.6 µg/spot. The precision of the procedure was confirmed by the finding that the %RSD values for the precision investigations were less than 2%. By comparing the Retention factor (R_f) values and peak regions for both the conventional Acyclovir and the acyclovir in tablet formulation, the specificity of the novel method was determined and established. The developed HPTLC method's adaptability is demonstrated by the low %RSD that was

acquired after making a few intentional modest adjustments in mobile phase composition and the saturation time. Hence it is evident that the proposed HPTLC method for the estimation of acyclovir in bulk and tablets is accurate, precise, sensitive, specific and robust.

CONCLUSION

The proposed HPTLC method for acyclovir estimation is robust, specific, accurate and precise. Validation demonstrates the method's selectivity and reproducibility for acyclovir analysis. The approach may be applied to regular quality control, laboratory-prepared blends and commercial formulations of acyclovir for qualitative and quantitative analysis since the suggested mobile phase efficiently resolves the titled constituent.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

API: Active Pharmaceutical Ingredient; **%:** Percentage; **µg:** Microgram; **mL:** Millilitre; **g:** Gram; **min:** Minute; **R^2:** Regression Coefficient; **R.S.D.:** Relative Standard Deviation; **HPTLC:** High-Performance Thin Layer Chromatography; **UV:** Ultra Violet; **nm:** Nanometre; **TLC:** Thin Layer Chromatography.

SUMMARY

Acyclovir, a synthetic purine nucleoside analogue belonging to the group of antiviral drugs, is prescribed to treat infections caused by the herpes simplex virus and the varicella-zoster virus. Since, satisfactory analytical methods were not available for estimating acyclovir, as many suffer from low sensitivity, simplicity issues, or a lack of selectivity, the objective of this study was to enhance sensitivity, improve efficiency, contribute to resource conservation, and overcome the limitations of the existing analytical methods. This study aimed to fill the gap in available analytical methods and provide a more cost-effective alternative for the detection and quantification of acyclovir.

Studies undertaken in the current project have created a novel HPTLC analytical technique for the determination of antiviral drug acyclovir. With the developed method, Acyclovir had a linear response within concentration ranging from 0.2-3.6 µg/spot. The precision of the procedure was confirmed by the low %RSD values. By comparing the Rf values and peak regions for both Acyclovir and the Acyclovir in tablet formulation, the specificity of the novel method was determined and established. The developed HPTLC method's adaptability is demonstrated by the low %RSD values obtained in robustness studies after making a few intentional modest adjustments in mobile phase composition and the saturation time. Hence it is evident that the HPTLC method developed for the estimation of acyclovir in bulk and tablets is accurate, precise, sensitive, specific and robust. Validation of the developed method demonstrates the method's selectivity and reproducibility for Acyclovir analysis. The approach may be applied to regular quality control, laboratory-prepared blends, and commercial formulations of Acyclovir for qualitative and quantitative analysis.

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