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Research Article

Development and validation of HPTLC method for estimation of acyclovir in formulations

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ABSTRACT

A new, simple, and rapid high-performance thin-layer chromatographic method was developed and validated for quantitative determination of Acyclovir. Acyclovir was chromatographed on silica gel 60 F254 TLC plate using chloroform: methanol: formic acid (6.5 + 3.5 + 0.1 *v/v/v*) as mobile phase. Acyclovir was quantified by densitometric analysis at 259 nm. The method was found to give compact spots for the drug (R_f = 0.25±0.01). The linear regression analysis data for the calibration plots showed good linear relationship with r^2 = *0.*9996 in the concentration range 100–600 ng/spot. The method was validated for precision, recovery, repeatability, and robustness as per the International Conference on Harmonization guidelines. The minimum detectable amount was found to be 100 ng/spot, whereas the limit of quantitation was found to be 300 ng/spot. Statistical analysis of the data showed that the method is precise, accurate, reproducible, and selective for the routine quality control of Acyclovir.

Keywords: Acyclovir; Estimation; HPTLC

INTRODUCTION

Acyclovir (ACI), 9-[(2-hydroxyethoxy)-methyl]-guanosine. It is an acyclic guanosine derivative which exhibits a selective inhibition of herpes viruses replication with potent clinical antiviral activity against the herpes simplex and varicella-zoster viruses Fig. 1. The literature survey reveals that few analytical methods for this drug are reported, which include chromatographic, and spectrophotometric and chromatographic methods. (C.Huang, et al., 2002; E.K.Oh et al., 2006; E.S. Wallker et al., 1988; F. Demirkaya et al., 2005; F. Jafery et al., 1983; J. Fellenberg et al., 1976; K.Vyas et al., 2006; L.E. Riad et al., 1986; M.E. Auer et al., 2003; M.J. Lawrence et al., 2000; M.S. C'amara et al., 2005; N.S. Barakat et al., 2006; N.S. Rajadhyaksha et al., 2007; Owen J. et al., 2001; R.B. Miller et al., 1993; S.Mennickent et al., 2009; T.D. Cyr et al., 1987; Z. Rezaei et al., 2005.) Most of the methods reported are highly sophisticated, costly, and time consuming and require special sample preparation. The HPLC technique is excellent with respect to selectivity and sensitivity, but it cannot be used for routine analysis because of their specialty requirement and cost. Further, HPLC-based separation methods may not be suitable for the determination of drug from lipid based delivery systems such as mucoadhesive microemulsion (MME) formulations. These formulations contain various lipophilic excipients that are not soluble in commonly used organic solvents used in HPLC methods. Further, extraction of drug from such lipophilic excipients may not be achieved easily, and such excipients may get adsorbed on stationary phase. Hence, analysis of ACI, particularly from lipid-based delivery systems, would be difficult with respect to identification of suitable solvents and stationary phase. In view of this, high-performance thin layer chromatography-(HPTLC) based methods could be considered as a good alternative, as they are being explored as an important tool in routine drug analysis. Major advantage of HPTLC is its ability to analyze several samples simultaneously using a small quantity of mobile phase. This reduces time and cost of analysis. In addition, it minimizes exposure risks and significantly reduces disposal problems of toxic organic effluents, thereby reducing possibilities of environment pollution.

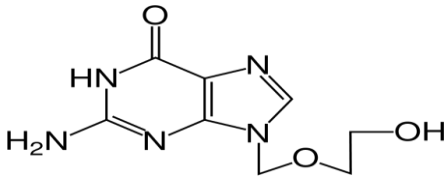

Figure 1: Chemical structure of Acyclovir

HPTLC also facilitates repeated detection of chromatogram with same or different parameters. Furthermore, in case of HPTLC, there are no restrictions on the choice of solvents and mobile phases; drug and lipophilic excipients can be dissolved in a suitable solvent that would evaporate during spotting on TLC plate, leaving behind analyte as a thin band. Therefore, for

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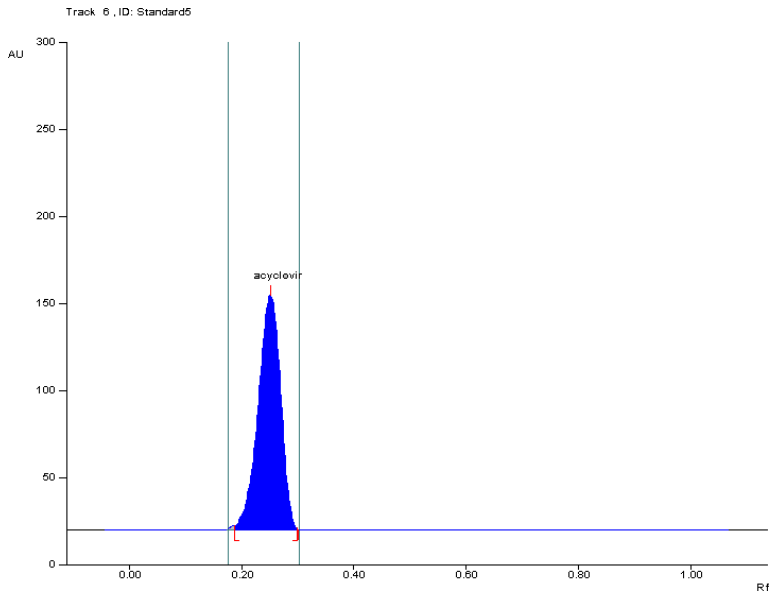

Figure 2: Typical HPTLC Chromatogram of Standard Drug Solution

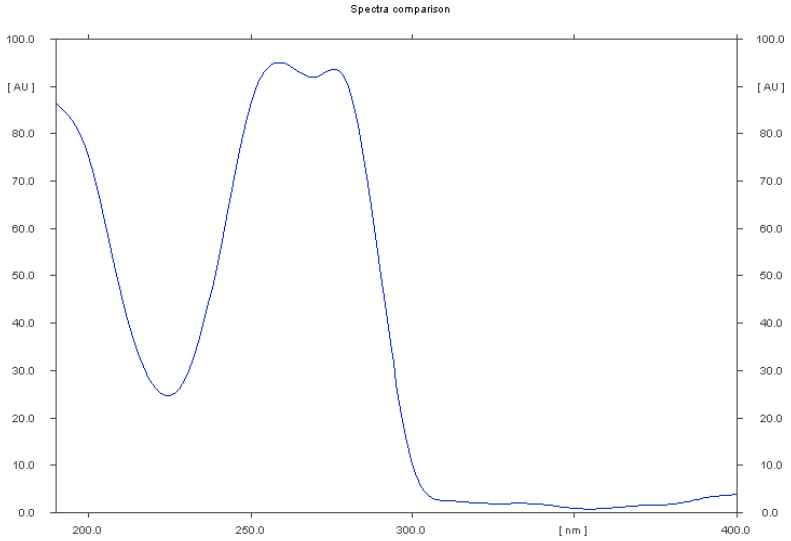

Figure 3: Typical Overlain Spectra of ACI Standard Drug Solutions

such methods, extraction procedure is not required always and could be developed for analyzing drug without any interference from excipients. The present investigation has been undertaken for the development and validation of HPTLC method for routine estimation of ACI from bulk and pharmaceutical dosage forms.

EXPERIMENTAL

2.1. Apparatus

The HPTLC system (Camag, Muttenz, Switzerland) consisted of Limomat V autosprayer connected to a nitrogen cylinder, a twin trough chamber (10 × 10 cm), a derivatization chamber, and a plate heater. Precoated silica gel 60 F_{254} TLC plates (10 × 10 cm, layer thickness 0.2mm (E. Merck KGaA, Darmstadt, Germany) was used as stationary phase. TLC plates were prewashed twice with 10 mL of methanol and activated at 80°C for 5 min prior to sample application. Densitometric analysis was carried out using a TLC scanner III with winCATS software.

2.2. Reagents and Materials

ACI pure powder was obtained as sample from Glenmark Pharmaceuticals Ltd., Mumbai (India) with 99.9% purity. All other chemicals and solvents were of analytical reagent grade and used as received without further purification.

2.3. HPTLC Method and Chromatographic Conditions

2.3.1. Sample Application.

The standard and formulation samples of ACI were spotted on Precoated TLC plates in the form of narrow bands of lengths 6 mm, with 10 mm from the bottom and left margin and with 9 mm distance between two bands. Samples were applied under continuous drying stream of nitrogen gas at constant application rate of 150 nL/s.

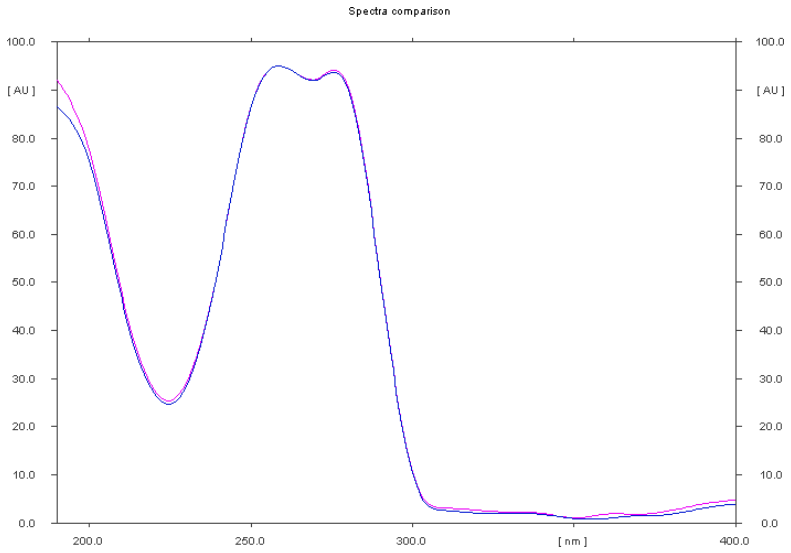

Figure 4: Peak purity spectra of ACI extracted from a tablet, scanned at the peak-start, peak-apex and peak-end positions of the spot (correlation > 0.99)

Table 1: Results of optical and regression characteristics (n = 6)

Parameters	ACI
Specificity	Specific
Concentration Range	100-600 ng/spot
LOD(ng/spot)	100
LOQ(ng/spot)	300
Regression Equation	203.6x + 9.662
Correlation Coefficient	0.9996

2.3.2. Mobile Phase and Migration

Plates were developed using mobile phase consisting of chloroform: methanol: formic acid (6.5 + 3.5 + 0.1 *v/v/v*). Linear ascending development was carried out in 10 cm × 10 cm twin trough glass chamber equilibrated with mobile phase. The optimized chamber saturation time for mobile phase was 20 min at 25±2°C. Ten milliliters of the mobile phase (5mL in trough containing the plate and 5 mL in other trough) was used for each development and allowed to migrate a distance of 70 mm, which required 10min. After development, the TLC plates were dried completely.

2.3.3. Densitometric Analysis and Quantitation Procedure

Densitometric scanning was performed on Camag TLC scanner III in absorbance mode and operated by winCATS planar chromatography version 1.3.4. The source of radiation utilized was deuterium lamp. The spots were analyzed at a wavelength of 259 nm. The slit dimensions used in the analysis were length and width of 5mm and 0.45 mm, respectively, with a scanning rate of 20 mm/s. These are selected as recommended by the CAMAG TLC Scanner III manual. It covers 70%–90% of the application band length, which in the present case is 6 mm. The monochromator bandwidth was set at 20 nm. Concentrations of compound chromatographed were determined from the intensity of diffusely reflected light and evaluated as peak areas against concentrations using linear regression equation.

2.3.4. Preparation of ACI Standard Stock Solution

Stock solution was prepared by weighing ACI (100 mg). Weighed powder was accurately transferred to a volumetric flask of 100 mL and dissolved in and diluted to the mark with methanol to obtain a standard stock solution of ACI (100 μg/mL).

2.3.5. Method Validation

Validation of the developed HPTLC method was carried out as per the International Conference on Harmonization (ICH) guidelines Q2 (R1) for specificity, sensitivity, accuracy, precision, repeatability, and robustness.

2.3.6. Specificity

The specificity of the developed method was established analyzing the sample solutions containing ACI sample and marketed tablets in relation to interferences from formulation ingredients. The spot for ACI in the sample was confirmed by comparing retardation factor (R_f) values of the spot with that of the standard.

2.3.7. Sensitivity

Sensitivity of the method was determined with respect to limit of detection (LOD) and limit of quantification

(LOQ). Noise was determined by scanning blank spot (methanol) six times. Series of concentrations of drug solutions (100–600 ng/spot) were applied on plate and analyzed to determine LOD and LOQ. LOD was calculated as 3 times the noise level, and LOQ was calculated as 10 times the noise level. LOD and LOQ were experimentally verified by diluting the known concentrations of ACI until the average responses were approximately 3–10 times the standard deviation (SD) of the responses for six replicate determinations.

2.3.8. Linearity and Calibration Curve

Linearity of the method was evaluated by constructing calibration curves at six concentration levels. Calibration curves were plotted over a concentration range of 100–600 ng/spot. Aliquots of standard working solution of ACI were applied to the plate (1, 2, 3, 4, 5, and 6 μL/spot). The calibration curves were developed by plotting peak area versus concentrations (n =6) with the help of the winCATS software.

2.3.9. Accuracy

Accuracy of the method was evaluated by carrying the recovery study at three levels. Recovery experiments were performed by adding three different amounts of standard drug, that is, 80%, 100%, and 120% of the drug, to the preanalyzed tablet formulations, and the resultant was reanalyzed six times.

2.3.10. Precision

Precision was evaluated in terms of Intraday and Interday precisions. Intraday precision was determined by analyzing sample solutions of ACI from formulations at three levels covering low, medium, and higher concentrations of calibration curve for five times on the same day. Interday precision was determined by analyzing sample solutions of ACI at three levels covering low, medium, and higher concentrations over a period of seven days (n = 5). The peak areas obtained were used to calculate mean and %RSD (relative SD) values.

2.3.11. Repeatability (System Precision)

Repeatability of measurement of peak area was determined by analyzing different amount of ACI samples covering low, medium, and higher ranges of the calibration curve seven times without changing the position of plate. Repeatability of sample application was assessed by spotting ACI samples covering similar range of calibration curve seven times and analyzing them once.

2.3.12. Robustness

By introducing small changes in mobile phase composition, its volume, chamber saturation time, and slight

Table 2: Recovery studies (n = 6)

Drug	Initial amount [ng]	Amount added [ng]	Amount recovered ± S.D. [ng]	% Recovered	%RSD
T1	300	240	239.11 ± 1.57	100.01	0.24
	300	300	298.52 ± 3.01	99.81	0.37
	300	360	358.41 ± 0.32	99.93	0.32
T2	300	240	238.08 ± 5.65	99.75	0.35
	300	300	299.04 ± 2.68	99.95	0.13
	300	360	358.44 ± 4.47	99.93	0.18

T1=ACIVIR™ 10 (FDC Pharmaceuticals Ltd., India) and T2=OCUVIR™ (USV Pharmaceuticals Ltd., India)

Table 3: Content of Acyclovir in various formulations

Drugs	Label claim [mg]	Amount found [mg] (n = 5)	Amount found [%]
T1	200	199.95	99.71
	200	199.95	99.22
	200	199.97	99.21
	200	198.94	99.52
	200	199.94	99.56
	Mean ± SD	199.95 ± 0.010	98.85 ± 0.23
	%RSD	0.25	0.23
T2	200	199.96	99.16
	200	199.94	99.10
	200	199.95	98.90
	200	200.05	100.54
	200	199.97	99.71
	Mean ± SD	199.97 ± 0.03	99.76 ± 0.40
	%RSD	0.30	0.40

T1=ACIVIR™ 10 (FDC Pharmaceuticals Ltd., India) and T2=OCUVIR™ (USV Pharmaceuticals Ltd., India)

change in the solvent migration distance, the effects on the results were examined. Robustness of the method was determined in triplicate at a concentration level of 300 ng/spot and the mean and %RSD of peak area was calculated.

2.4. Application of Developed Method in Formulations

Twenty tablets were weighed and finely powdered. Quantity equivalent to 100 mg of drug was weighed accurately and dissolved in 50 mL methanol. The solution was sonicated for 15 min and then filtered through Whatman filter paper no. 41. The residue was washed thoroughly with methanol. The filtrate and washings were combined and diluted suitably with methanol to obtain a 1 mg/mL concentration of ACI. An aliquot of this solution (1.0 mL) was further diluted to 10 mL with methanol to obtain a solution containing 100 μg/mL of ACI. On plates, 5 μL of these solutions were spotted and analyzed for ACI content using proposed method as described earlier. The possibility of interference from other components of the tablet formulation in the analysis was studied.

RESULTS AND DISCUSSION

To develop HPTLC method of analysis for ACI for routine analysis, selection of mobile phase was carried out on the basis of polarity. A solvent system that would give dense and compact spots with appropriate and significantly different R_f value for ACI was desired. Various solvent systems such as chloroform-methanol, methanol-toluene, methanol-ethyl acetate, toluene-ethyl acetate, toluene-chloroform-methanol, chloroform-methanol-formic acid were evaluated in different proportions. Among these, the solvent system comprising of chloroform: methanol: formic acid (6.5 + 3.5 + 0.1 *v/v/v*) gave good separation of ACI from its matrix with an R_f value of 0.25. It was also observed that chamber saturation time and solvent migration distance are crucial in chromatographic separation as chamber saturation time of less than 15min and solvent migration distances greater than 70 mm resulted diffusion of analyte spot. Therefore, chloroform: methanol: formic acid (6.5 + 3.5 + 0.1 *v/v/v*) proportion with chamber saturation time of 20min at 25°C and solvent migration distance of 70 mm was used as mobile phase. These chromatographic conditions produced a well defined compact spot of ACI with optimum migration at R_f = 0.*25* ± 0.01 at 259 nm Fig. 2 &3. It also gave a good resolution of analyte from excipients used in marketed tablet formulations.

Under the experimental conditions employed, the lowest amount of drug that could be detected was found to be 100 ng/spot and the lowest amount of drug that could be quantified was found to be 300 ng/spot, with RSD <5%.

Specificity is the ability of an analytical method to assess unequivocally the analyte in the presence of sample matrix. ACI was separated from excipients with an R_f of 0.25 ± 0.01 Fig. 4. Linearity of an analytical method is its ability, within a given range, to obtain test results that are directly, or through a mathematical transformation, proportional to concentration of analyte. Method was found to be linear in a concentration range of 100–600 ng/spot (n = 6), with respect to peak area. The regression data as shown in Table 1 reveal a good linear relationship over the concentration range studied demonstrating its suitability for analysis. No significant difference was observed in the slopes of standard curves (ANOVA, $P > .05$).

Accuracy of an analytical method is the closeness of test results to true value. It was determined by the application of analytical procedure to recovery studies, where known amount of standard is spiked in preanalyzed samples solutions. Results of accuracy studies were shown in Table 2; recovery values demonstrated the accuracy of the method in the desired range.

The precision of an analytical method expresses the degree of scatter between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions. Intraday precision refers to the use of analytical procedure within a laboratory over a short period of time using the same operator with the same equipment whereas Interday precision involves estimation of variations in analysis when a method is used within a laboratory on different days, by different analysts. In all instances, %RSD values were less than 5% confirming the precision of the method.

Ten-microliter aliquots of samples containing 100, 300, and 600 ng of ACI were analyzed according to proposed method. In order to control scanner parameters, that is, repeatability of measurement of peak area, one spot was analyzed without changing position of plate (n = 7). By spotting and analyzing the same amount several times (n =7), precision of automatic spotting device was evaluated. %RSD was consistently less than 5%, which was well below the instrumental specifications, ensuring repeatability of developed method as well as proper functioning of the HPTLC system.

The low values of %RSD obtained after introducing small deliberate changes in the developed HPTLC method confirmed the robustness of the method.

The ACI content of the marketed formulations were found to be within the limits (±5% of the theoretical value) and are mentioned in (Table 3). The low %RSD value indicated the suitability of this method for routine analysis of ACI in various formulations.

CONCLUSION

A new HPTLC method has been developed for the identification and quantification of Acyclovir. Low cost, faster speed, and satisfactory precision and accuracy are the main features of this method. Method was successfully validated as per ICH guidelines and statistical analysis proves that method is sensitive, specific, and

repeatable. It can be conveniently employed for routine quality control analysis of Acyclovir as bulk drug and in marketed formulations without any interference from excipients.

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