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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR QUANTITATIVE ESTIMATION OF ACYCLOVIR IN BULK DRUG AND TABLETS

[1]P. Ravisankar*, A. Niharika, S. Sireesha, O. Sai Koushik, V.Himaja
[1]Department of Pharmaceutical Analysis and Quality Assurance, Vignan Pharmacy College, Vadlamudi, Guntur, A.P, India.
***Corresponding Author: Email: banuman35@gmail.com**

ABSTRACT

The present work is aimed to develop a simple, rapid, reproducible, reliable and efficient reversed phase high performance liquid chromatographic (RP-HPLC) method for estimation of a antiviral drug Acyclovir in raw material and its tablet dosage form. Separation was done by using mobile phase consisting of HPLC grade water and methanol in the ratio of 50:50. The separations were carried out on Welchrom C_{18} column (250 x 4.6 mm; 5 µm) Shimadzu LC-20AT Prominence Liquid Chromatograph. The flow rate was set at 1 mL/min. The injection volume was 20 µL and the UV detector was operated at 250 nm using Shimadzu SPD-20A Prominence UV-Visible detector. The retention time of Acyclovir was found to be 3.077 minutes. The standard calibration plot was found linear over the range of 2 to 10 µg/mL and the coefficient of correlation was found to be (r^2 = 0.9999). The % RSD values of intraday and interday precision were found below two which indicates that the method was highly precise. The LOD and LOQ were found to be 0.247 and 0.74 respectively. The developed method was eventually applied for quantification of marketed formulation. Satisfactory results were obtained. The mean assay values of Acyclovir in good agreement with the label claim. The developed method was validated according to international conference on harmonization (ICH) guidelines for specificity, linearity, range, accuracy, precision, detection limit, quantitation limit, robustness and system suitability parameters and the results obtained were satisfactory. The method was can successfully utilized for the reliable quantification of Acyclovir in bulk and tablet dosage form.

KEYWORDS: RP - HPLC, Acyclovir, Validation, ICH guidelines.

1. INTRODUCTION

Acyclovir is a guanosine analogue antiviral medication (H. J. Schaeffer, 1978). It is mainly used for the treatment of herpes simplex virus infections, chickenpox and shingles. Other uses of Acyclovir include, prevention cytomegalovirus infections following transplant and infections due to epstein-barr virus. A thorough literature survey revealed that few methods have been described for the determination of Acyclovir such as Non-aqueous titration (K. Basavaiah, 2004), Spectrophotometry (Preeti Gandhi, 2006) (S. Ashok Reddy, 2011), LCMS/MS (Holkar G.2012), HPLC (H. Mascher 1992), (P. Nebinger 1993), (K. J. Swart 1994), (C. M. McMullin 1996), (N. M. Volpato 1997), (K. K. Peh 1997), (J. O. Svensson 1997), (C. Pham-Huy 1999), (R. A. Bangaru 2000), (M. Fernandez 2003), (D. Teshima 2003), (G. Bahrami 2005), (P. D. Tzanavaras 2007), (L. Zeng 2008) have been used for the estimation of Acyclovir. Most of the available reported methods for the estimation of Acyclovir are in human serum and plasma. Infact most of the available RP-HPLC methods has got disadvantages like peak tailing, long run time, less sensitivity, selectivity and expensive. Considering the already proposed methods in literature, the advantages of this new proposed method are keeping in view of this; an attempt has been made to develop a new RP-HPLC method with simple, convenient, rapid, precise, accurate, and economical and user friendly method with a simple and easily available mobile phase for the quantitative estimation of Acyclovir in tablet dosage form. The proposed method was validated as per International Conference on Harmonization (ICH Q2 (R1) 2005) guidelines. Figure 1 shows the structure of Acyclovir.

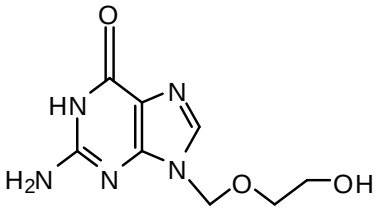

Figure 1. Chemical structure of Acyclovir.

2. MATERIALS AND METHODS

Materials used: An analytically pure sample of Acyclovir standard was gifted from Hetero Labs Ltd., Hyderabad India. All the chemicals were analytical grade. HPLC grade acetonitrile and triethylamine were obtained from Merck pharmaceuticals private Ltd., Mumbai, India. Methanol and water utilized were of HPLC grade and purchased from Merck specialties private Ltd., Mumbai, India. Commercial tablets of Acyclovir formulation was procured from local pharmacy. Aciver tablets containing Acyclovir with labeled amount of 200 mg per tablet are manufactured by Cipla Ltd. Mumbai, India.

Instruments used: Quantitative HPLC analysis was carried out on a isocratic high performance liquid chromatography (Shimadzu LC-20AT Prominence Liquid Chromatograph) with a LC-20AT VP pump, manual injector with loop volume of 20 µL (Rheodyne), programmable variable wavelength Shimadzu SPD-20A Prominence UV-Vis detector and Welchrom C_{18} Column (4.6 X 250 mm, 5µm particle size). The HPLC system was equipped with "Spinchrome" software. In addition an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), a sonicator (spectra lab, model UCB 40), UV-Visible Spectrophotometer (Systronics model-2203) were used in this study.

Preparation of solutions and reagents:

Mobile phase: Five hundred milliliters of HPLC grade methanol and five hundred milliliters of HPLC grade water were mixed. This mixture was sonicated for 10 minutes and filtered through 0.22 µm membrane filter and used as mobile phase.

Preparation of standard stock and working standard of Drug Solution: 100 mg of Acyclovir was accurately weighed and transferred to a 100 mL clean, dry volumetric flask with the addition of mobile phase, upto the mark and sonicate the solution to dissolve if necessary. This is primary stock standard solution of Acyclovir 1000 µg/mL concentration. This stock solution was further diluted to obtain desired concentrations (linearity range solutions containing 2 µg/mL, 4 µg/mL, 6 µg/mL, 8 µg/mL and 10 µg/mL of Acyclovir were prepared).

Preparation of stock solution for the commercially obtained tablets: Twenty tablets of Acyclovir (labeled claim 200 mg of Acyclovir) were weighed and average weight was calculated. The tablets were crushed to get fine powder. Then a quantity of the powder equivalent to 100 mg of Acyclovir was weighed in a 100 ml volumetric flask. The powder was then allowed to dissolve in mobile phase by sonication. Fill up the mark with mobile phase and filter the solution through 0.2 µm filters to remove insoluble materials. It was further diluted to obtained desired concentrations. The content of the tablets was calculated either from the previously plotted calibration graphs or by means of regression equations.

Optimization of mobile phase and method development: Optimization of mobile phase was performed based on trial and error method. A series of trials were conducted in order to get proper optimized HPLC conditions. In the first instance several mobile phase compositions were tried such as methanol: water, acetonitrile: HPLC grade water, methanol: acetonitrile: water in different ratio without adjusting pH. Finally the mobile phase comprising of Methanol and HPLC grade water in the proportion of 50:50 v/v was found to give best system suitability parameters and also obtained sharp, well-gaussian shape peak. This mobile phase was also selected as the diluent because the drug is freely soluble in the mobile phase. This mobile phase pH which is safe for column life and suitable for analyte stability, where as methanol is readily available solvent. The stationary phase made up of Welchrom C_{18} column with 4.6 X 250 mm, 5 µm were observed and they are found to be utmost suitable for Acyclovir. The ultra violet spectrum of diluted solutions of various concentrations of maximum absorption detection of Acyclovir was recorded by utilizing UV Systronic double beam SL 2203. An absorption maximum was found to be 250 nm. This wavelength was optimum for the detection of Acyclovir. Figure 2 shows the absorption maxima of Acyclovir. The developed method gave symmetric peak at retention time of 3.077 minutes and satisfied all the peak properties as pursuance of ICH guidelines.

Validation of analytical method: The developed analytical method was further subjected to validation in pursuance of ICH Q2 (R1) guidelines. The parameters evaluated were system suitability, specificity, linearity, precision, accuracy, robustness, limit of detection (LOD) and limit of quantitation (LOQ).

System suitability: The chromatographic systems used for analysis must pass system suitability limits before sample analysis can commence. Set up the chromatographic system, allow the HPLC system to stabilize for 40 minutes. Inject blank preparation (single injection) and standard preparation (six replicates) and record the chromatograms to evaluate the system suitability parameters like resolution (NLT 2.0), tailing factor (NMT 1.5), theoretical plate count (NLT 3000) and % RSD for peak area of six replicate injections of Acyclovir e standard (% RSD NMT 2.0).The parameters such as tailing factor, % RSD and theoretical plates were studied and found satisfactory. The system suitability data and the optimum chromatographic conditions are reported in Table 1.

Linearity: Under proposed experimental conditions, the relationship between the area and concentration of Acyclovir was studied. Linearity was checked by preparing standard solutions at 5 different concentration levels of each of Acyclovir. Acyclovir standard solutions (2, 4, 6, 8, 10 µg/mL) were injected into the HPLC system to get the chromatograms. The average peak area and retention time were recorded. The calibration curve was constructed between concentrations versus peak area by the prepared concentration of 2 -10 µg/mL of stock solution. The linearity range was found to be 2 -10 µg/mL and the results are presented in Table 2. The standard chromatograms of Acyclovir calibration standards are depicted in Figure 2 to Figure 6. Results show that a phenomenal correlation exists between peak area and concentration of drug within the linearity range. The calibration graph of Acyclovir is presented in Figure 7. Summary of validation parameters are shown in Table 3.

Specificity: The specificity of the method was determined by the prepared standard, sample solutions and the blank solution were injected and check any other excipients interference occurs or not. It was shown that the excipients present in pharmaceutical tablets of Acyclovir did not show any interference with Acyclovir peak because no excipients peaks appear in the chromatogram of the prepared tablet. Furthermore the well-shaped peaks also indicate the specificity of the method. The specificity results are tabulated in Table 4.

Precision: Precision of an analytical procedure is referred to as degree of scatterness between a series of observations obtained from multiple sampling of same homogenous sample in given conditions. The terms Intraday (repeatability) where as interday precision (intermediate precision) were investigated by replicating analysis for three concentrations (2 µg/mL, 4 µg/mL, 6 µg/mL) to the use of analytical procedure within same laboratory conditions over a short period of time by same analyst and same instrument. For interday precision, the analysis was carried out for three consecutive days at the same concentration levers as used in intraday precision. Regarding the intraday precision was carried out by using the 3 concentrations at different time interval in the day. The area was recorded as % RSD. The results of intraday and interday precision are shown in Table 5 and 6 respectively.

Accuracy/Recovery: The accuracy of the method was found out by standard addition method. For the previously analyzed sample 6 µg/mL. A known amount of standard drug was added at 50 %, 100 % and 150 % level. The concentrations were re-analyzed with the above described procedure. The percent recovery of the triplicate solutions was determined and average of the percent recovery was calculated. The recovery results are presented in Table 7.

Robustness: Robustness of the method is its ability to remain unaffected by small changes in variety of parameters such as the slight variation in acetonitrile percentage composition of the mobile phase, flow rate, detection wavelength. The results of robustness study is shown in Table 8 indicated that the small change in the conditions did not significantly affect the determination of Acyclovir.

LOD and LOQ: Limit of detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. Limit of detection and limit of quantitation were calculated using following formula LOD = 3.3 σ/S and LOQ = 10 σ/S, where, σ is the standard deviation of response and S is the slope of the calibration curve. The LOD and LOQ values are presented in Table 9. The results of LOD and LOQ supported the sensitivity of the developed method.

Application to commercial tablet: Using the developed RP-HPLC chromatographic method, assay of Acyclovir in tablet was carried out as mention in the experimental section. Six replicate determinations were made. Satisfactory results were obtained and were good agreement with the label claim and assay results were shown in Table 10. The results were very close to the labeled value of commercial tablets. The representative sample chromatogram of Acyclovir is shown in Figure 8.

3. RESULTS AND DISCUSSION

The present study was aimed at developing a precise, sensitive, rapid and accurate HPLC method for the analysis of Acyclovir in bulk drug and in pharmaceutical dosage forms. In order to achieve phenomenal retention time and peak asymmetry, C_{18} stationary phase column (250 mm X 4.6 mm i.d, 5 µm particle size) and mobile phase composed of methanol : water (50:50) v/v at a flow rate of 1mL/min was selected. The retention time for Acyclovir was found to be 3.077 minutes. UV spectra of Acyclovir showed that the drug absorbed maximum at 250 nm, so this wavelength was selected as the detection wavelength. The correlation coefficient (0.9999) of regression was found almost equal to one in the range of 2 - 10 µg/mL which states that the method was good linear to the concentration versus peak area responses. The comparison of chromatograms of placebo, standard and sample, there was no interference observed from the peaks of placebo, standard and sample. It shows that the method is specific. The precision studies were performed and the % RSD of the determinations was found to be 0.075 for intra-day precision and 0.089 for inter-day precision which are within the limits which indicates that the proposed method was found to be precise. The accuracy of the method was found to be good with the overall % RSD for recovery at 50 %, 100 % and 150 % levels were all within the limits which indicate that the proposed method was found to be accurate. Method validation following ICH guidelines indicated that the developed method had high sensitivity with LOD of 0.247µg/mL and LOQ of 0.748 µg/mL. The method was found to be robust even though on slight deliberate variation in the method conditions did have a tiny effect on the peak asymmetry, plate count and retention time and all are within the limits which indicated that the method is robust. Range is the minimum and maximum concentration of the sample at which the analytical procedure gives reproducible results. Range can be determined by linearity, accuracy and precision studies. The method was found acceptable across wide range of concentration 2-10 µg/mL. The retention time of the sample solution of Acyclovir tablet was found to be 3.077 minutes, which is similar to that of the standard solution of Acyclovir. This indicates that there is no drug-excipient interference and the drug is decorously resolved by the developed method. Robustness determines the reproducibility of the test result with small and deliberate variations in the method parameters. The experiment was carried out by slightly changing the ratio of methanol in mobile phase, detection wavelength and flow rate. The effectiveness of the deliberate little variations was observed on the flow rate

and mobile phase composition. The statistical data shows no significant variations in the above said parameters which indicate that the method is robust.

The developed method was successfully applied for the determination of Acyclovir in bulk drug and tablet dosage form. The assay result was complied in Table 10 and also shows that there is no interference of the tablet matrix with the drug. The assay results satisfactory results were obtained and were in a good agreement with the label claim. Very low % relative standard deviation shows that this method can be easily utilized for the estimation of Acyclovir in bulk drug and tablet dosage form.

Table.1.Optimum chromatographic conditions and system suitability data.

Parameter	Chromatographic conditions
Instrument	SHIMADZU LC-20AT prominence liquid chromatograph
Column	WELCHROM C_{18} Column (4.6 mm i.d. X 250 mm, 5 µm particle size)
Detector	SHIMADZU SPD-20A prominence UV-Vis detector
Diluents	Methanol : water (50 : 50)
Mobile phase	Methanol : water (50 : 50)
Flow rate	1 mL/min.
Detection wave length	UV at 250 nm.
Run time	5 minutes
Temperature	Ambient temperature (25 ºC)
Volume of injection loop	20 µL
Retention time (t_R)	3.077 min
Theoretical plates [th.pl] (Efficiency)	7568
Theoretical plates per meter [t.p/m]	151357
Tailing factor (asymmetry)	1.068

Table.2.Calibration data of the proposed method for the estimation of Acyclovir

Concentration, µg/mL.	Retention time, (t_R) min.	Peak area, mV.s.
0	-	0
2	3.077	325.628
4	3.077	620.794
6	3.077	930.411
8	3.077	1230.333
10	3.077	1532.481

Table.3.Summary of validation parameters.

Parameter	Result
Linearity range (µg/mL)	2-10 µg/mL
Liner Regression equation (Y = a + bX)	Y = 152.66 x + 9.979
Intraday precision (% RSD) (n=3)	0.075
Interday precision (% RSD) (n=3)	0.089
% Recovery	99.82
LOD (µg/mL)	0.2470
LOQ (µg/mL)	0.7480
Robustness	Robust

Table.4.Specificity study for Acyclovir

Name of the solution	Retention time, (t_R) min
Mobile phase	No peaks
Placebo	No peaks
Acyclovir 10 µg/mL	3.077 min.

Table.5.Results of precision study (intra-day) for Acyclovir.

Sample	Concentration (µg/mL)	Injection no.	Peak area (mV.s)	% RSD[#]
Acyclovir	6	1	930.511	0.075
		2	931.312	
		3	929.514	
		4	930.311	
		5	931.413	
		6	930.611	

[#] Acceptance criteria < 2.0.

Table 6. Results of precision study (inter-day) for Acyclovir.

Sample	Concentration (µg/mL)	Injection no.	Peak area (mV.s)	% RSD[#]
Acyclovir	6	1	931.411	0.089
		2	930.322	
		3	929.412	
		4	931.414	
		5	930.453	
		6	931.453	

[#] Acceptance criteria < 2.0.

Table 7. Recovery data for Acyclovir

Type of recovery in % level	Sample conc. (µg/mL)	Amount of the standard added (µg/mL)	Total conc. (µg/mL)	Found conc. (µg/mL)	% recovery	% RSD
50	6	3	9	8.98	99.77	0.22
			9	8.99	99.88	
			9	8.97	99.66	
100	6	6	12	11.99	99.91	0.16
			12	11.98	99.83	
			12	11.97	99.75	
150	6	9	15	14.97	99.8	0.13
			15	14.98	99.86	
			15	14.99	99.93	

RSD: relative standard deviation

Table 8. Robustness results of Acyclovir

Parameter	Optimized	Used	Retention time (t_R), min	Plate count[$]	Peak asymmetry[#]	Remark
Flow rate (±0.2 mL/min)	1.0 mL/min	0.8 mL/min	3.099	7599	1.060	*Robust
		1.0 mL/min	3.077	7551	1.068	*Robust
		1.2 mL/min	3.060	7498	1.063	*Robust
Detection wavelength (±5 nm)	250 nm	245nm	3.076	7558	1.060	Robust
		250 nm	3.077	7551	1.068	Robust
		255 nm	3.077	7555	1.060	Robust
Mobile phase composition (Methanol: Water)	50:50 v/v	55:45 v/v	3.071	7542	1.079	*Robust
		50:50 v/v	3.077	7551	1.068	*Robust
		45:55 v/v	3.080	7559	1.086	*Robust

Acceptance criteria (Limits): [#]Peak Asymmetry < 1.5, [$] Plate count > 3000, * Significant change in Retention time.

Table 9. Limit of detection and Limit of quantitation

Limit of Detection(LOD)	0.2470 µg/mL
Limit of Quantitation(LOQ)	0.7486 µg/mL

Table 10. Assay results of Acyclovir formulation.

Formulations	Labelled claim	Amount found (mg) (mean ± SD) (n=6)	Assay ± % RSD
Aciver (Cipla Ltd. Mumbai, India.)	200 mg/tablet	199.88 ±0.20 mg/tablet	99.94 ±0.41

SD: standard deviation. RSD: relative standard deviation.

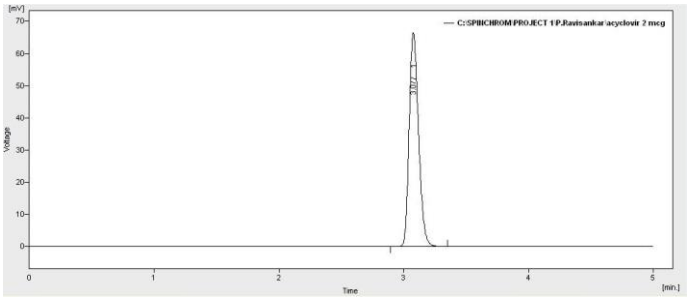

Figure 2. Standard chromatogram of Acyclovir (2 µg/mL)

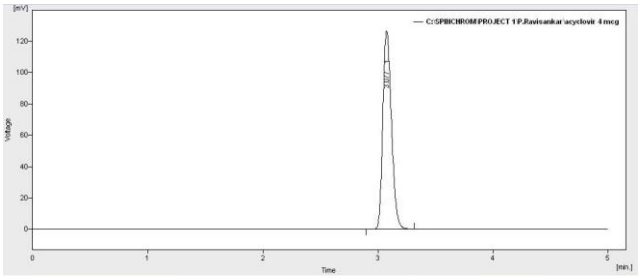

Figure 3. Standard chromatogram of Acyclovir (4 µg/mL)

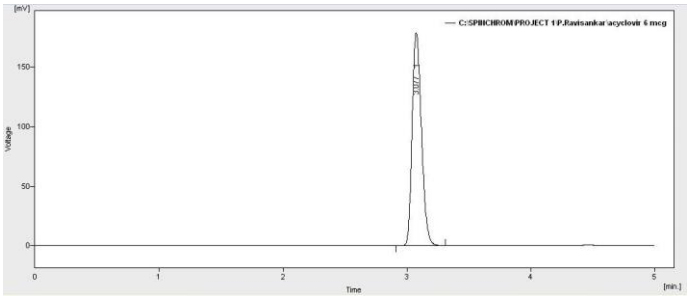

Figure 4. Standard chromatogram of Acyclovir (6 µg/mL)

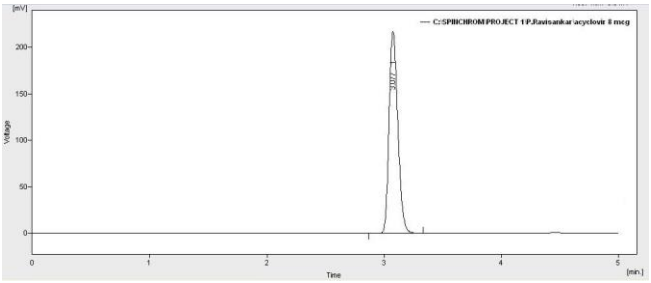

Figure 5. Standard chromatogram of Acyclovir (8 µg/mL)

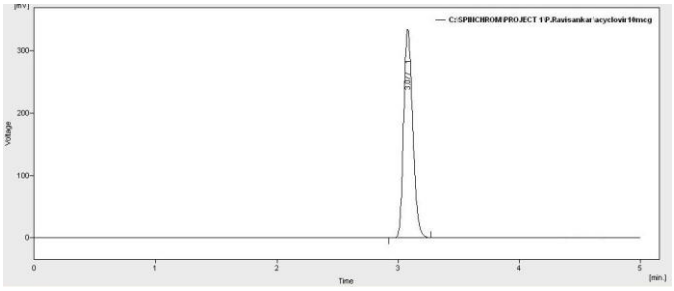

Figure 6. Standard chromatogram of Acyclovir (10 µg/mL)

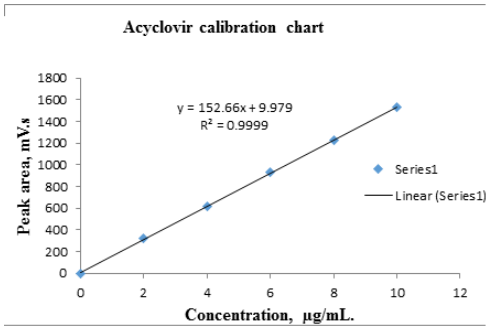

Figure 7. Calibration plot of Acyclovir

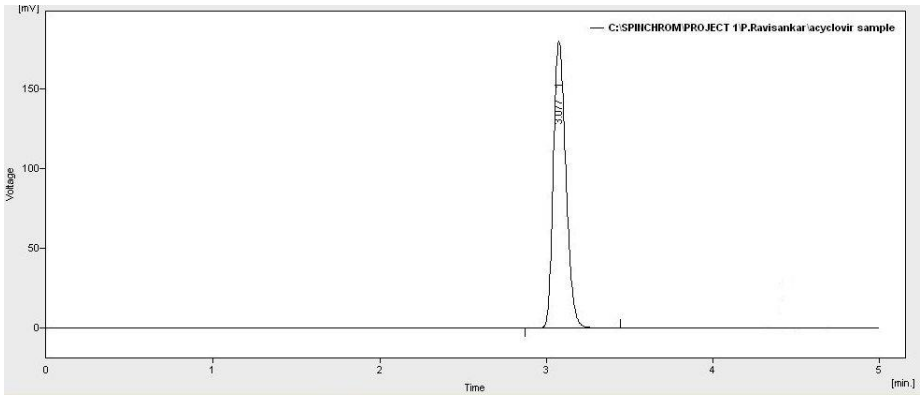

Figure.8.Chromatogram of marketed formulation Acivir

4. CONCLUSION

The developed RP-HPLC method provides a convenient and efficient method for the quantitative estimation of Acyclovir in bulk drug and pharmaceutical dosage form. This method has various advantages like less retention time (retention time only 3.077 min), quick analysis time (runtime only 5 minutes), low solvent consumption, outstanding peak symmetry, user friendly and convenient approach and also shown to specific, linear, accurate, precise, phenomenal sensitive and robust. The mobile phase can be easily prepared and diluents are economical and readily available and it does not need sample preparation with sophisticated techniques. All these key features proposed that this method can be considered as advantageous over other methods. The drug solutions employed in the study were stable up to 48 hours. These attribute the high quality of the method. This developed method for quantitative estimation of Acyclovir in bulk drug and tablet dosage form has been developed and found to be applicable for the routine analysis of Acyclovir in bulk and tablet dosage forms without any interference from the excipients. This HPLC method for assay of Acyclovir in bulk and tablet formulation was successfully developed and validated for its intended purpose. This method is recommended for routine quality control of the Acyclovir drug content in pharmaceutical preparations.

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