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HIGH PRESSURE LIQUID CHROMATOGRAPHIC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF ACYCLOVIR IN BULK AND MARKETED FORMULATION

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Key words:

RP - HPLC, Acyclovir, Validation, ICH guidelines.

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ABSTRACT: A simple, selective, rapid and economical High Performance Liquid Chromatography (HPLC) method was developed for estimation of Acyclovir in bulk and marketed formulation. Chromatographic separation and estimation was achieved in a run time of 6.482 minutes on Hemsil C_{18}- 5U (250 × 4.6 mm, 5µm) using Acetonitrile and pH adjusted water (6.74) in the ratio of 10:90 v/v at flow rate of 0.5ml/min. UV detector set at 254nm was used for detection. The linearity range for Acyclovir was found to be 4-40 µg/ml with coefficient of linear regression greater than 0.9948. The method was validated as per International Conference on Harmonization (ICHQ2 (R1) 2005) guidelines for accuracy, precision, specificity, LOD & LOQ, linearity and robustness. The method was successfully applied for estimation of Acyclovir from bulk and marketed formulation for routine analysis.

INTRODUCTION: Acyclovir is a nucleic acid analogue of guanosine which is used as antiviral medication. It is mainly utilized for the treatment of herpes simplex virus infections, chickenpox and shingles. Other utilizations of Acyclovir include, prevents cytomegalovirus infections following transplant and infections due to epstein-barr virus. So, as we see it, Acyclovir is widely used many antiviral infections. Hence, from social point of view it is essential that we could get an analytical method, which is easy to perform, reliable, robust and precise to separate, detect and test acyclovir from bulk and marketed preparations.

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An exhaustive literature survey revealed that few methods have been described for the resolution of Acyclovir such as non-aqueous titration, spectrophotometry, LC-MS/MS, HPLC, have been utilized for the estimation of Acyclovir. Most of the available reported methods for the estimation of Acyclovir concentration 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 emerging proposed method are keeping in view of this; an attempt has been made to develop an emerging RP-HPLC method with simple, convenient, rapid, precise and economical and usable strong method with a simple and easily available mobile phase for the quantitative estimation of Acyclovir in tablet dosage form. This method was validated as per

International Conference on Harmonization (ICHQ2 (R1) 2005) guidelines.

MATERIAL AND METHODS:

Materials and Marketed formulation:

Acyclovir and Marketed tablets were procured as generous gift sample for the purpose of academic research from Manish Pharmaceutical Ltd., Mumbai, Maharashtra, India.

Reagents:

Acetonitrile and triethanolamine were procured from SD Fine Chemicals Mumbai, India. Ultrapure water was obtained from ELGA water purification system having resistance of 18.2 MΩ.

Instrumentation and Chromatographic Conditions:

Agilent 1200 series HPLC system with auto sampler, quaternary pump and UV detector was acclimated to develop and optimize the chromatographic condition suitable for estimation of Acyclovir. The detector was set at 254 nm and EZ Chrome Elite software version 3.2.1 was utilized for chromatographic data processing. Chromatographic separation was achieved on HiQC18HS column (5µ, 4.6mmX250mm). Various mobile phases were screened and determinately the isocratic elution was performed with mobile phase consisting of Acetonitrile and pH adjusted water (pH-6.74) and 0.1% of Triethanolamine was used to adjust the pH of water in the ratio of 10:90 v/v as optimized mobile phase at flow rate of 0.5 ml/min.

Preparation of solutions and reagents:

All the mobile phase components were filtered through 0.45µ Ultipor®N® 68 Nylon 6, 6 membrane and sonicated for 25 minutes to abstract the dissolved air bubbles and utilized as mobile phase.

Preparation of standard stock and working standard of Drug Solution:

About 100 mg Acyclovir was accurately weighed and transferred to a 100 mL of dry volumetric flask with the addition of mobile phase, upto the mark and sonicate the solution to dissolve if indispensable. 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 4 µg/mL, 8 µg/mL, 12 µg/mL, 16 µg/mL, 20 µg/mL, 24 µg/mL, 28 µg/mL, 32 µg/mL, 36 µg/mL, 40 µg/mL of Acyclovir were prepared).

Preparation of stock solution for the commercially obtained tablets:

20 tablets of Acyclovir (labelled claim 200 mg of Acyclovir per tablet) were weighed and average weight was calculated. The tablets were crushed to get fine powder. Then a quantity of the powder equivalent to 200 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 abstract insoluble materials. It was further diluted to obtain desired concentrations.

Optimization of mobile phase and method development:

Optimization of mobile phase was performed and predicated on trial and error method. A series of trials were conducted in order to get suitable optimized HPLC conditions. In the first instance several mobile phase compositions were tried such as Methanol: pH adjusted water, Acetonitrile: water in different ratio without adjusting pH. Determinately the mobile phase comprising of Acetonitrile and pH adjusted water (pH 6.74) adjusted with triethanolamine and acetic acid in the proportion of 10:90 v/v was found to give best favourable parameters and in addition obtained sharp, well-gaussian shape peak.

This mobile phase was called as the diluent because the drug is liberatingly soluble in the mobile phase. This mobile phase pH which is safe for column life and advantageous for analyte stability, whereas methanol is easily available solvent. The stationary phase composed of HemsilC18 column with 4.6 X 250 mm, 5µm were observed and they are found to be atmostsuitable for Acyclovir. The ultra violet spectrum of diluted solutions of different concentrations of maximum absorption detection of Acyclovir was recorded by utilizing UV Systronic double beam SL 2203. An absorption maximum was found to be 254 nm. This wavelength was optimum for the detection of Acyclovir. The developed method gave symmetric peak at

retention time of 6.482 minutes and satisfied all the peak properties as pursuance of ICH guidelines.

Densitogram obtained using these optimised chromatographic conditions for Acyclovir is shown in **Fig.1**, R_t value for Acyclovir was found to be 6.482.

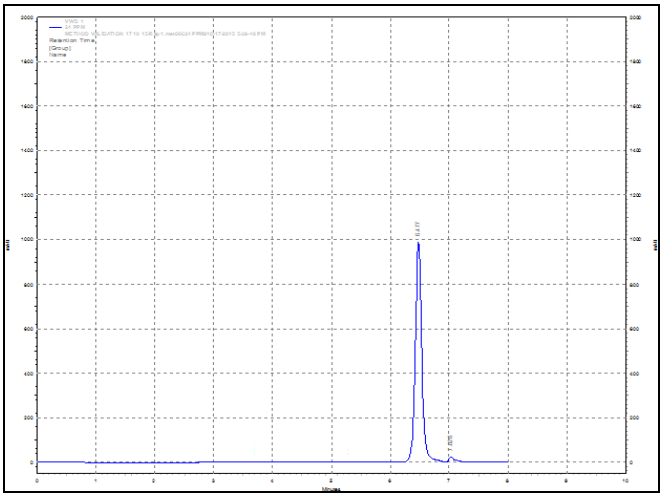

FIG 1: CHROMATOGRAM OF STD ACYCLOVIR (24ppm).

Analytical Method Validation(AMV):

Validation of analytical method:

The developed analytical method was further subjected to validation in pursuance of ICHQ2 (R1) guidelines. The parameters evaluated were Linearity, Specificity, Precision, Limit of Detection (LOD) and Limit of Quantification (LOQ), Accuracy, Robustness

Linearity:

Linearity was checked by preparing standard solutions at 10 different concentration levels of each of Acyclovir. Acyclovir standard solutions (4, 8, 12, 16, 20, 24, 28, 32, 36, 40 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 4 - 40µg/mL of stock solution (**Fig. 2**). The linearity range was found to be 4 -40 µg/mL and the results are presented in **Table 1**. Results show that a phenomenal correlation exists between peak area and concentration of drug within the linearity range.

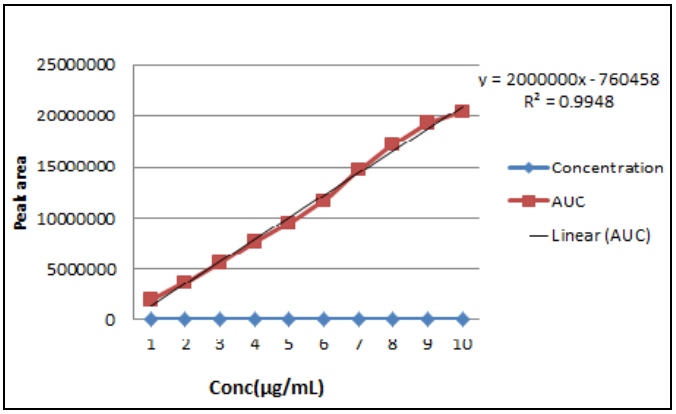

FIG. 2: CALIBRATION PLOT OF ACYCLOVIR.

TABLE 1: CALIBRATION DATA OF THE PROPOSED METHOD FOR THE ESTIMATION OF ACYCLOVIR

Concentration, µg/mL.	Retention time, (t_R) min.	Peak area, mV.s.
4	6.482	1579631.3
8	6.482	3003469
12	6.482	3935257.3
16	6.482	6085088.3
20	6.482	7406918.6
24	6.482	7916876.6
28	6.482	10094381
32	6.482	11202654.3
36	6.482	13230465.3
40	6.482	15096808.3

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 (**Fig.3**). Furthermore the well-shaped peaks also indicate the specificity of the method. The specificity results are tabulated in **Table 2**.

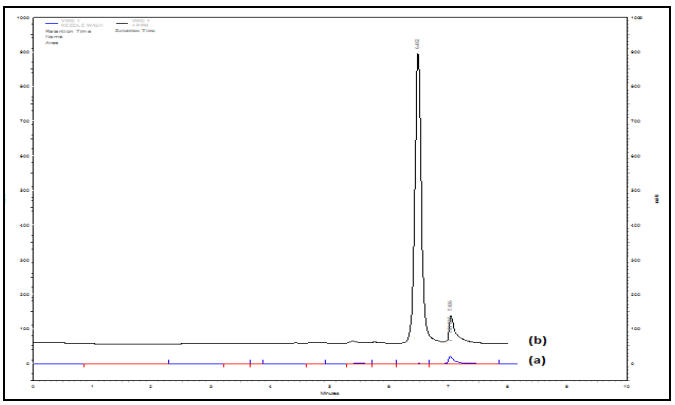

FIG. 3: AN OVERLAY OF BLANK AND ACYCLOVIR.(a) BLANK RUN,(b) ACYCLOVIR.

TABLE 2: SPECIFICITY STUDY FOR ACYCLOVIR.

Name of the solution	Retention time, (t_R) min
Mobile phase	No peak
Placebo	No peak
Acyclovir 4µg/mL	6.482

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 (4 µg/mL, 24 µg/mL, 36 µg/mL) to the utilization 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 move as utilized in intraday precision. Regarding the intraday precision was carried out by utilizing the three 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 3** and **4** respectively.

TABLE 3: INTRA-DAY PRECISION STUDIES.

		Acyclovir			Inference
Concentration levels		**Low**	**Mid**	**High**	
Concentration(µg/ml)		4	24	36	
Peak area	Session 1	1651287	7843557	13450889	
	Session 2	1654355	7833779	13427633	Acceptable %
	Session 3	1652670	7855684	13444989	RSD, Hence
Average Peak area		1652770.66	7844340	13441170.33	Precise
Standard Deviation		1536.48	10973.47	12089.13	
% RSD		0.09	0.14	0.09	

TABLE 4: INTER-DAY PRECISION STUDIES.

		Acyclovir			Inference
Concentration levels		**Low**	**Mid**	**High**	
Concentration(µg/ml)		4	24	36	
Peak area	**Session 1**	1588159	7850920	13237169	
	Session 2	1588073	7840905	13247340	
	Session 3	1587736	7850934	13237205	Acceptable % RSD, Hence Precise
Average Peak area		1587989.33	7847586.33	13240571.33	
Standard Deviation		223.56	5786.21	5861.86	
% RSD		0.01	0.07	0.04	

LOD and LOQ:

Limit of detection is the lowest concentration in a sample that can be detected, but not indispensably quantified under the verbally expressed experimental conditions. The circumscription of quantitation is the lowest concentration of analyte in a sample that can be resolute with acceptable precision and precision. Limit of detection and circumscribe of quantitation were calculated utilizing following formula LOD = 3.3 σ/S and LOQ = 10 σ/S, where, σ is the standard deviation of replication and S is the slope of the calibration curve. The LOD and LOQ values are presented in **Table 5**. The results of LOD and LOQ strengthen the sensitivity of the developed method.

TABLE 5: LOD AND LOQ OF ACYCLOVIR.

Parameters	Calculated values
LOD	0.016 µg/ml
LOQ	0.0486 µg/ml

Accuracy/Recovery:

The precision of the method was determined by standard integration method. A known amount of standard drug was integrated at 80 %, 100 % and 120 % level. The concentrations were re-analyzed with the above described procedure. The percent recovery of the triplicate solutions was resolute and average of the percent recovery was calculated. The recovery results are presented in **Table 6.**

TABLE 6: ACCURACY- RECOVERY STUDIES OF ACYCLOVIR.

Drug	Level of % recovery	Amount present in extract (*µ*g/mL)	Amount of standard added (*µ*g/mL)	Total amount (*µ*g/mL)	% recovery	Average % recovery	% RSD	Inference
Acyclovir	80	50	40	90	99.01%		0.70%	Acceptable
	100	50	50	100	99.25%	99.10%	0.53%	Recovery,
	120	50	60	110	99.06%		0.66%	Hence Accurate

Robustness:

Robustness of the method is its ability to remain unaffected to minute changes in variety of parameters such as the small variation in a percentage composition of the mobile phase, flow rate, detection wavelength. The results of robustness study is shown in **Table 7** designated that the minute changes in the conditions did not significantly affect the robustness of Acyclovir.

TABLE 7: ROBUSTNESS STUDIES OF ACYCLOVIR.

Method parameters and Variation	Levels of Variation	Modified Parameters	%RSD	Retention Time (Min)
Proportion of Mobile phase	-	Acetonitrile: pH adjusted Water(6.74), (8:92 v/v)	0.49	0.0641
	+	Acetonitrile: pH adjusted Water(6.74), (12:88 v/v)	0.70	0.0425
Flow rate (0.5±0.1) ml/min	-	0.4	0.60	0.231
	+	0.6	0.81	0.434

RESULTS AND DISCUSSION: This study was aimed at developing a precise, sensitive, rapid and precise 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 Acetonitrile: Double Distilled water of pH 6.74 (10:90) v/v at a flow rate of 0.5 mL/min was selected. The retention time for Acyclovir was found to be 6.482 minutes. UV spectra of Acyclovir showed that the drug absorbed maximum at 254 nm, so this wavelength is known as the detection wavelength.

The correlation coefficient (0.9948) of regression was found virtually identically equivalent to one in the range of 4 - 40 µg/mL which states that the method was good linear to the concentration versus peak area replications. The comparison of chromatograms of placebo, standard and sample, there was no interference observed from the top of placebo, standard and sample. It shows that the method is concrete. The precision studies were performed and the %RSD of the determinations was found to be 0.106 for intra-day precision and 0.04 for inter-day precision which are within the circumscriptions which indicate that the proposed method was found to be precise. The precision of the method was found to be good with the overall %RSD for recovery at 80 %, 100 % and 120 % levels were all within the constraints which denote that the proposed method was found to be precise.

Method validation following ICH guidelines designated that the developed method had high sensitivity with LOD of 0.016 µg/mL and LOQ of 0.0486 µg/mL. The method was found to be robust even though on remote deliberate variation in the method conditions did have a minute effect on the peak asymmetry, retention time and all are within the constraints which indicate 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 persistant by linearity, precision and precision studies. The method was found acceptable across wide range of concentration 4-40 µg/mL. The retention time of

the sample solution of Acyclovir tablet was found to be 6.482 minutes, which is comparable that of the standard solution of Acyclovir. This indicates that there is no drug-excipient interference and the drug is properly resolved by the developed method. Robustness determines the reproducibility of the test result with minute and deliberate variations in the method parameters.

The experiment was carried out by marginally transmuting the ratio of methanol in mobile phase, detection wavelength and flow rate. The efficacy of the deliberate little variations was observed on the flow rate and mobile phase composition. The statistical data shows no paramount variations in the above verbalized parameters which designate that the method is robust. The developed method was prosperously applied for the resolution of Acyclovir in bulk drug and tablet dosage form.

CONCLUSION: Until now, Most of the mobile phases reported for the HPLC separation of the acyclovir were ternary or quaternary. Only few reported were binary, but in that, phosphate buffer was used as one of the component, which can damage the column irreversibly. Therefore, we used the pH adjusted ultrapure water (adjusted with HPLC grade triethanolamine and acetic acid) instead of phosphate buffer to extend column life. For the second component in the binary mixture, we used acetonitrile, the solvent that has lowest density and column friendly. This binary mixture of Acetonitrile: pH adjusted water in the ratio 10:90 gave the best results with retention time of 6.48 minutes.

The significance of this method development is as follows:

1. Acyclovir is an antiviral drug and used in many conditions of infections and its method development for its detection from bulk and marketed preparations would help tremendously for its separation, testing and detection.

2. As one component, pH adjusted water is comparatively much better than phosphate buffer for column life.

3. Other component, Acetonitrile performs dual functions of separation as well as column preservation.

4. The ratio of 10:90 of Acetonitrile: pH adjusted Ultrapure water results in less usage of acetonitrile, lesser solvent use.

5. The Run time of maximum 8 minutes results in less solvent passing through the column, resulting in longer column life and less solvent usage.

6. The better column life results in lesser expenses and ultimately profit.

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