Development and Validation of a Stability-Indicating RP-HPLC Method for the Estimation of Aciclovir in Bulk and Ointment Dosage Form

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ABSTRACT

A simple, precise and gradient RP- HPLC method has been developed and validated for Aciclovir in bulk and ointment formulation. The proposed method was validated to obtain official requirements including stability, accuracy, precision, linearity and selectivity. The estimation was developed on C (18) column reversed-phase using the mobile phase composition as Acetonitrile: Methanol: Phosphate buffer (16:20:64 v/v). The flow rate was set as 1ml/minute, and the maximum absorption was observed at 290 nm using Shimadzu SPD-20A Prominence UV-Visible detector. The Aciclovir, drug showed a precise and good linearity at the concentration ranges of 20-100 µg/ml. The RP-HPLC assay showed the highest purity ranging from 99.79 % to 100.97 % for Aciclovir, ointment formulation, and 100.21 % was the mean percentage purity. The Aciclovir retention time was found to be 5.01 minutes. The method accuracy was shown by the statistical analysis. To examine the stability of the drug, various forced degradation studies were conducted on Aciclovir ointment. The developed method was validated according to the ICH guidelines.

Key Words: Aciclovir, Methanol, Buffer, HPLC and UV.

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INTRODUCTION

Aciclovir, 9-[(2-hydroxyethoxy)-methyl]-guanosine, is an acyclic guanosine derivative, which exhibits a selective inhibition of herpes viruses replication with the potent clinical antiviral activity against the herpes simplex and varicella-zoster viruses [1, 2]. Aciclovir, is available as tablet, ointment and IV injections. Aciclovir, is generally considered safe for use in pregnancy with no harm having been observed [3]. It appears to be safe during breastfeeding. Aciclovir is a nucleic acid analogue made of guanosine. It works by decreasing the production of the virus's DNA [4]. There are many works published for the determination of Aciclovir, in biological fluids of different species. Several HPLC methods have been reported for the determination of Aciclovir, in human serum using UV or fluorescence detection [5, 6]. According to the literature review, several methods have been developed for Aciclovir, like UV spectroscopy, fluorescence spectroscopy capillary electrophoresis, HPTLC, HPLC and voltammetry method [7-10]. Herein, a sensitive and selective HPLC method for the Aciclovir, has been presented for the first time. The method development and the validation of a stability-indicating RP- HPLC method for the estimation of Aciclovir, were new, which would fulfil all ICH guidelines requirements of validation. Increasing the importance of speed, time and reliability of analysis in pharmaceutical analytical laboratories, a new method for Aciclovir determination in the ointment formulation with a very short analysis time was described in this method. The proposed method was very fast, quick and accurate in terms of the chromatographic retention time and run time compared

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with other reported methods. The proposed aim of this study was to develop simple, accurate, specific and precise RP-HPLC method for the Aciclovir estimation in the bulk and pharmaceutical ointment formulation.

MATERIALS AND METHODS

Chemicals

The Aciclovir reference standard (RS) was purchased from Sigma, Germany. The Acivir ointment (Aciclovir 3% w/w) manufactured and marketed by Cipla Ltd, purchased from local Pharmacy from Vellore, India. The HPLC grade acetonitrile, water and methanol were purchased from Sigma, German.

RP-HPLC instrumentation

Shimadzu LC-20 AT HPLC system, using SPD-10 detector (SPD- M20A, Japan) was used. A Zorbax Eclipse Plus, Agilent Technology column (150mm x 4.6mm, 5µm) with Pore size of 95Å were also utilized. The column temperature was maintained at a 29ºC, and the flow rate was 1ml/min. The sample injection volume was 20µl, and the wavelength was set as 250nm, and the HPLC run time was set for 15 minutes.

Preparation of Mobile phase

Accurately weighed 1.35g of KH_2PO_4 was transferred into 1-liter volumetric flask and dissolved by 500 ml of HPLC grade water, and the pH was adjusted to 6 by the gradual addition of phosphoric acid, the resulting solution was filtered with 0.45µ membrane filter. The final mobile phase was prepared by adding the ratio of (16:20:64 v/v) Acetonitrile, Methanol and phosphate buffer.

Preparation of Aciclovir stock solution

Standard Aciclovir solution

Accurately 2mg Aciclovir reference standard was taken in 100 ml volumetric flask, and mixed with 100 ml of mobile phase solution, and the resulted solution was kept in the sonicator for 5 minutes. The concentration of 20-100 µg/ml was achieved by diluting the standard stock solution with the mobile phase. Aciclovir powder was freely soluble in methanol.

Preparation of sample solution

0.5 gm of marketed sample of Acivir 3% w/w ointment was weighed accurately which was equivalent to 30 mg of Aciclovir transferred into 25ml volumetric flask and dissolved with 25 ml of mobile phase and filtered a through Whatman 1 filter paper. Further dilutions were made based on the required concentrations.

Solution stability

The prepared drug solution stability was analysed during the time of analysis, and also the same analysis method was repeated on the same day with different time intervels. The same analysis was repeated after 24 hrs by keeping the drug solution under the laboratory temperature (37 ± 1°C) and in refrigeration (5 ± 1°C).

Method validation

The proposed method was preceded to achieve a new, sensitive and easy method for the estimation of Aciclovir by RP-HPLC from the ointment formulation. The experimental analysis was validated according to the ICH guidelines recommendations and USP-30.

System suitability

The resolution, retention time, tailing factor and column theoretical plates parameters were performed by six replicates of standards and three replicates of the sample preparation.

Forced degradation study

Aciclovir ointment was put into different stress conditions to perform degradation studies. The degradation study was conducted to examine the proposed assay technique. Aciclovir is freely soluble in methanol and also methanol is the portion of mobile phase, so menthol was used as a solvent. In all the experiments, Aciclovir ointment contents equivalent to 2mg Aciclovir were weighed. The solution was prepared according to the stock solution procedure. 60 µg/ml of Aciclovir was taken for every analysis. The drug solution was treated with acid, base, oxidative stress, dry and wet heat and direct sun light (photolytic stress). According to the proposed method, the resulted drug samples were examined.

Acid degradation study

The stability of Aciclovir ointment in acidic state was examined by treating with different strengths of Hydrochloric acid 0.1N to 4N HCL. The solution of 60 µg/ml Aciclovir taken in this study was treated with 4N hydrochloric acid in presence of methanol. The treated drug solution was kept in dark chamber at 35ºC for 12 hours.

Alkali degradation study

The stability of the Aciclovir ointment in alkaline condition was examined by treating with different strengths of sodium hydroxide 0.1N to 4N NaOH. The solution of 60 µg/ml Aciclovir taken for this study was treated with 4N sodium hydroxide in presence of methanol. The treated drug solution was kept in the dark chamber at 35ºC for 12 hours.

Oxidation study

The stability of Aciclovir ointment under the oxidative condition using hydrogen peroxide was examined. The solution of 60 µg/ml Aciclovir taken for this study was treated using 20 % H_2O_2 in methanol. The treated drug solution was incubated at 35ºC for 12 hours.

Wet heat study

The solution of 60 µg/ml Aciclovir taken in this study was treated with HPLC grade water, and the resulted solution was kept in the dark chamber at 35ºC for 12

hours.

Dry heat study

To conduct a dry heat analysis, the drug solution was prepared by 2gm of Aciclovir ointment approximately in a clean aluminum foil, and kept in an oven at 35ºC for 12. The resulted Aciclovir was weighed, and the solutions were prepared like the preparation of stock solution procedure; 60 µg/ml of Aciclovir was taken for the analysis.

Photo stability study (Sun light)

2gm of Aciclovir ointment was transfered in a glass dish, and exposed to the direct sunlight over a period of 4 hours. The resulted Aciclovir ointment was weighed, and the solutions were prepared like the preparation of stock solution procedure; 60 µg/ml of Aciclovir was taken for the analysis.

RESULTS AND DISCUSSION

Method optimization

Chromatogram with good shape peaks and good retention time showed a good resolution for Aciclovir, and forced the degradation of products. The proposed method was supposed to identify the number of the degradation products formed during the stressed conditions. The typical RP-HPLC conditions have been presented in Table 1. The good separation of Aciclovir and the products degraded under the stressed conditions showed the success of the method. The HPLC chromatograms of Aciclovir standard and Aciclovir ointment have been presented in figure 1 and 2.

Table 1. RP-HPLC conditions for the estimation of Aciclovir.

Parameters	Description
Column	Zodiac C(18) column (50mm x 4.6mm, 5µm)
Column temperature	27 ± 1°C
Mobile phase	(16:20:64 v/v) acetonitrile, methanol and phosphate buffer.
Detection	Photodiode array detection at 290 nm
Injection volume	20 µl
Flow rate	1 ml min^{-1}

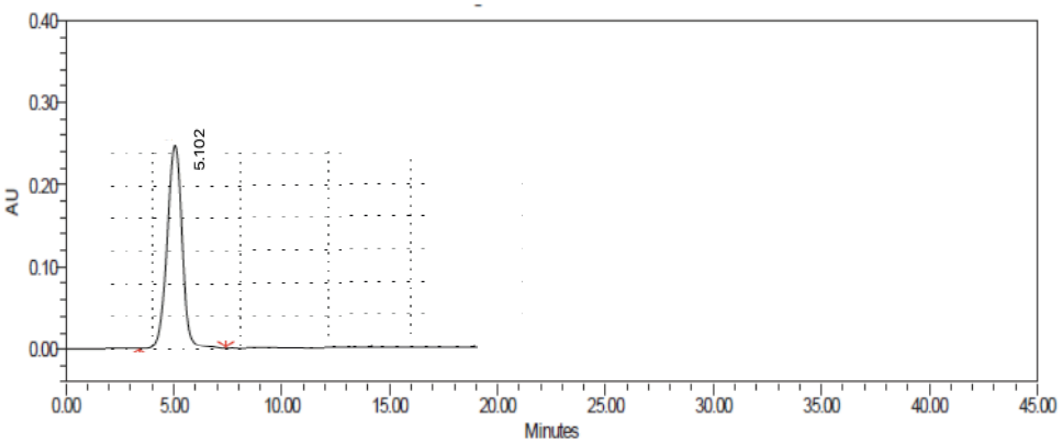

Fig. 1. A Chromatogram of Aciclovir Standard

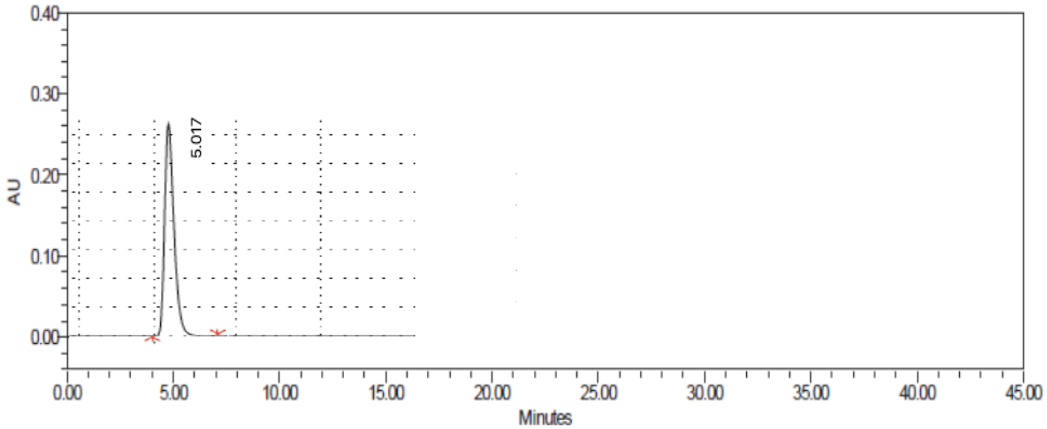

Fig. 2. A Chromatogram of Aciclovir ointment formulation

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Method validation

The method was proceeded to achieve sensitive, easy and economical determination and estimation by HPLC from the ointment formulation. Based on the ICH recommended guidelines, the experimental was validated.

Linearity

The proposed method's linearity was examined for five concentrations. The concentration ranged from 20-100 µg/ml. The Aciclovir standard linearity was determined by the plotting graph concentration vs the absorbance. Absorbance as a function of analyte concentration linearity was evaluated for Aciclovir. The linearity graph has been presented in figure 3, and the data has been shown in Table 2. The system suitability was demonstrated by the linearity analysis.

Table 2. RP- HPLC linearity for Aciclovir

Concentration (µg/ml)	Peak area
20	21567.67
40	42344.22
60	64351.32
80	85097.45
100	1077649.79

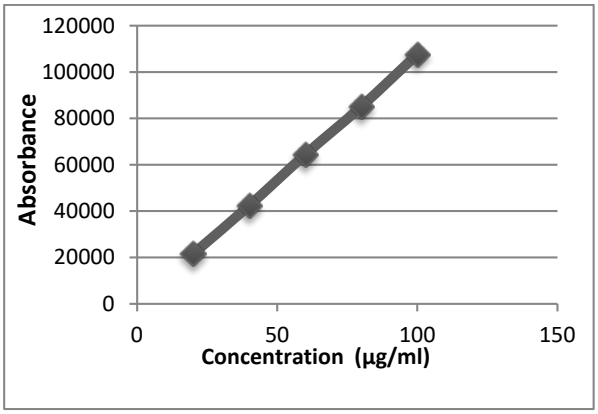

Fig. 3. Calibration graph of Aciclovir 20-100 µg/ml precision

Accuracy

The recovery experiment showed the accuracy of the method. The good recovery showed that the method was accurate. The analysis for the recovery was performed by the known amount of Aciclovir working standard added to the pre-analyzed solution of the formulation in the test concentration range of (40%, 60% and 100 %). For each recovery level, three samples were prepared and repeated for 3 consecutive days. The statistical results for the recovery study were well within the range (S.D. < 2.0). The Aciclovir ointment formulation recovery results have been presented in Table 3.

Table 3. Recovery studies of Aciclovir ointment formulation

Drug	% Level	Amount Added (µg/ml)	Amount Found (µg/ml)	% Recovery	Mean recovery
Aciclovir	40	60	99.98	99.67	99.27
	40	80	119	99.55	
	40	100	139	99.75	

Precision

The experimental results to check the precision (repeatability) of the proposed method have been shown in Table 4. In the proposed method, the intraday and interday precisions were examined by analyzing the responses of the sample on the same day for 4 repetitions and 3 alternate days for 60-100 µg/ml concentration range of Aciclovir. The obtained results were represented in % RSD. The % CV of the proposed method was precise as the values of < 1.0 % for the repeatability study. The precision data have been presented in Table 5.

Table 4. Method precision data of Aciclovir by RP-HPLC method

Aciclovir 20µg/ml (n=4)	Retention time	Area
1	5.11	59497.45
2	5.10	59897.75
3	5.07	59197.48
4	5.13	59297.40
Mean	5.10	59472.22
S.D [a]	0.0211	100.71
% CV[b]	0.57	2.02

n=4 observations

Table. 5. Intermediate precision data of Aciclovir by RP-HPLC method

Aciclovir µg/ml	Inter-day measured mean area ± S.D.[a]	%CV[b] (n^c=4)	Intra-day measured mean area ± S.D.[a]	%CV[b] (n^c=4)
60	5457.25± 2.19	0.0772	4197.44± 4.65	0.0115
80	10919.75±2.22	0.0717	7672.75±2.15	0.0147
100	169613.12±2.11	0.0622	9776.12±4.26	0.1036

n^c = 4 observations

Specificity

The standard reference and the drug formulation showed the specificity of the method. The RP-HPLC chromatogram of Aciclovir both bulk and the ointment formulation have been presented in figure 1, 2. The bulk and ointment formulation retention time was found to be

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5.1 minutes. For the ointment formulation, there was no excipient interference detected, which showed the specificity of the method. The proposed method showed the ability to determine the analyte in presence of the excipients.

Limit of detection and quantitation

The limit of detection and the quantification for Aciclovir has been presented in table 6. The limit of detection (LOD) and the limit of quantification (LOQ): LOD and LOQ were examined by the minimum detectable peak area by injecting the known concentration of the drug solution. As per the International Conference on Harmonization guidelines, the results were multiplied thrice to get LOD and 10 times to get LOQ. LOD and LOQ were found at the concentrations of 0. 57µg/mL and 1.25 µg/mL, respectively. The limit of detection and quantification for Aciclovir has been presented in table 6.

Table 6. Limit of detection and quantification

Parameters	Aciclovir
LOD (µg/ml)	0.57
LOQ (µg/ml)	1.25

System suitability

For the system suitability parameters, five repeats of the standards and two repeats of the sample preparation were injected, the data has been presented in table 7. The Assay data of Aciclovir has been presented in table 8.

Table 7. Results of the system suitability parameters

SNo	Parameters	Aciclovir
1.	Theoretical plates	8700
2.	Tailing factor	0.977
3.	Resolution factor	2.10
4.	Retention time	5.01± 0.1
5.	Calibration range or Linear dynamic range	20-100µg/ml

Table 8. Quantitative estimation (Assay) data of Aciclovir

Drug	Label claim (µg)	Amount found (µg)	Mean amount found (µg /ml)	Percentage purity (% w/w)	Mean purity (%w/w)	% Deviation
Aciclovir	300	300.11 299.67 300.11 301.33 300.17	300.01	99.91 99.79 100.21 100.97 100.24	100.21	+ 0.1 +0.2 +0.1 +0.1 +0.2

n= 4 observations

Statistical parameters

The assay's obtained results were subjected to the coefficient of variation; and the statistical analysis, the regression equation and the standard deviation have been presented in table 9.

Table 9. Results of statistical parameters Force degradation of Aciclovir in formulation

SNo	Parameters	Aciclovir
1.	Standard deviation (SD)	1.07
2.	Relative standard deviation (RSD)	0.0676
3.	% RSD	0.566
4.	Standard error (SE)	0.02117
5.	Correlation Coefficient (r)	0.9870
6.	Slope (a)	5032.3
7.	Intercept (b)	1102.9

Aciclovir showed slight and moderate degradation in dry heat, oxidative and Photo stability (Sun light) condition for a short period of time. Table 10 indicates the degradation of Aciclovir under different stress conditions. According to the ICH guidelines of the forced degradation, Aciclovir was examined.

Table 10. Summary of force degradation of Aciclovir by RP-HPLC method

SNo	Stress condition/ state	Time	% Assay ± S.D. [a](n[b]=5)
1	Acidic 4N HCL(35 °C)/ solution	12 hrs	98.27 ± 1.612
2	Alkali 4N NaOH (35 °C)/ solution	12 hrs	99.12± 0.7142
3	Wet heat (35 °C)/ solution	12 hrs	98.37±1.321
4	Dry heat (35 °C)/ solid	12 hrs	95.71±1.244
5	Oxidative 20 % H_2O_2 (35 °C)	12 hrs	98.67±1.138
6	Photo stability/ solid	4 hrs	83.76±1.349

S.D.[a] is standard deviation for n[b] = 5 observations

Alkali degradation

The stability of the Aciclovir ointment in alkaline condition was examined. There was no degraded product separated from Aciclovir. The chromatogram of alkali-degraded result was compared with the formulation and standard chromatogram. The result showed that around 1–2 % of the Aciclovir drug was degraded, and in alkaline condition, the drug was highly stable.

Acid degradation

The stability of the Aciclovir ointment in acid condition was examined. The chromatogram of the acidic condition product was compared with the formulation and standard chromatogram. The results showed that around 1–2 % of the Aciclovir drug was degraded. The results also showed that the drug in acidic condition was highly stable.

Oxidation

The stability of the Aciclovir ointment in oxidation condition was examined. It was observed that around 2-4% degradation was taken place on exposure to 20 % H_2O_2 for 12 hrs. The chromatogram of oxidation product was compared with the formulation, standard chromatogram and blank H_2O_2. No degradation product peaks were observed. The 20 % H_2O_2 peak time was observed at RT 10.27 and in the peak height and area, no significant decrease with time was observed as presented in figure 4. The results showed that the drug was highly stable to the oxidative conditions.

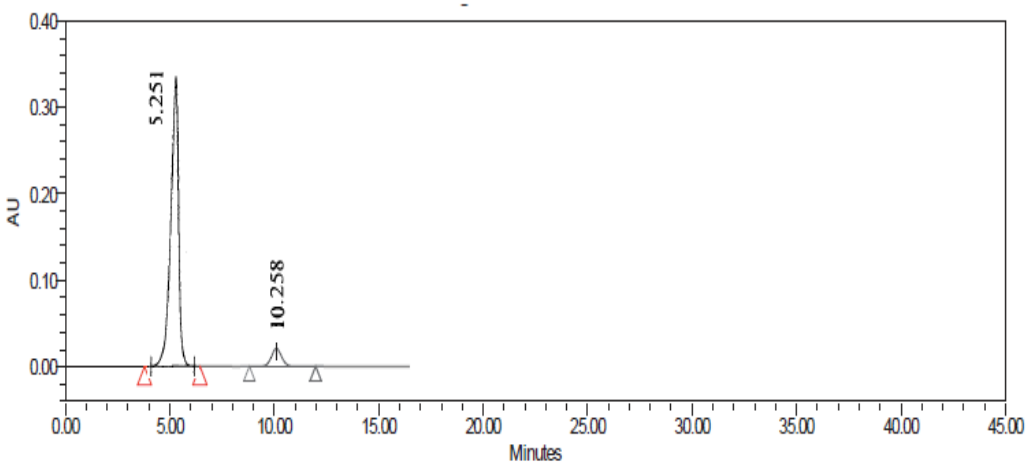

Fig. 4: Chromatogram of Aciclovir under oxidation condition by RP-HPLC method

Wet heat (Hydrolysis)

The stability of the Aciclovir ointment in neutral condition was examined, 1–3 % drug degradation was observed after 12 hrs of the incubation at 35ºC. The chromatogram of wet heat degraded product was compared with the formulation and standard chromatogram as presented in figure 5. In the wet degraded chromatogram Aciclovir peak area, the height was decreased and no degradation peak was observed.

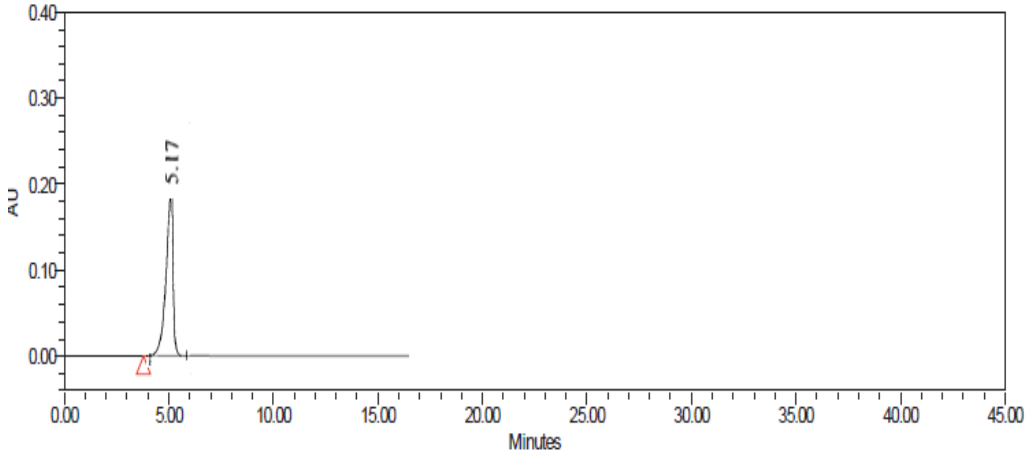

Fig. 5: Chromatogram of Aciclovir under wet heat condition by RP-HPLC method

Dry heat

The chromatogram for Aciclovir in dry heat showed that the drug was slightly unstable as compared to acid, base and wet degradation. Around 3-7% drug degradation was observed. The chromatogram of dry heat degraded product was compared with the formulation and the standard chromatogram. In the dry heat degraded chromatogram, the drug was decomposed into minor degradation product. The result showed no significant decrease in the peak height and peak area with time as presented in figure 6.

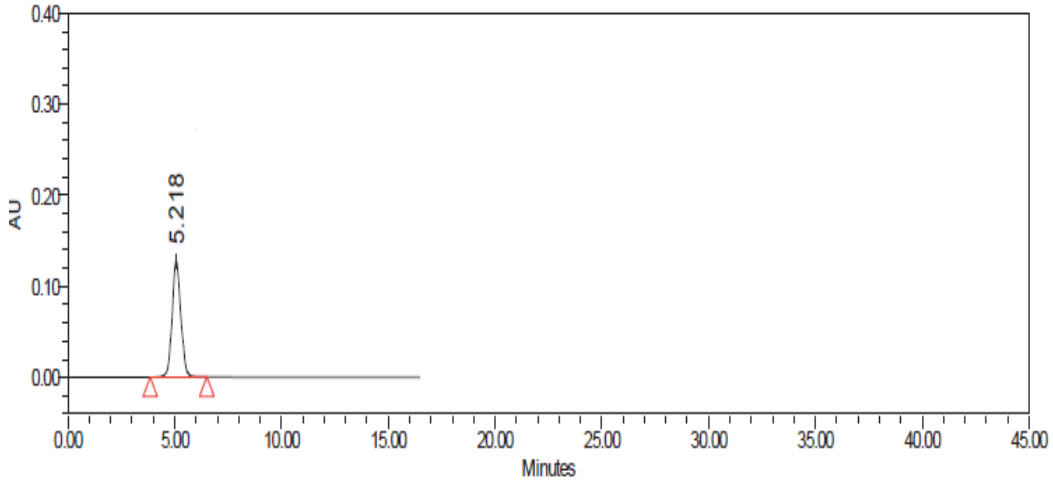

Fig. 6: Chromatogram of Aciclovir under dry heat condition by RP-HPLC method

Photo stability (Sun light)

The chromatogram of Aciclovir under photo stability study was found to be unstable after the exposure of the drug to direct sunlight for 4 hours. Almost 10-20% of the drug was degraded in 4 hours. One minor drug degradation peak was observed at 12.5 minutes, and also there was a significant increase in the drug peak height, and a decrease in peak area was observed as presented in figure 7.

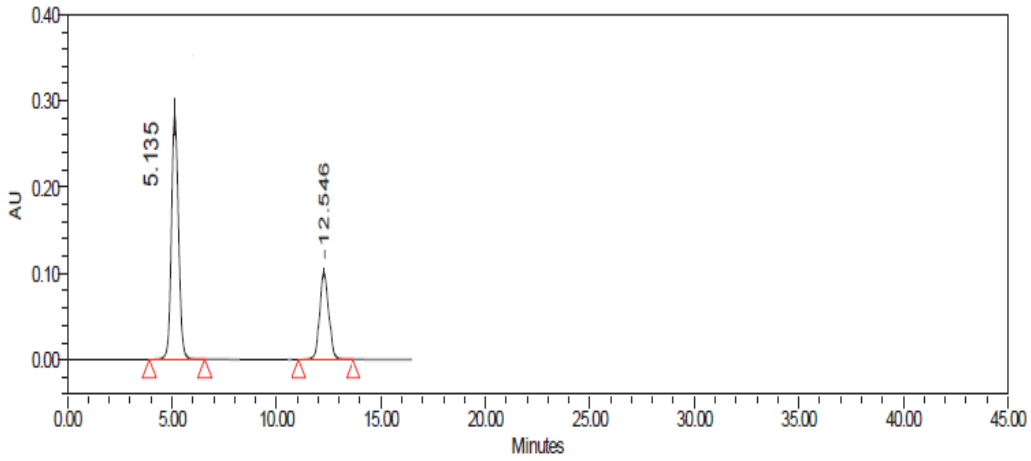

Fig. 7: Chromatogram of Aciclovir under Photo stability condition by RP-HPLC method

CONCLUSION

The force degradation study was performed according to the guidelines of the International Conference on Harmonization (ICH). The developed RP-HPLC method showed accuracy, sensitivity and stability. The developed method was rapid and reproducible. The developed method can be used for the routine analysis of Aciclovir, ointment formulations.

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