Stress Studies on Acyclovir

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Abstract

Acyclovir is an antiviral drug of choice in the treatment of many types of herpes virus infections, including genital herpes simplex infections, herpetic conjunctivitis, herpes simplex encephalitis, etc. The present study describes the degradation behavior of acyclovir under different International Conference on Harmonization recommended stress conditions (hydrolysis, oxidation, photolysis, and thermal decomposition) in order to establish a validated stability-indicating high-performance liquid chromatography method. Acyclovir is found to degrade extensively in acidic conditions and oxidative stress. Mild degradation of the drug occurs in alkaline and neutral conditions. The drug is stable to dry heat. The drug is found to be sufficiently stable after light exposure in a solid state; however, photolytic degradation is observed when the drug is exposed as a solution in water. The major degradation product in acidic hydrolysis and photolysis is identified as guanine through comparison with the standard. Separation of drug and the degradation products under various conditions is successfully achieved on a C-18 column utilizing water–methanol in the ratio of 90:10. The flow rate is 1 mL/min, and the detection wavelength is 252 nm. The method is validated with respect to linearity, precision, accuracy, selectivity, specificity, and robustness. The mean values of slope and correlation coefficient are 39.307 and 0.9998 with relative standard deviation values less than 2%. The recovery of the drug is found to be in the range of 97.34% to 102.35%. From the previous study it is concluded that the stability-indicating method developed for acyclovir can be used for analysis of the drug in various stability samples.

Introduction

Stability testing and stress testing (forced degradation studies) are critical components of drug development strategy (1). The studies help us understand the mechanism of drug's decomposition, which further helps in obtaining information on physical and chemical factors that result in instability (2). These factors are then controlled in order to stabilize the drug or drug formulation, resulting in increased shelf-life or improved efficacy.

Stress testing is defined as the stability testing of drug substances and drug products under conditions exceeding those used for accelerated testing. These studies are undertaken to elucidate the intrinsic stability of the drug substance. According to the International Conference on Harmonization (ICH) guideline Q1A (R2) on the stability testing of new drug substances and products, the stability testing of the drug substance should be carried out under different stress conditions to validate the stability-indicating supremacy of analytical methods used for the analysis of stability samples (3). The stress conditions should include extremes of pH, oxidative, photolytic degradation, and effect of temperatures. The standard conditions for photo stability testing are described in ICH guideline Q1B (4).

Acyclovir (Figure 1) is an important advancement in the field of antiviral therapy. It is used in the treatment of many types of herpes virus infections, including genital herpes simplex infections, herpetic conjunctivitis, herpes simplex encephalitis, and mucocutaneous herpes simplex infections (5). A number of chromatographic methods have been reported in the literature for the determination of acyclovir in pharmaceuticals. Sia et al. (6) reported the use of a thin-layer chromatography (TLC) method for the determination of acyclovir. Another TLC method was reported by Vasilescu and Nicolescu (7). Numerous reports are available in the literature regarding the use of high-performance liquid chromatography (HPLC) for the analysis of acyclovir (8–12). A reversed-phase (RP) HPLC method for the quantitation of acyclovir in eye drops was reported by Zhu and Huang (13). Other examples include the determination of acyclovir in eye gel (14) and in suspension (15). A fluorimetric method for the determination of acyclovir in pharmaceuticals and pure form has been developed (16). Some examples of methods for the analysis of acyclovir in biological samples
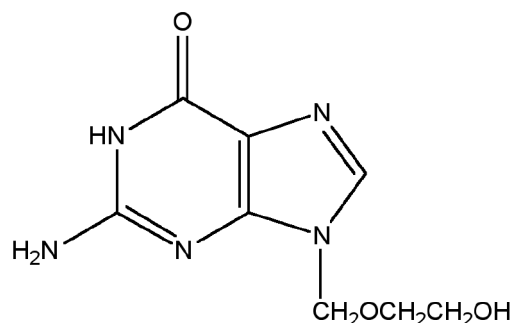
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Figure 1. Structure of acyclovir.

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include HPLC (17–22), RP-HPLC (23), high performance capillary electrophoresis (HPCE) (24), hydrophilic interaction liquid chromatography-electrospray mass spectrometry (MS) (25), and scintillation proximity radioimmunoassay (26).

There are no earlier reports in the literature involving stability study on acyclovir under all ICH recommended stress conditions. In the present study, stress testing of acyclovir was carried out under a variety of ICH recommended test conditions (3,27). The stress conditions used were hydrolytic, oxidative, photolytic, and thermal degradation. The method of analysis was validated with respect to linearity, accuracy, precision, specificity, and robustness as per ICH guidelines.

Experimental

Reagents and solutions

Acyclovir was provided by Ranbaxy Laboratories (Gurgaon, India) and was used without further purification. Methanol (HPLC grade) was purchased from Merck (Mumbai, India). Sodium hydroxide (S.D. Fine-chem., Mumbai, India), hydrochloric acid (Qualikems, New Delhi, India), and hydrogen peroxide (Qualigens Fine Chemicals, Mumbai, India) were of analytical reagent grade. The water used was triple distilled and membrane filtered HPLC-grade water.

Acyclovir stock standard solution (1 mg/mL)

Accurately weighed 100 mg of acyclovir powder was transferred to a 100-mL volumetric flask, 50 mL of water was added to it, and it was vortexed for 10 min. The final volume was made up with water, and the resulting solution was vortexed for 1 min.

Apparatus and operating conditions

The HPLC system consisted of a LC-10AT VP Shimadzu pump equipped with a SPD-10AV P Shimadzu UV–visible detector. The data were acquired and processed by the use of Spinchrom software. The separations were carried out on a C-18 RP column (YMC, Japan, 250 × 4.6 mm, S-5μ). The column was operated at ambient temperature. The mobile phase was made up of water–methanol (90:10) and the flow rate was 1 mL/min. The wavelength of detection was set at 252 nm. Specificity testing was done on another HPLC (Waters Delta 600) equipped with a Waters 600 controller pump, Waters 2996 PDA detector, and a degasser module (all from Waters, Milford, MA). Data acquisition and processing was performed by use of Waters Empower 2. The photostability studies were carried out in a stability chamber (KBF 240, WTB Binder, Tuttlingen, Germany) equipped with light sources as defined under option 2 in the ICH guideline Q1B. The light bank consisted of a combination of two blacklight OSRAM L73 lamps and four OSRAM L20 lamps. The blacklight lamp (L73) had a spectral distribution between 345 and 410 nm with a maximum at 365 nm. The output of white fluorescent lamps (L20) was similar to that specified in ISO 10977 (1993). Both UV and visible lamps were put on simultaneously. The study was performed by keeping the samples at a distance of ~ 25 cm from the light bank. The overall illumination at the point of placement was 5,000 lux, which was tested using a calibrated lux meter (Escorp, New Delhi, India). The chamber was maintained

at 40°C and 75% RH. The samples were exposed for a total period of 14 days. The thermal studies were performed in a hot air oven maintained at 70°C.

Method validation

Linearity

Linearity of the method was established by preparing a calibration curve in water. For this, stock solution of the drug (1 mg/mL) in water was prepared. A range of concentrations (10–200 μg/mL) was then prepared after suitable dilution of the prepared stock with water. The solutions (20 μL) in the range of 10–200 μg/mL of drug were injected in triplicate into the HPLC column. Water–methanol mixture (90:10) was used for elution. The linearity plots were constructed, and data was treated using linear regression analysis method.

Precision

To determine the intra- and inter-day precision of the method, repeatability studies were performed. For intra-day precision studies, drug solution at three different concentration levels (10, 50, and 100 μg/mL) was injected in hexplicate on the same day. These studies were also repeated on three different days to determine the inter-day precision. From the area obtained, concentration was calculated, and the results were expressed as % RSD.

Accuracy

Accuracy of the method was evaluated by spiking the drug at three different concentrations (10, 50, and 100 μg/mL) in a mixture of stressed samples. The percent recovery of added drug was then calculated using the linearity plots.

Specificity

The specificity of the method was established through determination of purity for the drug in the presence of degradation products using photo-diode array (PDA) detector.

Robustness

Robustness of the method was established by studying the separation studies on a different chromatographic system on a different day. Similarly, the studies were carried out by altering the chromatographic conditions. To study the effect of flow rate on the resolution, the same was altered by 0.2 units (i.e., from 0.8 to 1.2 mL/min). The effect of column temperature on resolution was studied at 25°C, 40°C, and 45°C.

Stress studies

Neutral (water) conditions

For studies in neutral conditions, the drug solution in water (1 mg/mL) was refluxed for 96 h. The samples were withdrawn at different time intervals and subjected to HPLC analyses.

Acidic conditions

Acidic hydrolysis of the drug was carried out in hydrochloric acid of different strengths. The drug was dissolved in 0.1 N HCl at a concentration of 1 mg/mL, and the solution was heated at 80°C for a period of 2 h. The same concentration of drug was subsequently exposed to 1 N HCl and 2 N HCl at 80°C for 2 h.

Alkaline conditions

Alkaline degradation studies were performed in 1 N NaOH at a

drug concentration of 1 mg/mL. The solution was then subjected to heating at 80°C for 2 h. Before carrying out the HPLC analysis, samples were suitably diluted and neutralized with 0.1 N HCl.

Oxidation

Oxidative degradation studies were done in hydrogen peroxide solutions at room temperature. The drug was initially exposed to 1% H_2O_2 for 30 min and subsequently for 3 h. Studies were also performed in 3% H_2O_2 for 8 and 24 h. Finally, the same concentration of drug was also exposed to 10% H_2O_2 and 30% H_2O_2 for 24 h.

Photostability

Photolytic studies in solution were performed in water and 0.1 N HCl. The drug solution (1 mg/mL) was exposed in a photostability chamber for 14 days. Photolytic studies in solid state were conducted by exposing the drug in thin layer in a photostability chamber for 14 days. Samples were withdrawn at different time periods and analyzed.

Thermal stress studies

To investigate the stability of the drug under thermal stress conditions, bulk drug was spread in a thin layer on a petri plate and subjected to dry heat at 70°C in a dry heat oven. The sample was analyzed after 15 days.

Identification of guanine as major degradation product

The formation of guanine as major degradation product in acidic hydrolysis and acidic photolysis was verified by spiking with the standard. The suspicion that guanine could be formed during degradation of acyclovir was based on information in the literature that guanine can be present as a synthetic contaminant or a degradation product in the acyclovir (28).

Results and Discussion

Initially, mobile phase consisting of water–methanol in a ratio of 80:20 was used for the separation of degradation products. The flow rate was 1 mL/min. But this resulted in non-satisfactory separation of degradation products. No improvement was observed on decreasing the percentage of methanol to 15%. Good separa-
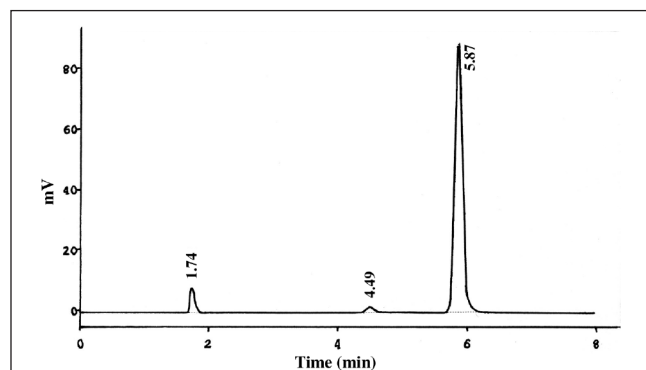
Figure 2. Chromatogram depicting the separation of degradation products of acyclovir: mobile phase, water–methanol (90:10); column temperature, 40°C; and flow rate, 1 mL/min.

tion could only be achieved on reducing the percentage of methanol to 10% (Figure 2). The peak obtained at 5.87 min represents the drug peak. The degradation product in acidic conditions corresponds to the peak at 4.49 min. The resolution between the drug and its major degradation product under deliberately changed chromatographic conditions was found to be greater than 2.5, indicating the robustness of the method.

Method validation

Linearity

The data obtained from linearity studies was plotted against the concentration, and the plots were subjected to linear regression analysis. The data from the linearity curve showed that the response of the drug was strictly linear in the studied concentration range. The equation of the line was $y = 39.307x$. A very high correlation of 0.9998 ($\pm$ 0.0002) was obtained with a slope of 39.307 and with an RSD 1.962%.

Precision

The results of intra- and inter-day precision studies are shown in Table I, respectively. The results revealed that percent RSD values for intra-day studies ranged from 0.2% to 0.36% and for inter-day precision from 0.62% to 2.7%.

Accuracy

The data from recovery studies is shown in Table II. Percentage recovery was calculated from the difference between

Table I. Precision Studies

Actual concentration (µg/mL)	Measured concentration (µg/mL) ± S.D.; RSD (%)	
	Repeatability (*n* = 6)	Intermediate precision (*n* = 3)
10	10.48 ± 0.04; 0.39	10.46 ± 0.11; 1.06
50	50.60 ± 0.18; 0.36	49.98 ± 1.35; 2.70
100	100.56 ± 0.20; 0.20	100.79 ± 0.62; 0.62

Table II. Recovery Studies (*n* = 3)

Spiked concentration (µg/mL)	Measured concentration (µg/mL) ± SD; RSD (%)	Recovery (%)
10	9.92 ± 0.13; 1.28	99.21
50	48.67 ± 1.01; 2.07	97.34
100	102.35 ± 0.58; 0.57	102.35

Table III. Results of Robustness Study

Parameters	Variations	Resolution between the drug and impurity peak
Temperature	at 25°C	3.1
	at 30°C	2.7
	at 45°C	2.5
Flow rate (± 20% of the set flow)	at 0.8 mL/ min	3.4
	at 1.2 mL/ min	2.5
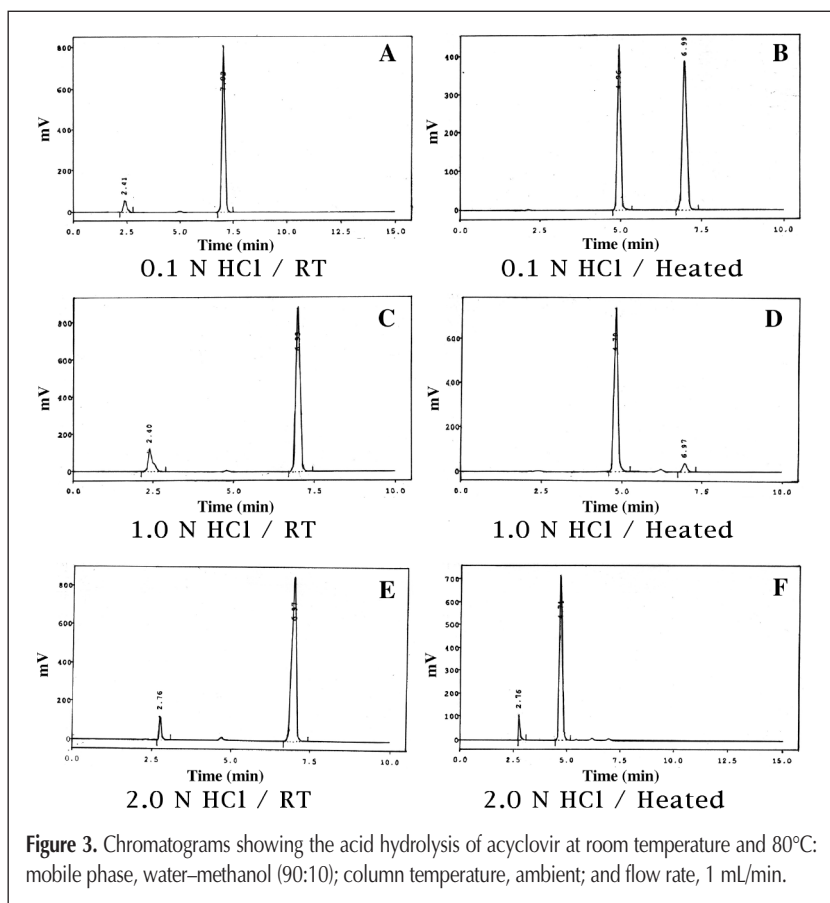
Figure 3. Chromatograms showing the acid hydrolysis of acyclovir at room temperature and 80°C: mobile phase, water–methanol (90:10); column temperature, ambient; and flow rate, 1 mL/min.
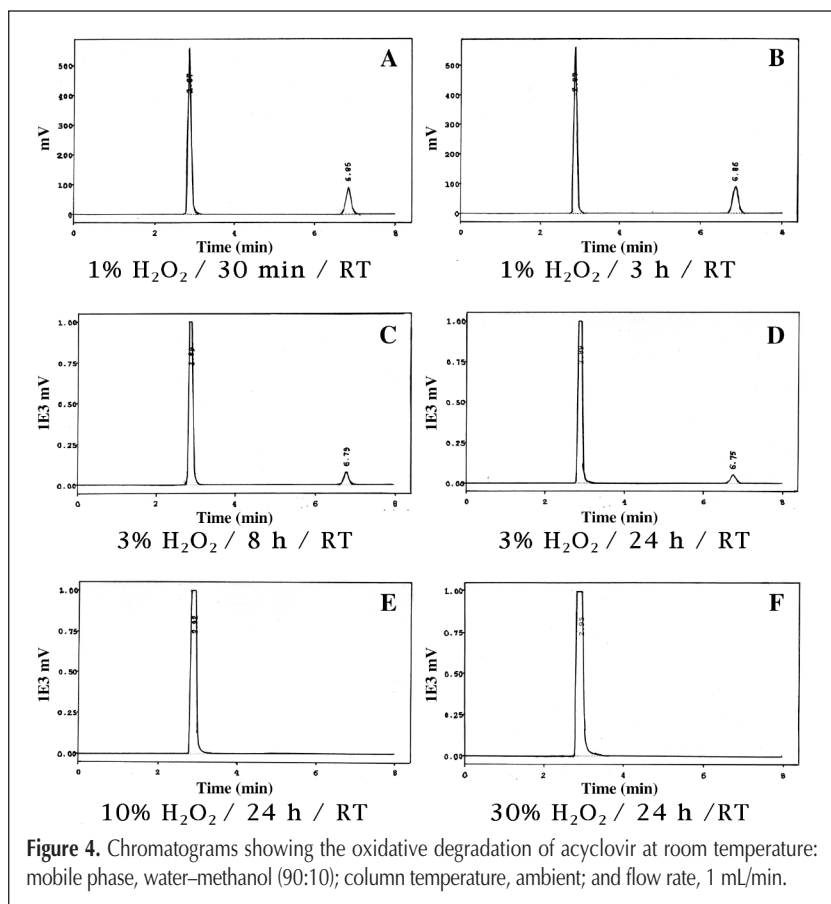
Figure 4. Chromatograms showing the oxidative degradation of acyclovir at room temperature: mobile phase, water–methanol (90:10); column temperature, ambient; and flow rate, 1 mL/min.

the peak areas obtained for fortified and unfortified solutions. Good recoveries were obtained in the range of 97.34% to 102.35%, which itself is an indication of high accuracy.

Specificity

Good resolution was obtained between the drug and the degradation products formed under different stress conditions, indicating the good specificity of the method (Figure 2). Studies performed to determine the purity of the drug peak using a PDA detector showed purity angle (PA) value of 0.264 and purity threshold (TH) value of 0.429. As the purity angle value was found to be less than purity threshold, the method was found to be specific to the drug.

Robustness

The method was found to possess robustness when the studies were repeated on two different systems. In all the deliberately altered conditions the resolution between the drug and its major degradation product was found to be greater than 2.5, indicating the robustness of the method (Table III).

Degradation behavior of acyclovir under different stress conditions

Neutral conditions

In neutral conditions, no significant degradation was noticed after refluxing for two days. However, approximately 15% of drug was degraded after refluxing for 96 h. As no new peak was obtained, it could be concluded that acyclovir was degraded to non-chromophoric compounds in neutral conditions (29).

Acidic conditions

The results showed that the drug was acid labile. When drug solution in HCl was heated, a new peak appeared in the chromatogram at 4.79 min due to hydrolysis of the drug (Figure 3). There was a rise in the area of degradation product peak with a corresponding increase in the strength of HCl. Only 45.67% of drug remained after heating in 0.1 N HCl for 2 h. This amount was further decreased to 4.44% after increasing the acid strength to 1.0 N while keeping other conditions the same. The entire drug degraded when drug solution in 2 N HCl was heated for 2 h at 80°C.

Alkaline conditions

The drug was found to be stable in alkaline conditions when compared with acidic conditions. After exposure to 1 N NaOH at 80°C for 1 h, only 5% of drug was degraded. Further exposure by 1 h under the same conditions reduced the drug content to 88.65%.

Degradation in oxidative conditions

The extent of degradation of drug under oxidative conditions at room temperature depended upon the length of exposure and concentration of oxidizing agent. Almost no degradation was seen when the drug was exposed to 1% H_2O_2 solution for 30 min. The exposure time was then increased to 3 h while keeping the concentration of H_2O_2 the same. No significant degradation was seen after also increasing the exposure time. Therefore, the drug was then exposed to a higher concentration of oxidizing agent. Only 88.91% of drug remained after exposure to 3% H_2O_2 solution for 8 h, and the amount decreased to 54.33% after increasing the exposure time to 24 h. Complete degradation of drug was observed in 10% and 30% H_2O_2 solutions with the disappearance of drug peak in the chromatogram (Figure 4).

Thermal degradation

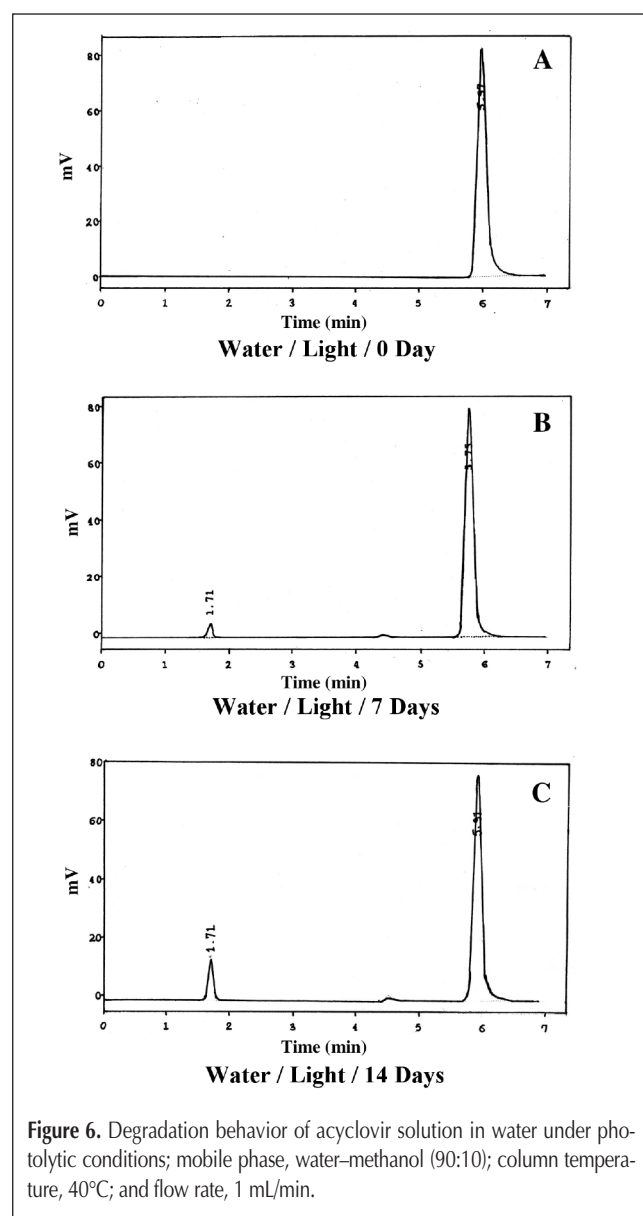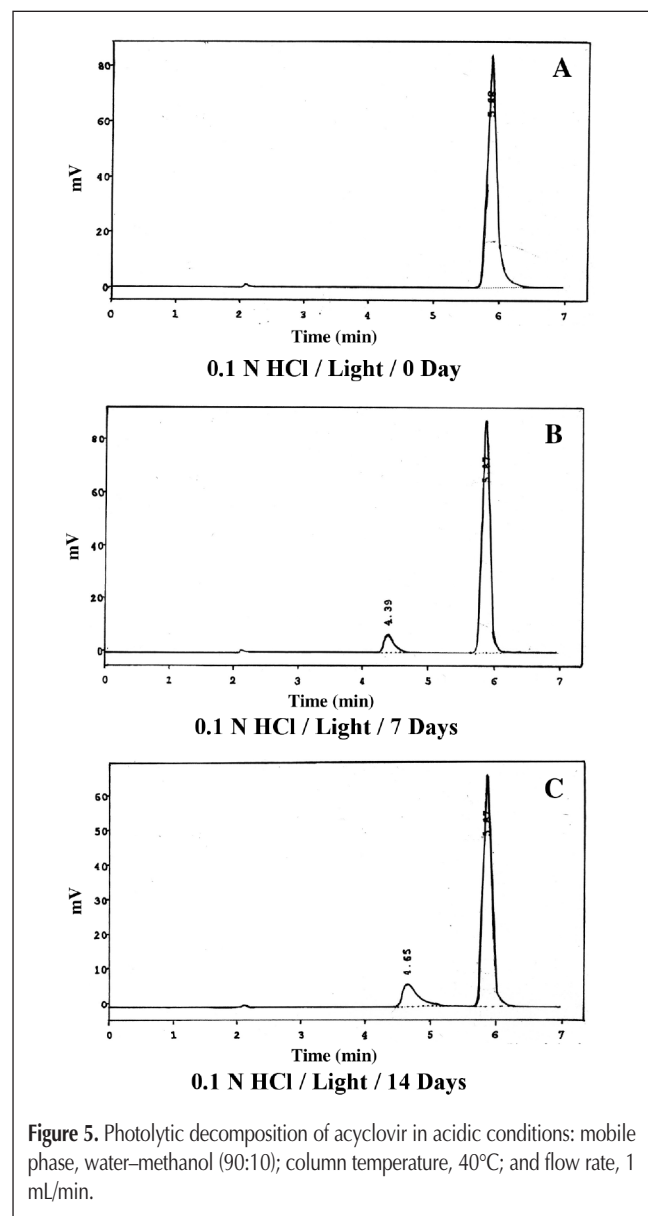No significant degradation was observed after subjecting the drug to dry heat at 70°C for 15 days. The percent amount of drug remaining after 15 days of dry heat exposure was 98.148%.

Photolytic degradation

From the results of the photodegradation studies, it was observed that the drug in the solid state was comparatively more photostable than in solution. No peak was seen in the degradation study of a solid sample. In acidic solution, approximately 11% degradation was seen after 7 days of light exposure. Further exposure of 7 days reduced the drug content to 72.66%. The degradation was accompanied with the formation of a single hydrolytic product, guanine (Figure 5). In the case of neutral solution approximately 11% degradation was seen after 15 days of light exposure (Figure 6).

Identification of the major degradation product

The spiking studies confirmed that guanine was the major degradation product formed during acidic hydrolysis and acidic photolysis.

Figure 5. Photolytic decomposition of acyclovir in acidic conditions: mobile phase, water–methanol (90:10); column temperature, 40°C; and flow rate, 1 mL/min.

Figure 6. Degradation behavior of acyclovir solution in water under photolytic conditions; mobile phase, water–methanol (90:10); column temperature, 40°C; and flow rate, 1 mL/min.

Conclusion

Acyclovir is a potent antiviral drug used in a number of viral diseases. The present study was conducted to understand the degradation behavior of acyclovir under ICH recommended stress conditions. The study conducted presents new and interesting aspects of acyclovir stability under a variety of stress conditions as no such systematic study has been previously reported. This study is an example of the development of a stability-indicating assay method for acyclovir where forced degradation was carried out under all stress conditions. Acyclovir showed degradation in all of the studied conditions. However, the extent of degradation was different. The drug was found to be acid labile, forming guanine as a degradation product that appeared in the chromatogram. In the case of neutral hydrolysis, the drug degraded to non-chromophoric compounds and was comparatively more stable. The drug degraded to mild extent in alkaline conditions and was also stable to thermal stress for the studied period. The drug showed extensive degradation under oxidative conditions. It was sufficiently stable after light exposure in a solid state for 14 days. However, photolytic degradation was observed when drug was exposed as solution in water.

The developed method proved to be simple, precise, specific, selective, and robust. The developed method can be employed for the analysis of drug in the stability samples, but it is not suggested for the establishment of mass balance as most of the degradation products were non-chromophoric in nature and did not appear during HPLC analysis.

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