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Review Article

A validated stability indicating reverses phase liquid chromatographic method for the determination of valacyclovir

Shoheb Shakil Shaikh[1],*, V. K. Padri[1]

[1]*Dept. of Quality Assurance, Siddhi's Institute of Pharmacy, Nandgaon, Maharashtra, India*

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ABSTRACT

A simple reverse phase liquid chromatographic method with ultraviolet detector was developed for the accurate determination of Valacyclovir using GracesmartRP18, C18 Column (250 mm × 4.6 mm, 5μm particle size). The mobile phase used for the determination was Methanol: Citric Acid buffer in a ratio of 60: 40 v/v at a flow rate of 1.0 mL per min. Valacyclovir was eluted at 2.2 ± 0.1 min and detected at 254 nm. The method is linear over the concentration range of 10-50 μg/mL with correlation co-efficient r^2 = 0.999. The plate count and tailing factor was found 3847 and 1.24 respectively. The developed method was proved to be robust after extensively validated with different parameters such as Linearity, Precision, Accuracy, Robustness, Ruggedness, Limit of Detection (LOD), Limit of Quantification (LOQ) and specificity. The validated method is definite, meticulous and reproducible and can be used for routine analysis of Valacyclovir in bulk form.

1. Introduction

Valacyclovir ($C_{13}H_{20}N_6O_4$) hydrochloride, 2-[(2-amino-6-oxo-3H-purin-9-yl) methoxy] ethyl 2-amino-3-methylbutanoate (Figure 1), is an antiviral drug. It rapidly converts to acyclovir which has antiviral activity against Herpes Simplex Virus Type-1 (HSV-1) and Type-2 (HSV- 2) and Varicella-Zoster Virus (VZV) both in vitro and in vivo. The mechanism of action of Valacyclovir is inhibition of the viral DNA polymerase and also involved in the viral DNA chain termination. This viral enzyme converts acyclovir into acyclovir monophosphate, a nucleotide analogue. The monophosphate is further converted into diphosphate by cellular granulate kinase and into triphosphate by a number of cellular enzymes. In-vitro, Acyclovir Triphosphate stops replication of herpes viral DNA. The greater antiviral activity of acyclovir against HSV compared with VZV is due to its more efficient phosphorylation by the viral thymidine kinase. A few analytical methods have been reported for the quantitative determination of Valacyclovir using UV,[1–6] colorimetric,[7] HPTLC,[8] LC-MS[9,10] and Few HPLC methods were reported for the determination of Valacyclovir in pharmaceutical formulations[11–19] in biological fluids.[20–23] The objective of the work is to develop HPLC method for its estimation and validation in bulk and tablet dosage form with good accuracy, simplicity, precision and economy. The present method was validated according to the International Conference on Harmonization (ICH)[24] for the determination of Valacyclovir hydrochloride in bulk and tablet dosage forms.

* *Corresponding author*.
E-mail address: shaikhshoheb91@gmail.com (S. S. Shaikh).

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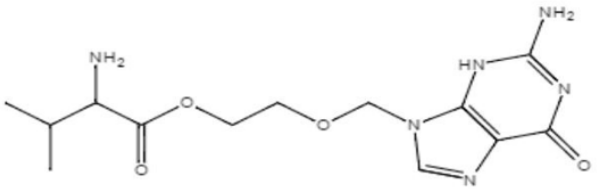

Fig. 1: Structure of valacyclovir

2. Experimental

2.1. Apparatus

The two LC systems, used for method development and validation were (i) The Agilent 1100 series (Agilent Technologies Inc., USA) connected to Variable Wavelength Detector (UV-Vis). The output signal was monitored and processed using chemstation software. (ii) Shimadzu LC-10AT (Shimadzu Corporation, Japan) connected to Variable Wavelength Detector (UV-Vis). Lab Solutions software was used for data acquisition and system suitability calculations. Metrohm Digital pH Meter, model 780 was used for the pH adjustments. The reproducibility of the measurements is within 0.01 pH. Mettler Toledo XP6 Excellence Plus XP Micro Balance having maximum capacity of 6.1 g, sensitivity of ± 0.01 mg was used for standard and sample weighing. Sartorius BS/BT 2245 model electronic analytical balance having maximum capacity of 220 g, sensitivity of ± 0.1 mg was used for chemicals weighing purpose.

2.2. Chemicals & reagents

Valacyclovir (API) gift sample was obtained from Cipla Limited India. HPLC grade of Citric Acid was obtained from Merck specialties Ltd, India. HPLC grade of Water and Methanol was obtained from Rankem Limited, India. Market samples of Valcivir 500 mg tablets of Valacyclovir were kindly supplied by Cipla Limited.

2.3. Chromatographic conditions

The column used for separation of analyte was Gracesmart RP18, C18 (250 mm×4.6 mm, 5μ). The mobile phase was composed of citrate buffer 0.005 M (pH=3) and Methanol (40: 60 v/v). This solution was filtered using a 0.45 micron Millipore filter paper membrane filter under vacuum filtration and pumped at ambient temperature, at a flow rate of 1.0 mL/min with UV detection wavelength at 254 nm.

2.4. Chromatographic parameters

Equipment: SHIMADZU LC-10ATvp Series HPLC system with UV detector Column: Grace Smart RP18 C18 (250 mm × 4.6 mm, 5μ)

Flow rate: 1.0 mL/minute.

Wavelength: 254 nm Injection volume: 20 μL

Column Oven Temperature: Ambient Run time: 10 Minutes.

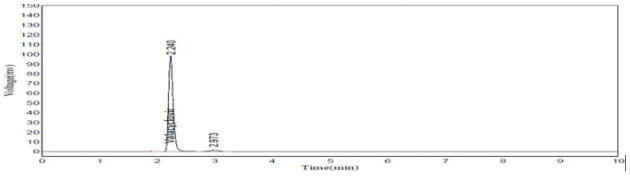

Fig. 2: Typical chromatogram of blank injection at 254 nm.

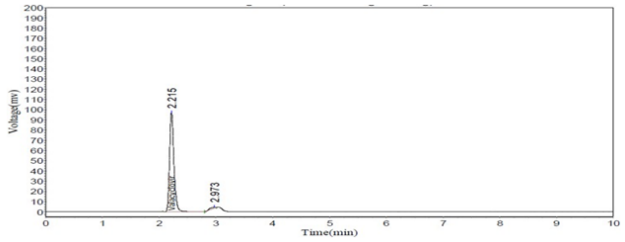

Fig. 3: Typical chromatogram of valacyclovir standard solutionat 254 nm.

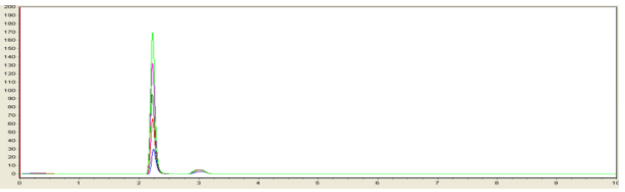

Fig. 4: Typical chromatogram of valacyclovir sample solution at 254 nm.

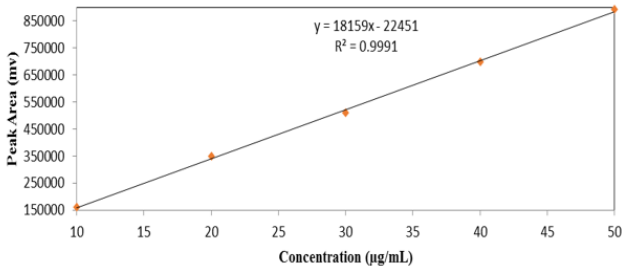

Fig. 5: Overlay zoom chromatogram of valacyclovir (different aliquots 10 to 50 μg/mL) at 254 nm.

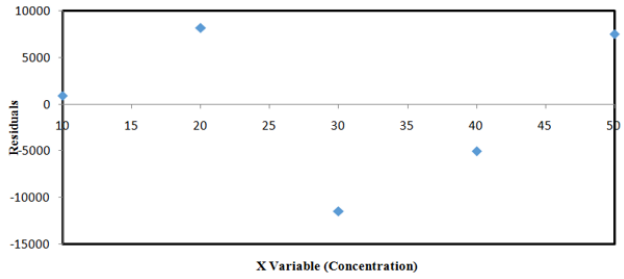

Fig. 6: Calibration curve of Valacyclovir.

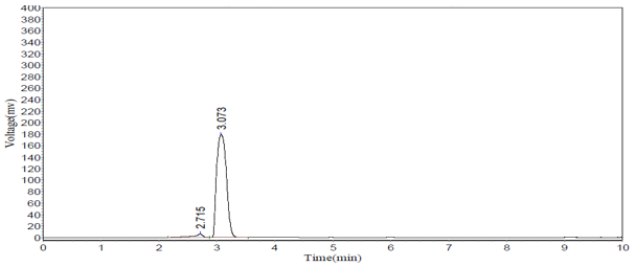

Fig. 7: A typical chromatogram representing acid hydrolysis degradation behavior of Valacyclovir.

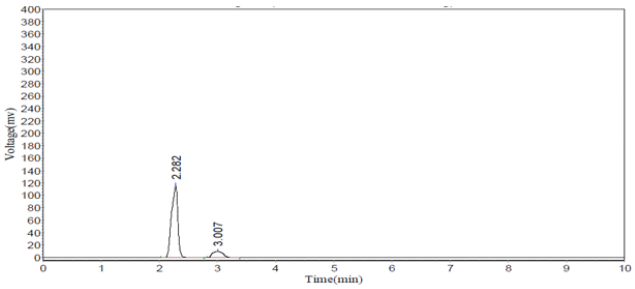

Fig. 8: A typical chromatogram representing alkaline hydrolysis degradation behavior of Valacyclovir.

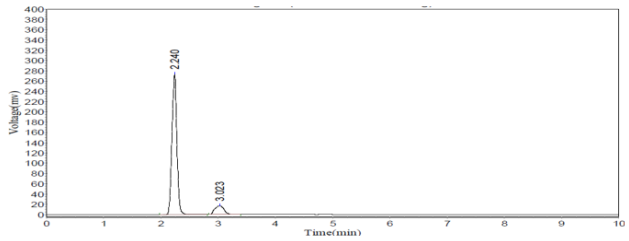

Fig. 9: A typicalchromatogram representing oxidative degradation behavior of Valacyclovir.

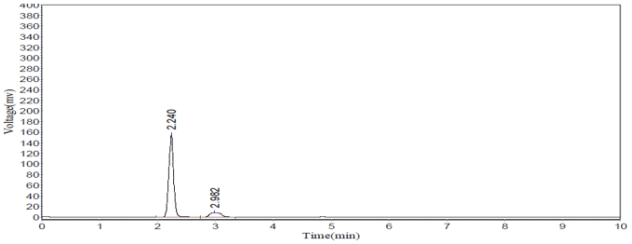

Fig. 10: A typical chromatogram representing irradiation with UV light degradation behavior of Valacyclovir.

3. Solutions and Sample Preparation

3.1. Preparation of citrate buffer

A 0.005 M sodium salt of citric acid buffer was prepared by dissolving 0.96 gm of citric acid in 1000 mL of HPLC grade water and pH was adjusted to 3.0 with 1.0 N orthophosphoric acid. The buffer was filtered through 0.45μ Millipore filter paper to remove all fine particles and gases.

3.2. Preparation of mobile phase

Mobile phase was prepared by mixing 40% of citrate buffer and 60% of Methanol HPLC grade (v/v) and filtered through 0.45μ Millipore filter paper and degassed by Sonication.

3.3. Preparation of diluent

Methanol was used as diluent.

3.4. Preparation of standard stock solution (1000 µg/mL)

Standard stock solution was prepared by dissolving 50 mg of Valacyclovir in 50 mL of Methanol taken in a clean and dry 50-mL volumetric flask and diluted up to the volume with Methanol (the concentration of resulting solution is 1000 μg/mL) and sonicated for 8 mins, filtered using 0.45 μ Millipore filter paper.

3.5. Preparation of standard working solution for assay (100 µg/mL)

Above Standard stock solution of 1000 μg/mL of Valacyclovir, further dilution to 10 folds (1.0 mL of stock solution transferred into a 10 mL volumetric flask and diluted up to mark with Methanol to get 100 μg/mL Valacyclovir. All other further dilutions were carried out using the working solution (100 μg/mL Valacyclovir) for the method development and validation parameters.

3.6. Preparation of sample solutions

10 tablets were taken in motor and crushed finely. Tablet powder equivalent to 50 mg Valacyclovir were taken in 50

mL clean and dry volumetric flask, few mL of diluents was added and sonicated to dissolve it completely and volume was made up to mark with Methanol. Resulting solution was sonicated for 8 min then the solution was filtered using 0.45μ Millipore filter paper. Further pipette out 300 μL from the above Valacyclovir sample stock solution into a 10 mL volumetric flask and diluted up to the mark with Methanol to get the 30 μg/mL concentration.

3.7. Procedure

Standard and sample solution were injected (20 μL) into the chromatographic system and the peak areas were measured for Valacyclovir and the % assay was calculated by comparing the peak area of standard and sample chromatogram by using the formula given below.

$$Assay\ \% = \frac{AT}{AS}\ X\ \frac{WS}{WT}\ X\ \frac{DT}{DS}\ X\ \frac{P}{100}\ X\ \frac{Avg.WT}{Label\ Claim}\ X100$$

Where,

AT = Average peak area of Test or Sample preparation, AS= Average peak area of standard preparation,

WS = Weight of the standard taken in mg,

WT=Weight of the Test or sample taken in mg,

P = Percentage purity of working standard,

DS= Dilution factor for standard preparation, DT=Dilution factor for sample preparation.

4. Force degradation studies

To assess the stability indicating property of the developed HPLC method stress studies were carried out under ICH recommended conditions. Forced degradation of Valacyclovir was carried out by exposing the bulk sample to acidic, alkaline, oxidative, photolytic, dry heat and neutral conditions. The aim was to study the ability of the proposed method to measure the analyte response in presence of its degradation products.

4.1. Acid and alkali hydrolysis

Aliquot of 1.0 mL of Valacyclovir solution (1000 μg/mL) was transferred to a small round bottom flask. The solution was mixed with 9 mL of 0.1N Hydrochloric Acid or 0.1 N Sodium Hydroxide. The prepared solutions were maintained at 35 °C for 48 h. The samples were cooled to room temperature (25 ℃), neutralized with an amount of acid or base equivalent to that of the previously added. Diluted with methanol and filtered through a 0.45 μm membrane.

4.2. Oxidation

Aliquot of 1.0 mL of Valacyclovir solution (1000 μg/mL) was transferred to a small round bottom flask. The contents were then mixed with 9 mL of 30% hydrogen peroxide solution, and the reaction mixture was maintained at 35 °C with intermittent shaking for 48 h. The samples were cooled to room temperature (25 ℃). Diluted the sample with methanol and filtered through a 0.45 μm membrane.

4.3. Irradiation with UV light

Valacyclovir sample was exposed to UV light (365 nm) for 48 h. The material was dissolved in 10 mL Methanol. The stressed sample was suitably diluted with methanol and filtered through a 0.45 μm membrane.

4.4. Thermal degradation

Valacyclovir sample was exposed to a temperature of 70℃ for 48 h in a refluxing apparatus. The stressed sample was suitably diluted with methanol and filtered through a 0.45 μm membrane.

5. Results and Discussion

5.1. Method development

Reverse phase liquid chromatography method was tried to develop using various ratios of Methanol and Buffer as mobile phase. To improve the peak shape and tailing factor the pH ofmobile phase becomes important factor. Improved peak shape and separation was achieved at wasratio and flowrate of 1.0mL/min was employed. Gracesmart C18 column 250 mm×4.6mm, 5μ particlesize wass electedas the stationary phase to improve separation and the Valacyclovir 254 nm of wave length; therefore 254 nm was selected as the detection wavelength. The retention time was found to be2.24+0.1min with plate count and tailing factor as 3847 Valacyclovir were shown in Figure 2, Figure 3 and Figure 4 respectively.

5.2. Analytical method validation

As per ICH Q2A and Q2B guidelines (Validation of Analytical Procedures: Text and Methodology Q2 (R1), Geneva, 2005) the developed method was validated for different parameters like System Suitability, precision (Intra and Inter-day), linearity, accuracy, robustness, Limit of detection (LOD) and Limit of Quantification (LOQ).[24]

5.3. System suitability

At first the HPLC system was optimized as per the chromatographic conditions. One blank followed by a single calibration standard solution of 30 μg/mL (100% Test Concentration) of Valacyclovir was injected to check the system suitability. To ascertain the system suitability for

the proposed method, the parameters such as retention time, capacity factor, plate count, peak asymmetry were taken and results were presented in Table 1.

Table 1: System suitability parameters for valacyclovir

Parameter (n = 6)	System Suitability Results
Retention Time (minutes)	2.24 ±0.1
Capacity Factor (k)	2.2
Plate Count (N)	3847
Peak asymmetry (Tailing or Symmetry Factor)	1.24

5.4. Precision

Precision is a measure of the degree of repeatability of the analytical method. Precision of an analytical method is usually expressed as the standard deviation. Precision is determined by injecting six replicates of a single calibration standard solution of 30 μg/mL concentration. The intra-day and inter-day precision studies (intermediate precision) were carried out by estimating the corresponding responses on the same day and on other day for same concentration. The results are reported in terms of relative standard deviation of Peak Area, Retention time and Height were presented in Table 2.

Table 2: Intraday and inter-day precision data for valacyclovir

Parameters	Intra Day (Day-1) 1^{st} System,1^{st} Columnand 1^{st} Analyst	Inter Day (Day-2) 2^{nd} System, 2^{nd} Columnand 2^{nd} Analyst
Capacity Factor (k)	2.2	2.2
Plate Count (N)	3847	4075
Peak asymmetry (Tailing Factor)	1.24	1.28
% RSD of Retention Time (min) (n = 6)	0	0
% RSD of Peak Area (n = 6)	0.81	0.89
% RSD of Peak Height(n = 6)	0.86	0.87

5.5. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of the analyte.[24–26] 20 μL of each calibration standard solutions containing 10, 20, 30, 40 and 50 μg/mL of Valacyclovir was injected and chromatograms were recorded at 254 nm. A linear regression equation y = 17839x - 10691 and regression line was established by least squares method and correlation coefficient (r^2) for Valacyclovir is found to be greater than 0.999. The overlay chromatogram of different aliquots (10 to 50 μg/mL) of Valacyclovir was shown in Figure 5. Calibration Curve and residual plot obtained for Valacyclovir was shown in Figure 6 and Figure 7 respectively. Linearity data and Analysis of variance (ANOVA) were tabulated in Table 3.

Table 3: Linearity data and analysis of variance (ANOVA) data of Valacyclovir

Statistical Parameter	Results
Slope	17839
Intercept	10691
Correlation Coefficient	0.999
Regression Sum of Squares	329762079708
Residual Sum of Squares	283793078
Total Sum of Squares	330045872787

6. Accuracy or (% Recovery)

To pre-analysed sample solution, a definite concentration of standard drug (80%, 100% & 120 % level) was added and recovery was studied. 80% and 120% levels were prepared by considering 30 μg/mL concentrations as 100%. The % Mean recovery for Valacyclovir is 101.72 and these results are within acceptable limit of 98 - 102. The % RSD for Valacyclovir was 0.72 is within limit of ≤2. Hence, the proposed method is accurate and the % recovery results were summarized in Table 4.

Table 4: Recovery study data of Valacyclovir

Sample Name	Amount of Drug Concentration (μg/mL) Spiked	Found	% Recovery
S1:80%	24	24.29	101.21
S2:80%	24	23.96	99.85
S3:80%	24	24.29	101.21
S4:100%	30	30.19	100.62
S5:100%	30	29.98	99.93
S6:100%	30	29.98	99.93
S7:120%	36	36.60	101.66
S8:120%	36	36.14	100.38
S9:120%	36	36.60	101.66
		Mean	101.72
		Standard Deviation	0.74
		% RSD	0.73

6.1. Robustness

The robustness was established by changing the flow rate, pH, and Column temperature and mobile phase composition within allowable limits from actual chromatographic

conditions. The obtained results (Table 5) shows that there were no marked change in mean retention time (Rt), Assay and RSD is within limit of ≤ 2 .The tailing factor and plate count were found to be in acceptable limits. Hence, the method is reliable with variations in the analytical conditions and the results of Valacyclovir are shown in Table 5.

6.2. Stability of standard sample solution and mobile phase

Established the stability of standard, sample solution and mobile phase which was used in estimation of Valacyclovir over a period of 3 days. The sample and standard solutions injected at initial time, 24hr and 48 (stability sample) by keeping at controlled room temperature 25°C. Prepared the mobile phase as per the test method and kept it in well-closed condition, prepared the standard and sample solution, injected in chromatography and evaluated the system suitability parameters on each day. The system suitability results were shown in Table 6. RSD results are within limit of ≤2 and hence the sample and standard stock are stable for 48 hr at Controlled room temperature.

6.3. Limit of detection (LOD) and limit of quantification (LOQ)

Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated as 3.3×SD/S and 10×SD/S respectively as per ICH guidelines, Where SD is the standard deviation of the response (Y-intercept) and S is the slope of the calibration curve. The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The LOQ is the smallest concentration of the analyte which gives response that can be accurately quantified (signal to noise ratio of 10). The LOD and LOQ were obtained as 0.0029 μg/mL and 0.0087 μg/mL respectively. The lowest values of LOD and LOQ as obtained by the proposed method indicate that the method is sensitives.

6.4. Tablet analysis

The Content of Valacyclovir in the Valcivir 30 mg tablets was determined by the proposed method. RSD results 0.30 % is within limit of ≤2 and the results were presented in Table 7.

6.5. Specificity

The HPLC chromatograms of Valacyclovir were recorded by injecting the 20 μL of each resultant solution of acid hydrolysis, Base hydrolysis, oxidative degradation, irradiation with UV & thermal degradation samples into the HPLC system and shown in Figure 8 respectively. The Retention time and the % of Assay for the Valacyclovir were tabulated in Table 8. The results of stress testing studies reveals that, the proposed method has the ability to separate the analyte from its degradation products indicated a high degree of specificity of this method.

6.6. Conclusions

The method for the determination of Valacyclovir by High Performance Liquid Chromatography using Variable wavelength detector (UV) was developed and validated as per the ICH guidelines. Linearity was achieved in the range of 10 to 50 μg/mL, with the correlation co-efficient (r2 = 0.999). The percentage RSD (0.87) was within the acceptance criteria not more than 2. The percentage recovery was achieved in the range of 98-102%, which was within the acceptance criteria. The simplicity and short run time (10 minutes) enables through output analysis of Valacyclovir. The standard solutions and mobile phase are found to be stable up to 48 hours at controlled room temperature. Forced degradation studies revealed that possible degradation products do not interfere with the determination of Valacyclovir. The method is proved as accurate, precise, robust and rugged. Hence the method can be used for the regular and stability analysis.

7. Source of Funding

None.

8. Conflict of Interest

None.

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Table 5: Results of robustness for Valacyclovir.

Parameter	Analytical Condition							
	Flow Rate (mL/min)		Effect of pH		ColumnTemperature (°C)		Mobile phaseComposition (%)	
	0.98	1.02	2.9	3.1	33	37	-10	+ 10%
Mean Rt*	2.26	2.17	2.22	2.22	2.23	2.22	2.16	2.28
Mean Assay*	99.19	98.38	98.38	98.58	100.25	98.15	100.20	98.46
Std. Dev	0.59	0.59	0.21	0.27	0.65	0.35	0.65	0.68
% RSD	0.60	0.60	0.21	0.27	0.65	0.36	0.65	0.69
Tailing factor	1.27	1.41	1.27	1.27	1.28	1.27	1.22	1.40
Theoretical Plates	4062	3893	3913	3913	3790	3762	3893	4062

*Average of three determinations.

Table 6: Solution stability results for Valacyclovir

System Suitability Parameter	Results		
	Initial (0 hrs.)	24 hrs.	48 hrs.
% RSD of Area (n = 3)	0.96	0.67	0.73
Pale Count	3847	4075	4246
Tailing Factor	1.24	1.28	1.28

Table 7: Tablet analysis results of Valacyclovir

Name of the Formulation & label claim	Equivalent amount	Assay Found (%) (n = 3)	% RSD
Valcivir	30 mg	100.81	0.30

Table 8: Summary of forced degradation results of valacyclovir

Parameters	Retention time	Assay (%)
Acid Hydrolysis	2.65	40.85
Base Hydrolysis	3.07	96.83
Oxidation	2.28	87.74
Irradiation with UV light	2.24	89.51
Thermal degradation	2.24	89.05

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Author biography

Shoheb Shakil Shaikh, Assistant Professor

V. K. Padri, Assistant Professor

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