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Development and Validation of Stability Indicating RP-Liquid Chromatographic Method for the Quantitative Determination of Valganciclovir

Sreenivasulu Sura[1*], Rameswara Rao Modalavalasa[2], Chandra Sekhar KB[3]

[1]Department of Chemistry, Research Scholar, Jawaharlal Nehru Technological University Anantapur, Ananthapuramu. 515002, A.P., India
[2]Department of Quality Control, Chemical Technical Operations, Dr. Reddy's Laboratories Ltd., IDA Bollaram-502325, Hyderabad, Telangana, India
[3]Department of Chemistry, Oil Technological & Pharmaceutical Research Institute, Jawaharlal Nehru Technological University, Anantapur, Ananthapuramu -515001, A.P., India

ABSTRACT

A new simple stability indicating and user friendly RP-Liquid Chromatographic method was developed for the accurate and precise determination of Valganciclovir using Phenomenex Gemini-NX C18 Column (250 mm Length × 4.6 mm diameter × 5 µm particle size) and variable wavelength (UV) detector. The mobile phase used for the study was sodium Citrate buffer:Methanol in a ratio of 40:60 v/v with a flow rate of 1.0 ml/min. Valganciclovir was detected at wavelength 254 nm and eluted at 2.18 ± 0.5 min. The developed method is linear over 10-80 µg/ml concentration range and correlation co-efficient (r^2) obtained as 0.9996. The tailing factor and plate count was found 1.32 and 3649 respectively. The proposed method was proven as robust after its extensive validation, which involves various parameters such as Precision, Linearity, Accuracy, Ruggedness, Robustness, Limit of Detection (LOD), Limit of Quantification (LOQ) and specificity. The developed and validated method is definite and reproducible, can be used for routine analysis of Valganciclovir in bulk form.

Keywords: Valganciclovir, C18 column, Methanol:citrate buffer (60:40), Specificity

INTRODUCTION

IUPAC name of valganciclovir is [2-[(2-amino-6-oxo-3H-purin-9-yl) methoxy]-3-hydroxypropyl] 2-amino-3-methylbutanoate (Figure 1). Valganaciclovir is a white to off-white crystalline powder with molecular formula $C_{14}H_{22}N_6O_5$ and molecular weight of 354.367 g/mol. Valgancyclovir is the L-valyl ester i.e., pro-drug of gancyclovir. It is an antiviral medication used to treat cytomegalovirus infections. The mechanism of action of valganciclovir is that it is a pro-drug of gancyclovir that exists as a mixture of two dia-stereomers. After administration, these dia-stereomers are rapidly converted to gancyclovir by hepatic and intestinal esterase. Gancyclovir inhibits replication of viruses, including Cytomegalovirus (CMV) and Herpes Simplex Virus (HSV). Gancyclovir terminates the viral DNA elongation by incorporating into viral DNA, resulting inhibition of viral replication [1].

Few literature references are available related to analysis or analytical methods such as UV spectrophotometric method for the assay of valganciclovir in bulk drugs and formulation [2,3]. Electrochemical sensor based on electro reduced graphene oxide for the determination of valganciclovir [4], determination of valganciclovir and its metabolite gancyclovir in plasma samples by High Performance Liquid Chromatography (HPLC) method, [5,6] Liquid Chromatography–Mass Spectrometry (LC-MS) [7-9] and reactivity of Valganciclovir in aqueous solution [10], a HPLC method for dia-stereomers ratio of Valganciclovir [11,12], HPLC method for the chiral purity determination of "(S)-2-azido-3-methylbutanoic acid, a key raw material of valganciclovir" [13], residual determination of Valganciclovir Hydrochloride on stainless steel surface of Pharmaceutical manufacturing equipment's [14] and analytical methods for determination of Valganciclovir in dosage forms [15,16]. The objective of the work is to develop and validate a UV method for its estimation in bulk and formulated dosage form with good precession, accuracy, economy and simplicity. The proposed method was validated as per recommendations of the International Conference on Harmonization (ICH) [17] for the determination of valganciclovir in bulk and formulated dosage forms.

Apparatus

The two LC systems, used for method development studies and validation were (i) The Agilent 1100 series (Agilent Technologies Inc., USA) connected to Variable Wavelength (UV-Vis) detector. Chemstation software was used for data acquisition and system suitability calculations.

(ii) Shimadzu LC-10AT (Shimadzu Corporation, Japan) connected to Variable Wavelength Detector (UV-Vis). The output signal was monitored and processed using Lab Solutions software (Version). 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 model electronic micro balance having maximum capacity of 6.1 g, sensitivity of ± 0.01 mg was used for standard and sample weighing. For chemicals weighing purpose Sartorius BS/BT 2245 model electronic analytical balance having maximum capacity of 220 g, sensitivity of ± 0.1 mg was used.

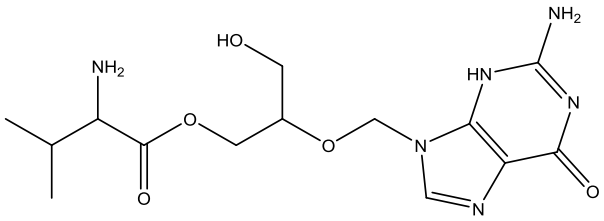

Figure 1: Structure of valganciclovir

MATERIALS AND METHODS

Chemicals and reagents

Valganciclovir (API) gift sample was obtained from Aurobindo Pharma Limited, India. HPLC grade of Methanol was obtained from Rankem Limited, India. HPLC grade of Sodium Citrate was obtained from Merck specialties Ltd, India. HPLC grade Water and HPLC grade Methanol was obtained from Rankem Limited, India. Market samples of Valacept 450 mg tablets of Valganciclovir were kindly supplied by Cipla Ltd.

Chromatographic conditions

The chromatographic column used for the separation of analyte was Phenomenex Gemini-NX C18 Column (250 mm length × 4.6 mm diameter, 5 µ particle size). The mobile phase composed of sodium citrate buffer 0.005 M, pH=3.0 and Methanol in the ratio of 40:60 v/v. Prepared mobile phase was filtered using a 0.45 micron millipore membrane filter paper under vacuum filtration and pumped at ambient temperature, using a variable wave length (UV) detector at wavelength 254 nm and with a flow rate of 1.0 ml/min.

Chromatographic parameters

Equipment	Shimadzu LC-10A Tvp series HPLC system with UV detector
Column	Phenomenex Gemini-NX C18 Column (250 mm × 4.6 mm × 5 µ)
Flow rate	1.0 ml/min
Wavelength	254 nm
Injection volume	20 µl
Column oven Temperature	Ambient
Run time	10 min

Solutions and sample preparation

Preparation of buffer

A 0.005 mM sodium citrate buffer was prepared by dissolving 0.96 g of sodium salt of citric acid in 1000 ml of HPLC grade water and pH was adjusted to 3.0 with 1.0 N ortho-phosphoric acid. The prepared buffer was filtered through 0.45 µ millipore membrane filter paper to remove fine particles and gases.

Preparation of mobile phase

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

Preparation of diluents

Directly, methanol used as diluent.

Preparation of standard stock solution (1000 µg/ml)

Standard stock solution was prepared by dissolving 50 mg of valganciclovir in 50 ml of methanol taken in a 50 ml dry and clean volumetric flask and diluted up to the volume with methanol (the concentration of resulting solution is 1000 µg/ml) and sonicated for 5 min (s), filtered using 0.45 µ millipore membrane filter paper.

Preparation of standard working solution for assay (100 µg/ml)

Above Standard stock solution (1000 µg/ml solution) of valganciclovir, further diluted 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 valganciclovir). All other further dilutions were carried out using the working solution (i.e., 100 µg/ml valganciclovir) for the method development and validation parameters.

Preparation of sample solutions

In a motor, 5 tablets were taken and crushed finely. Tablet powder equivalent to 100 mg valganciclovir was taken in 100 ml dry and clean volumetric flask, to it a few ml of diluent was added, dissolved by sonication and made up to volume with methanol. Resulting solution was

sonicated for 5 min then the solution was filtered using 0.45 µ millipore membrane filter paper. Further pipette out 400 µl from the above Valganciclovir sample stock solution into a 10 ml volumetric flask and diluted up to the mark with Methanol to get the 40 µg/ml concentration.

Procedure

20 µl of each blank, standard and sample solution were injected into the chromatographic system. Peak areas were measured for Valganciclovir and the percentage assay was calculated by comparing the peak areas of standard and sample, obtained from the respective chromatograms using below formula.

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

Where: AT=Average peak area of principle peak from test or sample chromatogram, AS=Average peak area of principle peak from standard chromatogram, WS=Amount of the standard taken in mg, WT=Amount of the Test or sample taken in mg, P=Percentage purity of standard, DS=Dilution factor for standard preparation, DT=Dilution factor for sample preparation.

Force degradation studies

Stress studies were carried out under ICH recommended conditions to assess the stability indicating property of the developed HPLC method. Forced degradation of valganciclovir was carried out by exposing the bulk sample to acidic hydrolysis, alkaline hydrolysis and oxidative, photolytic, thermal and neutral conditions. The purpose of the study is to determine the ability of the proposed method to measure the analyte response in presence of its degradation products.

Acid and alkali hydrolysis

Aliquot of 1.0 ml of valganciclovir solution (1000 µg/ml) was transferred into a small round bottom flask. To it 9.0 ml of 0.1 N HCl or 0.1 N NaOH was added and mixed well. The prepared solutions were maintained at 35°C for 48 h. The samples were cooled to room temperature (25°C), neutralized by adding an equal amount of base or acid. Diluted with methanol and filtered through a 0.45 µm membrane filter.

Oxidation

Aliquot of 1.0 ml of valganciclovir solution (1000 µg/ml) was transferred to a small round bottom flask. To the contents in flask, 9.0 ml of 30% hydrogen peroxide solution were added and the reaction mixture was maintained at 35°C with intermittent shaking for 48 hr. The samples were cooled to room temperature (25°C). Diluted the sample with methanol and filtered through a 0.45 µm membrane filter.

Irradiation with UV light

Valganciclovir sample was exposed to ultra violet light (wavelength=365 nm) for 48 h. The sample was dissolved in 10 ml methanol. Resultant sample solution was suitably diluted with methanol and filtered through a 0.45 µm membrane filter.

Thermal degradation

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

RESULTS AND DISCUSSION

Method development

Various ratios of Methanol and buffer as mobile phase were tried to develop a simple reverse phase liquid chromatography method. pH of mobile phase becomes important factor to improve the peak shape and tailing factor. Improved peak shape and separation was achieved at pH=3.0 with sodium citrate buffer. There after sodium citrate buffer and methanol were taken in isocratic ratio (40:60 v/v) and flow rate of 1.0 ml/min was employed. Phenomenex Gemini-NX C18 Column 250 mm length × 4.6 mm diameter, 5 µ particle size was selected as the stationary phase to improve separation and the tailing of the peak was reduced considerably. Valganciclovir showed maximum absorption at wavelength 254 nm. Therefore 254 nm was kept constant as the detection wavelength throughout the study. The retention time was found to be 2.18 ± 0.1 min with tailing factor and plate count as 1.32 and 3649 respectively. The resulting chromatograms of blank, Valganciclovir standard and sample were shown in Figures 2-4 respectively.

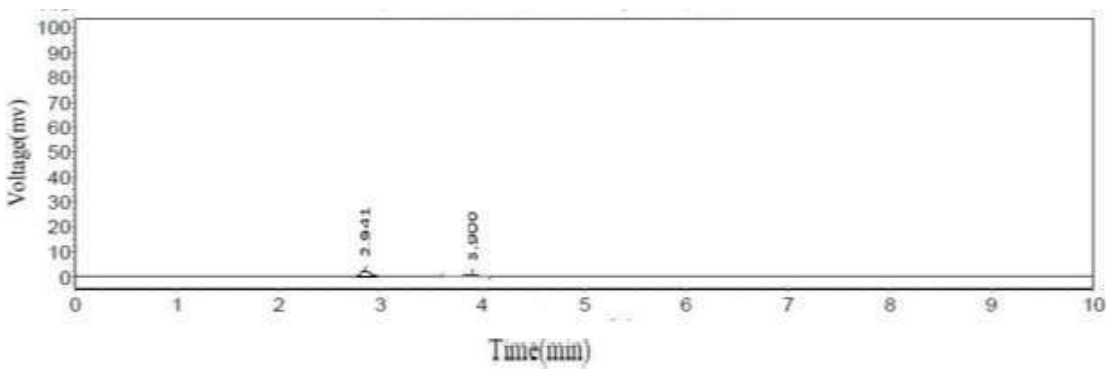

Figure 2: Typical chromatogram of blank injection at 254 nm

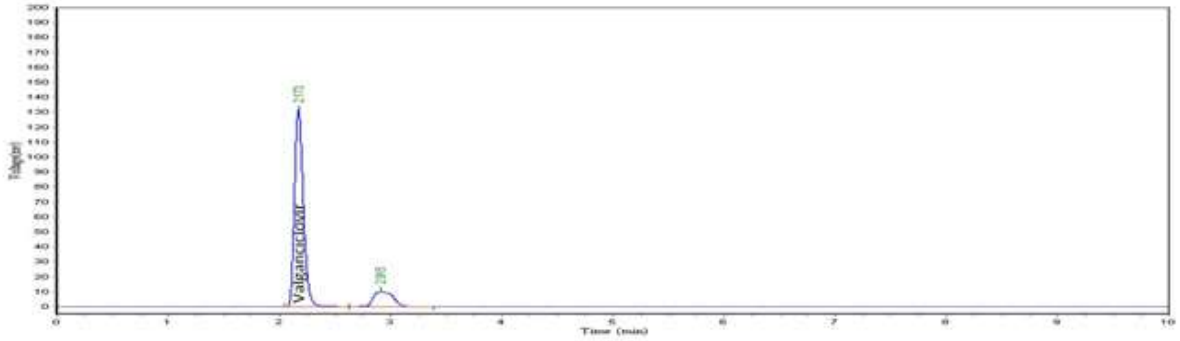

Figure 3: Typical chromatogram of valganciclovir standard solution at 254 nm

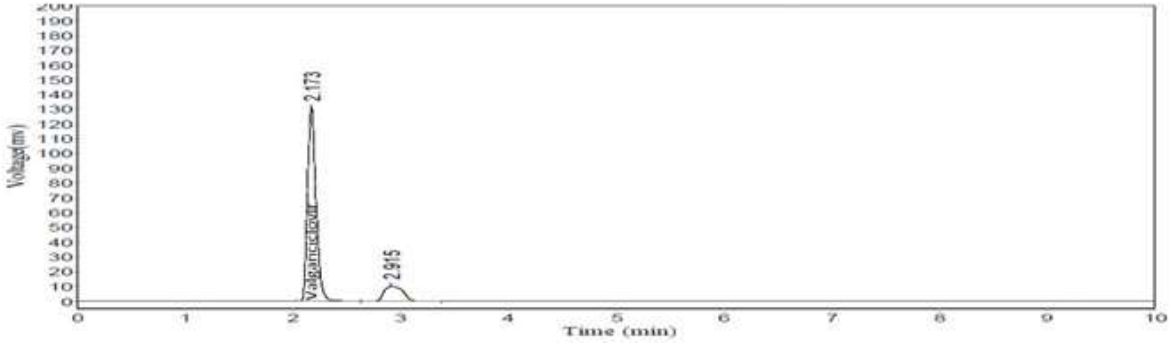

Figure 4: Typical chromatogram of valganciclovir sample solution at 254 nm

Analytical method validation

The developed Analytical method was validated with different parameters like system suitability, precision (intra and inter-day), linearity, accuracy, robustness, Limit of Detection (LOD) and Limit of Quantification (LOQ) [18] as per ICH Q2A and Q2B guidelines ("Validation of Analytical Procedures: Text and Methodology Q2 (R1)", Geneva, 2005) [17].

System suitability

HPLC system was optimized as per the chromatographic conditions. One blank injection was injected followed by a single calibration standard solution of 40 µg/ml (100% test concentration) of valganciclovir was to check the system suitability. The parameters such as retention time, capacity factor, plate count and peak asymmetry (Tailing factor) were taken to ascertain the system suitability for the proposed method and results were presented in Table 1.

Table 1: System suitability parameters for valganciclovir

Parameter (n=6)	System suitability results
Retention Time (min)	2.18 ± 0.1
Capacity Factor (k)	2.1
Plate Count (N)	3649
Peak asymmetry (Tailing or Symmetry Factor)	1.32

Precision

Precision of the developed method is determined by injecting six replicates of a single calibration standard solution of 40 µg/ml concentration. Precision is a measure of the degree of repeatability of the analytical method. Precision of an analytical method is usually expressed in terms of standard deviation. The intra-day and inter-day precision studies (intermediate precision) were carried out by estimating the corresponding responses on the same day and on different day for same concentration. The results are reported in terms of percentage relative standard deviation of retention time, peak area and height were presented in Table 2.

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

Parameter	Intraday (Day-1) 1st system, 1st column and 1st analyst	Interday (Day-2) 2nd system, 2nd column and 2nd analyst
Capacity Factor (k)	2.1	2.2
Plate Count (N)	3649	3649
Peak asymmetry (Tailing factor)	1.32	1.36
%RSD of retention time (min) (n=6)	0	0
%RSD of Peak Area (n=6)	0	0.00
%RSD of Peak Height (n=6)	0.76	1.04

Linearity

The linearity of an analytical method is to determine the ability (within a given range) of method to obtain test results which are directly proportional to the analyte concentration [17-22]. 20 µl of each calibration standard solutions containing 10, 20, 40, 60 and 80 µg/ml valganciclovir was injected and chromatograms were recorded at 254 nm. Regression line was established by least squares method and a linear regression equation obtained as Y=17062, X=+11674. Correlation coefficient (r^2) for valganciclovir is found to be 0.9996. The comparison overlay chromatograms of different concentrations (10-80 µg/ml) of valganciclovir was shown in Figure 5. Calibration curve and residual plot obtained for valganciclovir were shown in Figures 6 and 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 valganciclovir

Statistical parameter	Results
Slope	17062
Intercept	11674
Correlation coefficient	0.9996
Regression Sum of Squares	954835032740
Residual Sum of Squares	777479957
Total Sum of Squares	955612512696

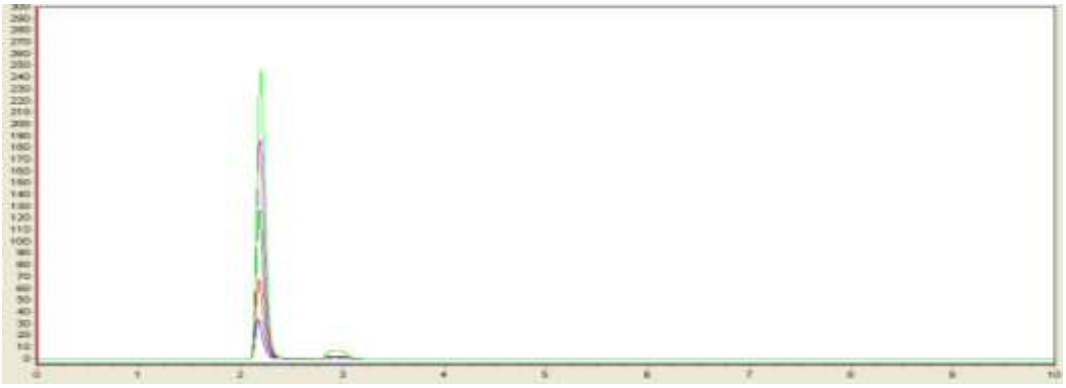

Figure 5: Overlay zoom chromatogram of valganciclovir (different aliquots 10-80 µg/ml) at 254 nm

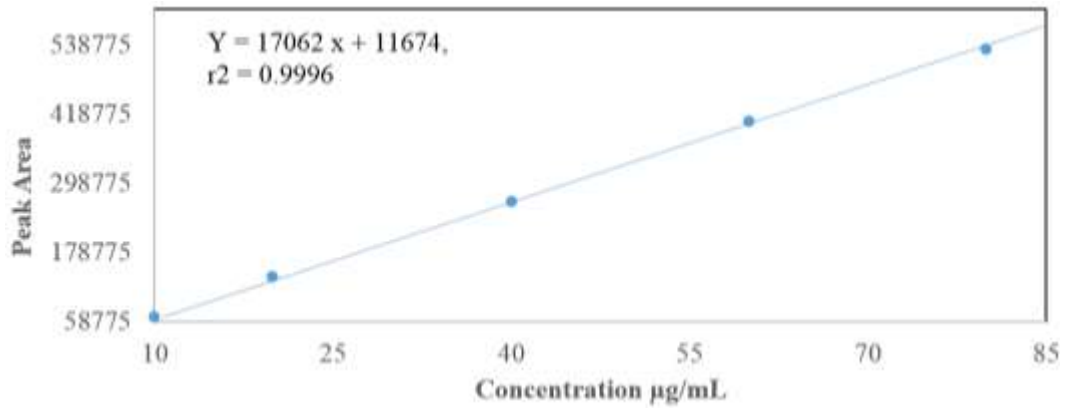

Figure 6: Calibration curve of valganciclovir

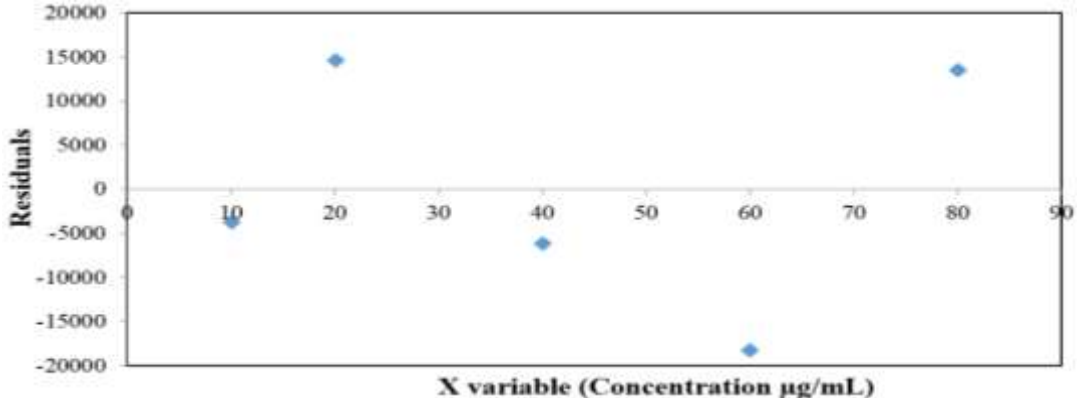

Figure 7: Residual plot of valganciclovir

Accuracy or (%Recovery)

A definite concentration of standard drug (80%, 100% and 120% level) were added to pre-analysed sample solution and the percentage recovery was studied. 80% and 120% levels were prepared by considering 40 µg/ml concentrations as 100%. The Mean % recovery for valganciclovir was 99.50%, which is within acceptable limit of 98-102. The % RSD for Valganciclovir was 0.46 which is within acceptance limit of ≤ 2. Hence, the developed analytical method is accurate and the %recovery results were tabulated in Table 4.

Table 4: Recovery study data of valganciclovir

Sample name	Amount of drug concentration (µg/ml)		%Recovery
	Spiked	Found	
S1: 80%	32	31.82	99.43
S2: 80%	32	31.60	98.75
S3: 80%	32	31.64	98.88
S4: 100%	40	39.93	99.84
S5: 100%	40	40.00	100.00
S6: 100%	40	40.03	100.09
S7: 120%	48	47.84	99.66
S8: 120%	48	47.71	99.39
S9: 120%	48	47.74	99.46
Mean			99.50
Std. Dev.			0.46
%RSD			0.46

Robustness

The robustness of proposed method was established by varying the flow rate, pH, and column temperature and mobile phase composition within allowable limits from established chromatographic conditions. The obtained results (Table 5) shows that Assay result of the samples are within acceptable limit of ≥ 90, also there were no obvious change in mean Retention Time (Rt), and %RSD obtained for assay results is within acceptable limit of ≤ 2. The plate count and tailing factor were found to be in acceptable limits. Hence, the method is consistent with variations in the analytical conditions and the results of valganciclovir are shown in Table 5.

Table 5: Results of robustness for valganciclovir

Parameter	Analytical condition							
	Flow rate (ml/min)		Effect of pH		Column temperature (°C)		Mobile phase composition (%)	
	0.98	**1.02**	**3.4**	**3.6**	**22**	**33**	**-10%**	**+ 10%**
Mean RT*	2.27	2.14	2.19	2.20	2.18	2.24	2.18	2.18
Mean Assay*	96.00	93.33	92.20	93.68	93.42	95.97	94.56	93.52
Std. Dev.	0.92	0.81	1.05	1.23	1.02	0.45	0.64	0.40
%RSD	0.96	0.87	1.14	1.31	1.10	0.46	0.68	0.42
Tailing factor	1.34	1.34	1.31	1.31	1.35	1.35	1.37	1.33
Theoretical Factor	3669	3653	3537	3330	3511	3701	3650	3650

*Average of three determinations

Stability of mobile phase, standard and sample solution

Established the stability of mobile phase standard and sample solution, which were used in estimation of valganciclovir over a period of 3 days. The standard and sample solutions injected at initial time, 24 h and 48 h (stability sample) by storing 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 given in Table 6. The %RSD results are within acceptance limit of ≤ 2. Hence, the standard stock and sample solutions are stable for 48 h at controlled room temperature.

Table 6: Solution stability results for valganciclovir

System suitability parameter	Results		
	Initial (0 h)	24 h	48 h
%RSD of area (n=3)	0.83	0.53	0.47
Pale count	3650	3650	3650
Tailing factor	1.37	1.33	1.33

LOD and LOQ

LOD and LOQ were calculated based on the ICH guidelines as 3.3 × SD/S and 10 × SD/S respectively. Where, 'S' refers to the slope of the calibration curve and 'SD' refers to the standard deviation of the response (Y-intercept). 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 0.006 µg/ml and 0.055 µg/ml respectively. By using the proposed method lowest values for LOD and LOQ were obtained, which indicates that the method is sensitive.

Tablet analysis

The content of valganciclovir in the valacept 450 mg tablets was determined by the newly proposed method. RSD results 0.12% is within acceptance limit of ≤ 2% and the results were tabulated in Table 7.

Table 7: Tablet analysis results of valganciclovir

Name of the formulation	Label claim	Amount found (mg) (n=3)	%RSD
Valacept	450 mg	436.23 mg	0.12

Specificity

The HPLC chromatograms of valganciclovir were recorded by injecting the 20 µl of each resultant solution of acid & base hydrolysis, oxidative degradation, irradiation with UV and thermal degradation samples into the HPLC system, which were shown in Figures 8-12 respectively. The retention time and the % of degradation of the valganciclovir were tabulated in Table 8. The results of stress testing studies reveal that, the proposed method has a high degree of specificity due to its ability to separate the analyte from its degradation products.

Table 8: Summary of forced degradation results of valganciclovir

Parameters	Retention time (min)	Degradation (%)
Acid Hydrolysis	2.60	48.12
Base Hydrolysis	2.71	83.31
Oxidation	2.34	25.10
Irradiation with UV light	2.19	1.30
Thermal degradation	2.20	4.31

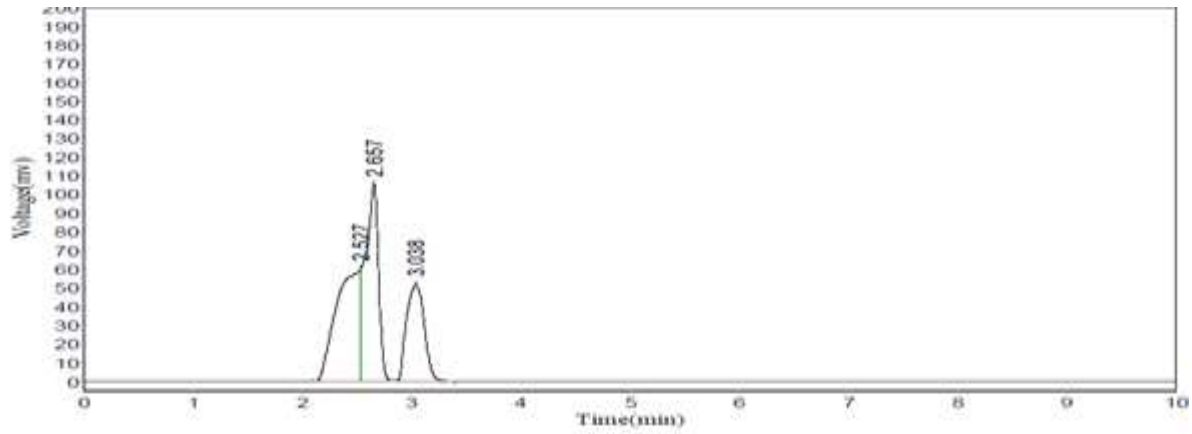

Figure 8: A typical chromatogram representing acid hydrolysis degradation behaviour of valganciclovir

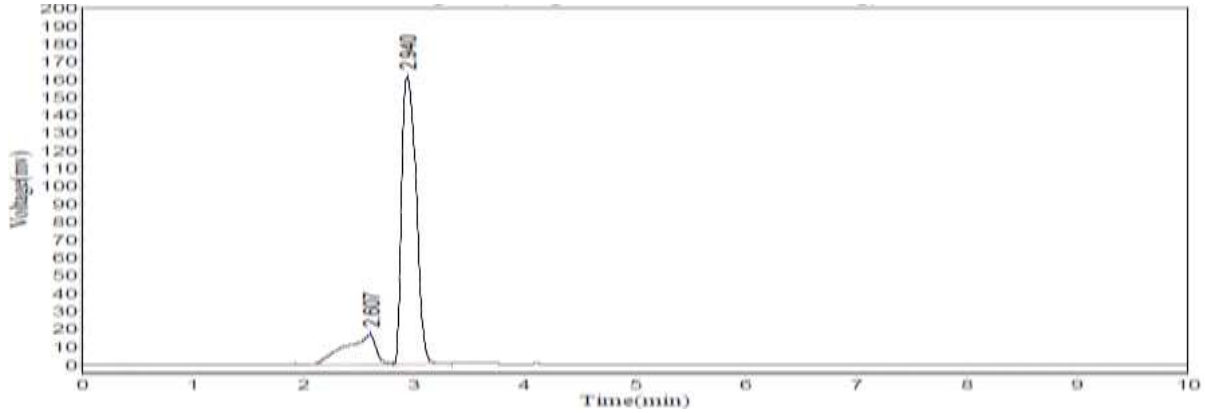

Figure 9: A typical chromatogram representing alkaline hydrolysis degradation behaviour of valganciclovir

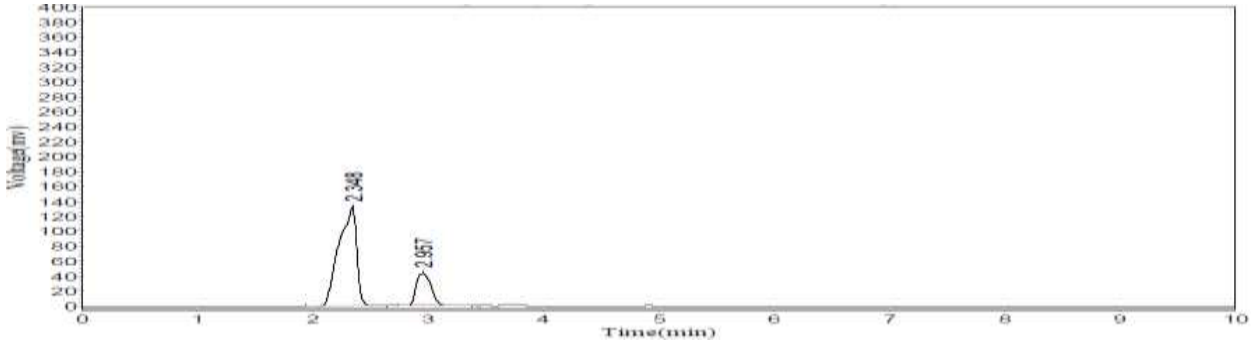

Figure 10: A typical chromatogram representing oxidative degradation behaviour of valganciclovir

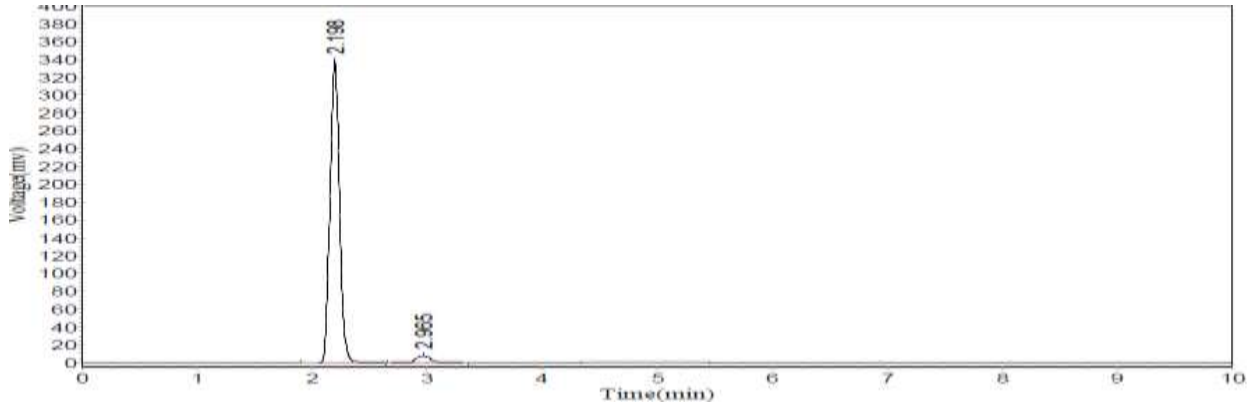

Figure 11: A typical chromatogram representing irradiation with UV light degradation behaviour of valganciclovir

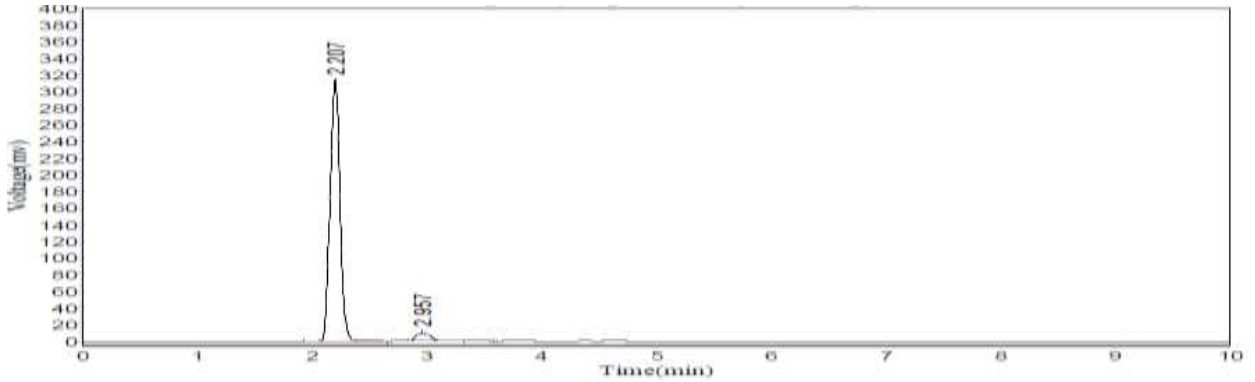

Figure 12: A typical chromatogram representing thermal degradation behaviour of valganciclovir

CONCLUSION

The proposed new method for the determination of valganciclovir by HPLC using Variable Wavelength Detector (UV) and Phenomenex Gemini-NX C18 Column was developed and validated based on the ICH guidelines. The method is linear over the range of 10-80 µg/ml, with the correlation co-efficient (r2=0.9996). The percentage RSD obtained was within the acceptance criteria i.e., not more than 2. The percentage recovery in terms of accuracy was achieved in the range of 98-102%, which was within the acceptance limit. The simplicity and short run time (10 min) enables through output analysis of Valganciclovir. The mobile phase and standard solutions are found to be stable up to 48 h at controlled room temperature. The method is proved as specific, accurate, precise, robust and rugged. Hence, the newly developed and validated method can be used for the regular and stability analysis.

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