Research Article

Development and Validation of RP-HPLC Method for the Determination of Ganciclovir in Bulk Drug and in Formulations

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A simple, rapid, accurate, and precise gradient reversed-phase HPLC (RP-HPLC) method has been developed for the determination of ganciclovir (GNC) in pharmaceuticals. Chromatographic separation was carried out on inertsil ODS C₁₈ (4.6 mm i.d × 250 mm, 5.0 μm) LC column using ammonium acetate buffer, sodium salt of hexane sulfonic acid as ion-pairing reagent in 1000 mL water, and acetonitrile (90 : 10) (v/v) as mobile phase at a flow rate of 1.0 mL min⁻¹ and with UV detection at 245 nm at column temperature (30°C). The runtime under these chromatographic conditions was 10 min. The method was linear over the range of 0.02–75 μg mL⁻¹. The limits of detection (LOD) and quantification (LOQ) values were 4.1 and 20 ng mL⁻¹, respectively. The method was successfully extended to study the effect on GNC upon treatment with 2 N NaOH, 2 N HCl, and 5% H₂O₂ for 2 hrs at 80°C and upon exposure to UV (1200 K lux hrs) for 72 hrs and thermal (105°C) for 5 hrs. The proposed method was further applied to the determination of GNC in pharmaceuticals, with good percent recovery. The accuracy and the precision of the method were validated on intraday and interday basis in accordance with ICH guidelines.

1. Introduction

Ganciclovir (GNC), chemically known as 2-amino-9-[[[(1,3-dihydroxypropan-2-yl)oxy]methyl]-6,9-dihydro-3H-purin-6-one (Figure 1), is a nucleoside analogue widely used in the treatment of cytomegalovirus infections.

It has proved to be effective against cytomegalovirus in immunocompromised patients, mainly in those with the acquired immunodeficiency syndrome (AIDS), congenital immunodeficiency, or in individuals following organ transplantation [1, 2]. Various techniques have been developed for the determination of GNC in pharmaceuticals. It is official in the United States Pharmacopoeia [3], which describes an HPLC method for its determination in injections and in oral suspension.

The literature is enriched with several methods for the determination of GNC in pharmaceutical dosage forms including body fluids. The most extensively used technique for the quantitation of ganciclovir is HPLC, but most of the procedures using this technique are devoted to body fluids like plasma [4–14], plasma and tissues [15], serum

[16], and blood samples [17]. There is only one report [18] dealing with the application of HPLC for the determination of pharmaceutical formulations, that is eye drops. GNC in bulk drug and in its formulations has been assayed by UV spectrometry by measuring the absorbance of 0.1 M HCl and 0.1 M NaOH at 253 and 266 nm, respectively [19]. The methods reported are moderately sensitive with molar absorptivity values of 2.0×10^3 . Other reported methods for pharmaceuticals include visible spectrophotometry [20–22], flow injection luminescence spectrometry [23], and radioimmunoassay [24–26]. The aim of this study was to develop an analytical HPLC method for sensitive, specific, and rapid determination of GNC in its tablets.

2. Materials and Methods

2.1. Materials and Standards. HPLC-grade acetonitrile (Lab-scan Asia Co. Ltd, Bangkok, Thailand), analytical reagent grade ammonium acetate (Rankem, Bangalore, India), and sodium salt of hexane sulfonic acid (Rankem, Bangalore,

2-amino-9-[[(1,3-dihydroxypropan-2-yl)oxy] methyl]-6,9-dihydro-3H-purin-6-one

FIGURE 1: Structure of ganciclovir.

FIGURE 2: Linearity graph.

India) were used. Deionised, Milli Q water (Millipore, Bangalore, India) was used to prepare the mobile phase. Ganciclovir was obtained from Lotus Pharmaceuticals Ltd., Bangalore, India. Tablets were purchased from local commercial sources. A stock-standard solution equivalent to 1000 µg mL⁻¹ GNC was prepared by dissolving accurately weighed amount of pure drug in the mobile phase.

2.2. Equipment. HPLC analysis was performed on an Alliance Waters HPLC system equipped with Alliances 2657 series low-pressure quaternary pump, a programmable variable wavelength UV-visible detector, Waters 2996 photodiode array detector, and auto sampler. Data were collected and processed using Waters Empower 2.0 software.

2.3. Chromatographic Conditions. Chromatographic analysis was carried out on an inertsil ODS-3V C₁₈ (4.6 mm i.d × 250 mm, 5.0 µm) LC column. The mobile phase consisted of 0.025 M ammonium acetate (pH ≈ 6.8) containing 0.4 g sodium hexane sulphonate and acetonitrile (90 : 10 v/v). The mobile phase, filtered through 0.45 µm membrane filter and degassed before use, was pumped at 1 mL min⁻¹ flow rate. The column was thermostated at 30°C. Under these conditions the runtime was 10 min.

FIGURE 3: Typical chromatogram (pure GNC, 50 µg mL⁻¹).

TABLE 1: Regression and sensitivity parameters.

Parameters	Value
Linearity range, µg mL ⁻¹	0.02–75
Slope (b)	59682.7532
Intercept (a)	–295296.2843
Standard deviation of intercept (S _a)	235822.5161
Standard deviation of Slope (S _b)	11483.1236
Correlation coefficient (r)	0.9999
Limit of detection (LOD, ng mL ⁻¹)	4.1
Limit of quantification (LOQ, ng mL ⁻¹)	20

** Y = a + bX, where Y is the area and X is the concentration in µg mL⁻¹.

3. Procedures

3.1. Calibration Graph. Ten µL of working standard solutions (0.02–75 µg mL⁻¹ GNC) was injected automatically onto the column in triplicate and the chromatograms were recorded. The calibration graph was prepared by plotting the mean peak area percentage versus concentration in µg mL⁻¹ (Figure 2).

3.2. Pharmaceutical Preparations. Twenty tablets were accurately weighed and crushed into a fine powder and mixed using a mortar and pestle. A quantity of tablet powder equivalent to 100 mg of GNC was weighed accurately into a 100 mL calibrated flask, 50 mL of mobile phase as diluent added and was sonicated for 20 min to complete dissolution of the GNC, and the solution was then diluted to the mark with the diluent and mixed well. A small portion of the tablet solution (say 10 mL) was withdrawn and filtered through a 0.2 µm filter to ensure the absence of particulate matter. The filtrate was appropriately diluted with the diluent before injection into the column.

3.3. Results and Discussion. Mobile phases consisting of several buffering systems such as phosphoric acid, sodium acetate, potassium dihydrogen orthophosphate, sodium dihydrogen ortho phosphate or ammonium acetate with acetonitrile, methanol, and isopropanol as organic modifiers in different volumetric ratios failed to meet the required system parameters. Finally, the mobile phase consisting of 2.0 g ammonium acetate and 0.40 g of sodium salt of hexane

FIGURE 4: Chromatogram after forced degradation with 2 N HCl at 80°C for 2 hrs (pure GNC, 50 $\mu\text{g mL}^{-1}$).

FIGURE 5: Chromatogram after forced degradation with 2 N NaOH at 80°C for 2 hrs (pure GNC, 50 $\mu\text{g mL}^{-1}$).

FIGURE 6: Chromatogram after forced degradation with 5% H_2O_2 at 80°C for 2 hrs (pure GNC, 50 $\mu\text{g mL}^{-1}$).

FIGURE 7: Chromatogram after exposure to UV at 1200 K lux hrs for 72 hrs (pure GNC, 50 $\mu\text{g mL}^{-1}$).

sulphonic acid in 1000 mL water and acetonitrile (90:10, v/v) was found to give better theoretical plates (>2000) and tailing factor peak (<1.2). The analysis was carried out at 30°C, which besides being economical, offers many advantages like low-column back pressure, good chromatographic peak shape, improved column efficiency (1.14 tailing factor), higher theoretical plates (4732), and consistency in retention time. Under the stated chromatographic conditions, the mean retention time was 3.183 min ($n = 6$). A model chromatogram is shown in Figure 3. Further, the optimized chromatographic conditions were used to study the effect on GNC after treatment with various stress conditions. Upon treatment with 2 N NaOH, 2 N HCl, and 5% H_2O_2 , for 2 hrs at 80°C, separately, there was no change in the retention time but there was a slight change in mean peak area in both 2 N HCl (Figure 4) and 2 N NaOH (Figure 5), and there was a significant change in both peak areas but no change in retention time in 5% H_2O_2 (Figure 6). There was no effect upon exposure to UV at 1200 K lux (Figure 7) and thermal treatment at 105°C (Figure 8), for 5 hrs.

4. Method Validation

4.1. Linearity. Working standard solution of GNC ($1000 \mu\text{g mL}^{-1}$) was appropriately diluted with the diluent solution to obtain solutions in the concentration range 0.02–75 $\mu\text{g mL}^{-1}$ GNC. Ten μL of each solution was injected

in triplicate onto the column under the operating chromatographic conditions described above. Calibration curve was constructed by plotting the mean peak area percentage versus concentration. The least squares method was used to calculate the slope (b), intercept (a), and the correlation coefficient (r) of the regression line. The relation between mean peak area Y ($n = 3$) and concentration, X expressed by the equation $Y = a + bX$, was linear. Values of slope, intercept, and correlation coefficient (r) were 59682.7532, -295296.2843 , and 0.9999, respectively. A plot of log peak area versus log concentration was a straight line with the slope of 0.9969 indicating excellent linearity between mean peak area and concentration in the range 0.02–75 $\mu\text{g mL}^{-1}$ GNC as shown in Table 1.

4.2. Limits of Detection (LOD) and Quantification (LOQ). LOD and LOQ were estimated from the signal-to-noise ratio. The LOD defined as the lowest concentration that gave a peak area with signal-to-noise ratio between 2–3 was found to be 4.1 ng mL^{-1} . The lowest concentration that provided a peak area with a signal-to-noise ratio 9.68, which is called LOQ, was found to be 20 ng mL^{-1} .

4.3. Specificity. Method specificity was checked by comparing the chromatograms obtained for pure GNC solution, synthetic mixture, tablet solution, and placebo blank. An examination of the chromatograms of the above solutions

TABLE 2: Intraday and interday accuracy and precision.

GNC Injected, $\mu\text{g mL}^{-1}$	Intraday accuracy and precision				Interday accuracy and precision			
	GNC found ^a , $\mu\text{g mL}^{-1}$	RE, %	RSD ^b , %	RSD ^c , %	GNC found ^a , $\mu\text{g mL}^{-1}$	RE, %	RSD ^b , %	RSD ^c , %
25.0	25.4	1.6	0.85	0.06	25.3	1.2	1.12	0.33
50.0	50.9	1.8	0.20	0.25	51.0	2.0	0.86	0.42
75.0	76.1	1.5	0.62	0.16	77.2	2.9	1.14	0.26

^aMean value of seven determinations, ^bbased on peak area, ^cbased on retention time.

FIGURE 8: Chromatogram after thermal treatment at 105°C for 5 hrs (Pure GNC, 50 $\mu\text{g mL}^{-1}$).

FIGURE 10: Chromatogram of tablet solution (GNC, 50 $\mu\text{g mL}^{-1}$).

FIGURE 9: Chromatogram of synthetic mixture (GNC, 50 $\mu\text{g mL}^{-1}$).

shown in Figures 3, 9, 10, and 11 reveals the absence of peaks due to additives present in tablet preparations.

4.4. Precision and Accuracy. Method precision was determined from the results of six independent determinations of GNC at three different concentrations, 25, 50, and 75 $\mu\text{g mL}^{-1}$ on the same day. The interday and intraday relative standard deviation (RSD) values for peak area and retention time for the selected concentrations of GNC were less than 2.0 and 0.5%, respectively. The method accuracy, expressed as relative error (%), was determined by calculating the percent deviation found between concentrations of GNC injected and concentrations found from the peak area. This study was performed by taking the same three concentrations of GNC used for precision estimation. The intraday and interday accuracy (expressed as %RE) values which are <2.9% are also compiled in Table 2.

4.5. Robustness. To determine the robustness of the method, small deliberate changes in the chromatographic conditions like flow rate and column temperature were made, and the results were compared with the original chromatographic conditions. The results indicated that changing the flow rate (± 0.1 mL) had some effect on the chromatographic behavior of GNC. However, the alteration in the column temperature ($\pm 2^\circ\text{C}$) had no significant effect. The results of this study expressed as %RSD are summarized in Table 3.

4.6. System Suitability. System suitability parameters were measured to verify the system performance and the values of retention time, number of theoretical plates and tailing factor were 3.17 ± 0.1 , 4732 per column and 1.14, respectively. All the values were within the acceptable range.

4.7. Solution Stability. The stability of standard and sample solutions was determined by monitoring the peak area and retention time over a period of 24 hrs by injecting the solutions at the intervals of 0, 8, 16, and 24 hrs. The standard and sample solutions were stored at ambient temperature (25°C) and protected from light during the stability study. No changes in drug concentrations were observed over a period of 24 hrs as shown by the small %RSD values. The %RSD for peak area ($n = 3$) was 1.93% for pure drug solution and the value for retention time ($n = 3$) was 1.43%. The results are presented in Table 4. No significant changes in concentration of the active ingredient were observed in the tablet solution as well.

4.8. Method Application. The developed and validated method was successfully applied to determine GNC in

TABLE 4: Solution stability (GNC concentration was $50 \mu\text{g mL}^{-1}$).

Time, hrs	Peak area	Mean $\pm$ SD	%RSD	Retention time (min)	Mean $\pm$ SD	%RSD
0	2989840	2929008 ± 52766	1.80	3.195	3.164 ± 0.05	1.43
	2895622			3.185		
	2901562			3.112		
8	2845671	2831673 ± 37143	1.31	3.158	3.139 ± 0.02	0.07
	2859782			3.145		
	2789565			3.115		
16	2946830	2936679 ± 54740	1.86	3.118	3.141 ± 0.02	0.64
	2985633			3.156		
	2877574			3.148		
24	2908480	2855884 ± 54189	1.90	3.149	3.119 ± 0.03	0.83
	2858942			3.102		
	2800231			3.106		

TABLE 5: Results of determination of GNC in tablets and statistical comparison with the reference method.

Tablet brand name**	Nominal amount, mg	Found* (percent of label claim $\pm$ SD)			
		Reference method	Proposed method	Student's <i>t</i> -value (2.77)	<i>F</i> -value (6.39)
Ganguard ¹	500	98.51 ± 1.05	99.62 ± 1.85	1.05	3.10
Natclovir ²	250	99.06 ± 0.88	100.1 ± 1.61	2.31	3.34

* Mean value of five determinations, ** marketed by: ¹Ranbaxy Lab, Ahmedabad, India, ²Natco Pharma Ltd., Pune, India, figures in the parenthesis are the tabulated values for four degree of freedom at 95% confidence level.

FIGURE 11: Chromatogram of placebo blank.

tablets. The results obtained tallied closely with the labeled amount in the case of tablets (Table 5), thus indicating the utility of the method for content uniformity evaluation. The results were statistically compared with those obtained by reported method [19] for accuracy and precision by applying the Student's *t*-test and variance ratio *F*-test. The reference method consisted of the measurement of the tablet extracts absorbance at 253 nm. The calculated *t*- and *F*-values were less than the tabulated values of 2.77 and 6.39 at the 95% confidence level and for five degrees of freedom suggesting that there was significant difference between the proposed method and the reference method with respect to accuracy and precision.

4.9. Recovery Study. To the preanalyzed tablet powder, pure GNC was added at three levels and the total was found by the proposed method. Each measurement was triplicated. When

the test was performed on 500 mg and 250 mg tablets, the percent recovery of pure GNC was 106.15 and 102.10 with standard deviation of 0.86 and 0.46, respectively. The results indicate that the method is very accurate and that common excipients found in tablet preparations do not interfere and the results are summarized in Table 6.

5. Conclusions

Very simple, cost effective, accurate, and precise RP-HPLC method for determining ganciclovir was developed and validated as per the ICH guidelines Q2 (2005). Analysis was carried at ambient temperature. Besides, short retention time (3.117), runtime (10 min), and flow rate (1 mL min^{-1}) make the method attractive since these features help in saving cost and time of analysis. Ammonium acetate has many properties which are particularly interesting for use on a general purpose buffer [27] the most significant being low toxicity and cost, easy availability, good buffering capacity in the chosen pH range, and ability to provide greatly improved separations without column deterioration. In summary, this method is selective, sensitive, rapid, and reproducible for ganciclovir in its tablets. The precision and accuracy are within acceptable ranges, the quantification limit is as low as 20 ng mL^{-1} [27], and finally the assay is robust and rugged.

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TABLE 6: Results of recovery study by standard addition method.

Tablet	GNC in tablet, $\mu\text{g mL}^{-1}$	Pure GNC added, $\mu\text{g mL}^{-1}$	Total found, $\mu\text{g mL}^{-1}$	Pure GNC recovered*, percent $\pm$ SD
Ganguard 500 mg	26	12.5	39.12	101.90 $\pm$ 0.16
	26	25	52.05	100.55 $\pm$ 0.72
	26	37.5	64.18	106.15 $\pm$ 0.86
Nadclovir 250 mg	24.9	12.5	38.03	101.30 $\pm$ 0.64
	24.9	25	51.88	102.10 $\pm$ 0.46
	24.9	37.5	62.88	100.10 $\pm$ 0.22

* Mean value of three determinations.

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