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METHOD DEVELOPMENT AND VALIDATION OF VALACYCLOVIR HYDROCHLORIDE AND RITONAVIR IN TABLET DOSAGE FORM USING REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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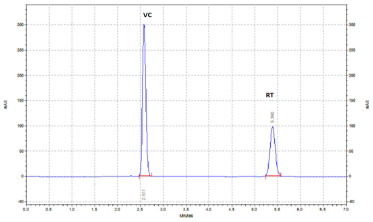
Graphical abstract



Abstract

Our main objective is to develop an accurate and precise RP-HPLC method for the simultaneous determination of Valacyclovir HCl and Ritonavir in tablet dosage form. An Agilent TC-C18 (2) column is used for the Separation of drugs by a mobile phase consisting of methanol, acetonitrile and water mixture in the ratio of 35:41.5:23.5v/v. The flow rate maintained was 1.3 mL/min and the wavelength used for detection was 222 nm. The linearity was observed in the range of 12.5-125µg/ml for Valacyclovir HCl (VC) and Ritonavir (RT) with a correlation coefficient of 0.9987 and 0.9981 respectively. The mean percentage recoveries for 80%, 100% and 120% accuracy were found to be 101.7%±2.09, 100%±2.49 and 101.5%±1.61 respectively for VC. The mean percentage recoveries for 80%, 100% and 120% accuracy were found to be 104.3%±0.99, 100%±1.77and 99.0%±1.22 respectively for RT. Linearity, accuracy, precision and robustness parameters for the suggested method were estimated for validation. The developed method can be utilized in the analysis of VC and RT tablets.

Keywords: Valacyclovir HCl, Tablet, HPLC, ritonavir, validation

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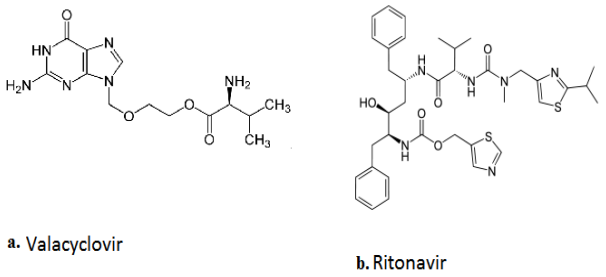
1.0 INTRODUCTION

Valacyclovir (VC) which is a prodrug, being converted Invivo to acyclovir, is an antiviral drug used in the management of herpes simplex, herpes zoster (shingles), and herpes B. C13H20N6O4 and 360.80 g/Mol are chemical formula and molecular weight of VC. Acyclovir is converted into the active triphosphate form, acyclo-GTP, by cellular kinases. Acyclo-GTP is a very potent inhibitor of viral DNA polymerase [1]. The structural formula of VC is shown in Figure 1(a). It is soluble in water (174g/L) [2, 3]. Literature survey revealed that various analytical methods such as UV spectrophotometry (4-6), HPLC (7-12) and LCMSMS (13) methods have been reported for the estimation of VC from its formulations and biological fluids.

Ritonavir (RT) is an antiretroviral drug from the protease inhibitor class used to treat HIV infection and AIDS. The molecular formula is C37H48N6O5S2 and the structural formula of RT is in Figure 1(b). RT is a yellow crystalline substance, practically insoluble in water, but soluble in ethanol [2, 3]. It has a molecular weight of 720.95 g/Mol. Detailed survey of literature for RT revealed several methods that have been reported for the assay of it either alone or in combined form in drug formulations. These analytical techniques include UV visible (Vis) spectrophotometry (14-18), RP-HPLC (19-21) and HPTLC (22).

The development and validation of an analytical method is to ensure a specific, accurate and precise method for a particular analyte. The principal objective for that is to enhance the conditions and parameters, which should be observed in the evolution and establishment. From the literature review it was found that there are no methods for the simultaneous estimation of VC and RT, but many methods for individual analysis of the drugs are present [4-19]. Hence it is aimed to acquire novel methods for the estimation of VC and RT in tablet dosage form using available analytical technique HPLC. The aim of the suggested method is to develop a simple and accurate method for the simultaneous determination of VC and RT using RP-HPLC technique in tablets.



a. Valacyclovir

b. Ritonavir

Figure 1 Structures of drugs

2.0 EXPERIMENTAL

VC and RT obtained from Matrix Laboratories (Hyderabad, India) were of analytical grade 99.9% pure). Commercial samples of VC and RT tablets were procured from the local medical store and applied within their shelf-life period. Methanol, water and acetonitrile of HPLC grade were obtained from Rankem R. F. C. L. Limited, Haryana, S. D. Fine Chem. Limited, Mumbai and MARCK Specialities private limited, Mumbai respectively. Quantitative HPLC was performed on AGILENT 1120 COMPACT LC, with Variable Wavelength detector. Agilent TC-C18 (2) column (250 x 4.6 mm, packed with 5 µm particles) is utilized for the chromatographic separation. Manual injections (20 µl) were applied. The column was kept at ambient temperature. The wavelength was set at 222nm for detection. To produce a suitable RP-HPLC method for the determination of VC and RT, different mobile phases methanol, water and acetonitrile were used in different compositions at different flow rates. Lastly, the mobile phase methanol: acetonitrile: water mixture in the proportion of 35:41.5:23.5v/v at a flow rate of 1.3 mL/ min gave peaks with satisfactory resolution for VC and RT. VC and RT got eluted at retention times 2.61 and 5.64 minutes respectively with symmetric peaks. The mobile phase was degassed and then filtered through 0.25 µm Micro filtration unit before it was pumped into the RP-HPLC system. By pumping the mobile phase through the column for at least 30 minutes before injecting the drug solution, equilibrium in the column was achieved. 7 minutes were the run time. Ezchrome software in a computer system is used for the collection and analysis of the data. Chromatogram showing the separated drugs is shown in Figure 2.

2.1 Mobile Phase Preparation

100ml volumetric flask was taken and 35 ml of methanol, 41.5 ml of acetonitrile and 23.5 ml of water, which are of HPLC grade were added.

2.2 Preparation of Standard Stock Solution:

5 mg of VC and 2mg of RT working standards was weighed accurately and added to 10 ml volumetric flasks individually. 7.5ml of mobile phase to each volumetric flask was added and sonication carried out for 15 minutes. Later it made up to the volume with the mobile phase.

2.3 Standard Preparation:

2ml of VC stock solution and 2ml of RT stock solution were taken and added to a 10ml volumetric flask, then made up to volume with mobile phase, producing 100µg/ml of VC and 40µg/ml of RT respectively. Then final solutions were filtered using micro filtration unit of 0.25 µm.

2.4 Standard Preparation for Linearity:

0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 ml of the VC stock solution were taken and added to the 10 ml volumetric flask individually, then made up to volume with mobile phase, producing 12.5, 25, 50, 75, 100 and 125 µg/ml of VC respectively. 0.625, 1.25, 2.5, 3.75, 5.0 and 6.25 ml of the RT stock solution were taken and added to the 10 ml volumetric flask individually, then made up to volume with mobile phase, producing 12.5, 25, 50, 75, 100 and 125 µg/ml of RT respectively. All the solutions were filtered using micro filtration unit of 0.25 µm.

2.5 Sample Stock Solution:

20 tablets of VC and RT were individually taken and powdered and the mean weights were estimated. VC powder weight equivalent to 5mg and the RT powder weight equivalent to 2mg was added to two 10ml volumetric flasks individually. The few ml of the mobile phase was added and sonicated for about 15 mins then the volume made up to the mark with mobile phase individually.

2.6 Sample Preparation:

2 ml of VC sample stock solution and 2 ml of RT sample stock solution were taken and added to 10 ml volumetric flask. Then made up to volume with mobile phase, producing 100µg/ml of VC and 40µg/ml of RT respectively. Then final solutions were filtered using micro filtration unit of 0.25µm. All determinations were

injected five times. In sonicator ambient temperature was maintained.

2.7 Validation:

The proposed method was validated for the analysis of VC and RT using following parameters. System-suitability studies are an intact part of method development and are practiced to ensure satisfactory performance of the chromatographic system. For five replicate injections of the drugs of 100µg/ml concentration, Number of theoretical plates (N) and tailing factor (T) were assessed. 12.5 -125 µg/ml was the linear range of both drugs. To obtain proportionality, the slope and intercept of the regression line and correlation coefficient were calculated statistically from the calibration curve of the VC and RT. To find out variations in the test methods precision was studied for VC and RT of 100 µg/ml concentration when analysis carried out by 2 different analysts (ruggedness). The standard solution was injected five times and the area was measured for all five injections in HPLC. The % relative standard deviation (%RSD) and %content results were used for assessment of precision and ruggedness. The accuracy of the method was demonstrated by analyzing VC and RT mixtures of 80%, 100% and 120%. After injection, recovery values for individual drugs were estimated. Specificity is the ability of a method to differentiate the analyte(s) of interest from other components in the sample. Placebo was prepared as per the marketed product formulas of both drugs. Placebo interference from excipients was studied. Robustness of the method were determined by varying flow rate, mobile phase ratio and wavelength parameters. Bench top stability (300C & 60 % RH) and Refrigerator (4-50C & 55%RH) stability were determined on the 1st and 2nd day.

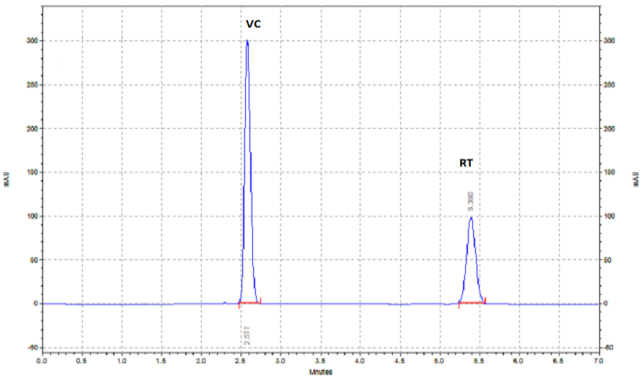


Figure 2 Chromatogram for Valacyclovir and Ritonavir

3.0 RESULTS AND DISCUSSION

An isocratic reverse – phase HPLC procedure was suggested as a suitable method for the analysis of VC and RT in tablets. Methanol: acetonitrile: water mixture in the proportion of 35:41.5:23.5v/v at a flow rate of 1.3 ml/ min was found to be a suitable mobile phase for complete and rapid separation of analytes. 2.61 and 5.64 minutes were the retention times for VC and RT respectively. System suitability studies for the VC and RT reported that the % relative standard deviation values of five replicate injections of different solutions of VC and RT were found to be 1.56 and 1.92. The theoretical plates for the VC and RT were found to be 5437 and 9708 respectively. The resolution was found to be 17.2. The related data were presented in Table 1.

As given in the Figure 2, the drugs got eluted giving symmetrical single peaks, well removed from the solvent front. The % relative standard deviation (%RSD) of the peak areas for five injections of the standard solution of VC and RT was used for determination of the precision of the HPLC system. %RSD for the VC and RT were found to be 1.77 and 0.66 respectively. The %RSD of both the drug under this method was not more than 2. In order to validate the accuracy of the described method, recovery studies were carried out by analyzing mixtures of VC and RT. The recoveries of VC and RT were evaluated for 80%, 100% and 120% concentrations. The mean percentage recoveries for 80%, 100% and 120% accuracy were found to be 101.7%±2.09, 100%±2.49 and 101.5%±1.61 respectively for VC. The mean percentage recoveries for 80%, 100% and 120% accuracy were found to be 104.3%±0.99, 100%±1.77and 99.0%±1.22respectively for the RT. The results of percentage recovery data were within the limit. Accuracy data were presented in Table 2.

Table 1 System suitability studies data

System suitability parameters	Observed value		Acceptance criteria
	Valacyclovir HCl	Ritonavir	
Percentage relative standard deviation	1.565158	1.925048	% RSD should not be more than 2.0
Theoretical plates for Valacyclovir HCl and Ritonavir in standard solution	5437	9708	Not less than 3500
Resolution between Valacyclovir HCl and Ritonavir peaks in standard solution	17.2		Not less than 2

Table 2 Accuracy data

Number of injection = 6	Peak area		Percentage Content		Percentage Recovery	
	Valacyclovir	Ritonavir	Valacyclovir	Ritonavir	Valacyclovir	Ritonavir
			Accuracy 1 (80%)			
Mean	18835507	10157317	81.42105	83.48716	101.78	104.3589
SD	361479.2	211147.2	1.674437	0.792524	2.093046	0.990656
%RSD	1.919137	2.07877	2.056516	0.949277	2.056516	0.949277
			Accuracy 2 (100%)			
Mean	23133461	12166322	100	100	100	100
SD	408303.1	206246.4	2.493996	1.779629	2.493996	1.779629
%RSD	1.764989	1.695224	2.493996	1.779629	2.493996	1.779629
			Accuracy 3 (120%)			
Mean	28182909	14459224	121.8275	118.8463	101.5229	99.03858
SD	448742	178569	1.939796	1.467732	1.616497	1.22311
%RSD	1.592249	1.234983	1.592249	1.234983	1.592249	1.234983

Table 3 Linearity data

	Valacyclovir		Ritonavir	
Conc (µg/ml)	mean	SD	Mean	SD
12.5	2070548.5	22718.6338	3455027	28398.8225
25	4685918.5	91677.1013	6340437	112101.881
50	10968711.5	150238.271	15023337	209841.008
75	18851640.5	223582.214	25192050.5	91288.1926
100	25947981.5	157047.709	34145485.5	246601.369
125	31492189.5	266473.898	40788646	322241.288
Slope(S)	269823.614	2036.14207	345708.9102	2433.93082
Intercept	-1756610.073	12882.5847	-1502869.953	1754.45397
Correlation coefficient	0.998715119	0.0001408	0.998141444	0.00022607

A linear calibration curve was obtained over the concentration range from 12.5-125µg/ml for VC and RT for quantitative application purpose. The correlation coefficient for VC and RT were 0.9987 and 0.9981 respectively. The regression equation of VC was found to be y=269823.614x-1756610.073 with a coefficient of correlation 0.9987 where x is concentration and y is absorbance.The regression equation of RT was found to be y=345708.9102x-1502869.953 with a coefficient of correlation 0.9981 where x is concentration and y is

absorbance. The curve fittings of VC and RT were found to be 99.87% and 99.81% respectively. The calibration curve of VC and RT drugs was present in Figure 3 and related linearity data in Table 3.

Based on the standard deviation of y-intercepts of regression lines (σ) and slope (S), limit of detection and limit of quantitation were determined. Limit of detection was calculated by 3.3 σ/S and for a limit of quantitation 10 σ/S. Limit of detection of VC and RT were found to be 0.1575 and 0.0167 µg/ml respectively. Limit of quantitation of VC and RT were found to be 0.4774 and 0.0507 µg/ml, respectively. Ruggedness for VC and RT determined by varying analysts carrying out the procedure. Totally 2 analysts carried out the procedure and the results were within the limits. Robustness of the method were determined by varying wavelength, flow rate and mobile phase ratio. The optimized method detection wavelength was 222nm and robustness were determined by varying wavelength to 220nm and 224nm. The optimized flow rate was 1.3ml/min and it was varied to 1.2ml/min and 1.4ml/min. The optimized mobile phase ratio is methanol: acetonitrile: water (35:41.5:23.5) and it was varied to 33:42.5:24.5 and 37:40.5:22.5.The variation of the wavelength (±2nm), flow rate (±0.1ml/min) and the mobile phase ratio was not shown any deviation from the true value and %RSD of all variations were within the limit. Robustness and stability data were presented in Table 4. Stability studies (Refrigerator stability and Benchtop stability) have reported the percentage deviation from the true value within the limit for the both drugs. All validation parameters results of the proposed method were presented in Table 5.

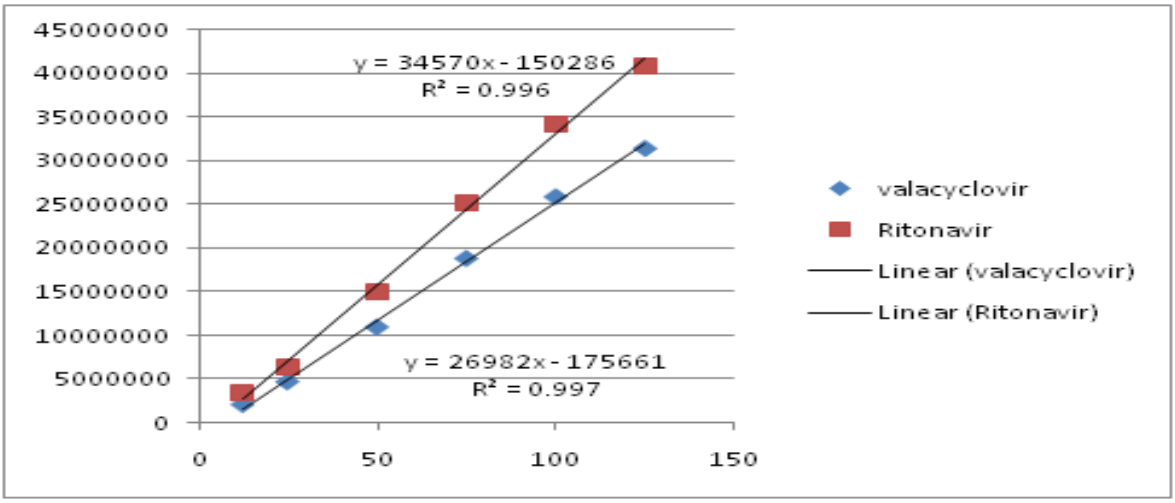


Figure 3 Linearity curve for Valacyclovir and Ritonavir

Table 4 Robustness and stability data

Robustness N=6	Peak area			Peak area	
	Valacyclovir	Ritonavir		Valacyclovir	Ritonavir
	Flow rate1.2min			Flow rate1.4min	
Mean	27786779	37065317.5	Mean	27026437	35937462
SD	304736.1526	207519.5768	SD	119980.4644	233369.2794
%RSD	1.096694772	0.559875352	%RSD	0.44393741	0.649376073
	Mobile phase 37:63			Mobile Phase 33:67	
Mean	28951390.5	38218852.5	Mean	25241914.5	34772158.5
SD	219922.2298	480344.0004	SD	456719.5629	544967.9034
%RSD	0.759625793	1.256824758	%RSD	1.80936974	1.567253593
	Wavelength detection at 220 nm			Wavelength detection at 224 nm	
Mean	27228577	41964446	Mean	28947644	28865697.5
SD	466310.0521	272955.9455	SD	73090.79954	475566.787

%RSD	1.712575917	0.650445726	%RSD	0.252493086	1.647515315
	Bench top stability data			Refrigerator stability data	
Mean	25920862	35094340	Mean	27438806	35841730
SD	415587.2	471823.4	SD	95571.14	30668.64
%RSD	1.603292	1.344443	%RSD	0.348306	0.085567

Table 5 Results of validation parameters

Validation	Parameters	Valacyclovir HCl	Ritonavir
System suitability	%RSD	1.56	1.92
	Theoretical plates	5437	9708
	Resolution	17.2	
Linearity	Correlation coefficient	0.998	0.998
	Slope	269823.614	345708.9102
	Intercept	-1756610.073	-1502869.953
Precision	%RSD	1.7	0.6
Ruggedness	%RSD for Analyst 1 variation	1.065392	1.6472
	%RSD for Analyst 2 variation	0.558351	0.350979
Accuracy	Mean % Recovery for 80%, 100% and 120% respectively	101.7%, 100%and 101.5 %	104.3%, 100% and 99.0%
Refrigerator stability	%RSD	0.348306	0.085567
Benchtop stability	%RSD	1.603292	1.344443
Specificity		No interference	No interference
Limit of detection		0.1575µg/mL	0.0167µg/mL
Limit of quantitation		0.4774µg/mL	0.0507µg/mL
Robustness	% RSD for flow rate 1.2ml/min	1.096694772	0.559875352
	% RSD for flow rate 1.4ml/min	0.44393741	0.649376073
	% RSD for M.P ratio 37:40.5:22.5	0.759625793	1.256824758
	% RSD for M.P ratio 33:42.5:24.5	1.80936974	1.567253593
	% RSD for Wavelength 220nm	1.712575917	0.650445726
	% RSD for Wavelength 224nm	0.252493086	1.647515315

4.0 CONCLUSION

The developed method is uncomplicated, accurate, sensitive and precise. The positive traits of the proposed method are its short duration for analysis and a simple process for sample preparation. The satisfying % recoveries and low % RSD Values confirmed the suitability of the developed method for the usual analysis of mixtures of Valacyclovir HCl and Ritonavir in pharmaceuticals.

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