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Method Development and Validation for Multicomponent Analysis of Emtricitabine and Ritonavir in Bulk Drug by RP-HPLC

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Abstract

A simple, sensitive, economic and specific reverse phase liquid chromatographic method was developed for the simultaneous estimation of Emtricitabine and Ritonavir in bulk drug. Chromatographic conditions consisted of C-18 Column (Shim-pack) 250 x 4.6 mm, particle size 5 µm , mobile phase combination of methanol and water (80:20), flow rate 1ml per minutes, run time 15 minutes and UV detection at 251nm. . The retention time for Emtricitabine and Ritonavir were found to be 3.25 and 7.8 min and average percentage recoveries 99.42% and 99.63% respectively. The validation parameters were found to comply with ICH guidelines. These methods can be further employed in future for the routine determination of Emtricitabine and Ritonavir in bulk drug and formulation.

Keyword: Emtricitabine, Ritonavir, RP-HPLC, accuracy and linearity.

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1. INTRODUCTION[1-10]:

Emtricitabine (Figure 1) is a synthetic fluoro derivative of thiacytidine with potent antiviral activity. Emtricitabine is phosphorylated to form Emtricitabine 5'-triphosphate within the cell. This metabolite inhibits the activity of human immunodeficiency virus (HIV) reverse transcriptase both by competing with the natural substrate deoxycytidine 5'-triphosphate and by incorporation into viral DNA causing a termination of DNA chain

Ritonavir (Figure 2) is an antiretroviral protease inhibitor that is widely used in combination with other protease inhibitors in the therapy and prevention of human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS).

Literature review revealed only few efficient methods was available for multicomponent analysis. Hence, an attempt has been made to develop cost effective, simple and precise RP-HPLC method to estimate both drugs in bulk drug.

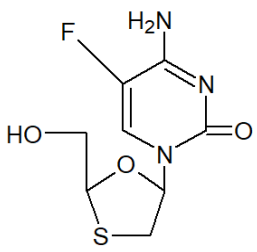

Figure 1: Structure of Emtricitabine

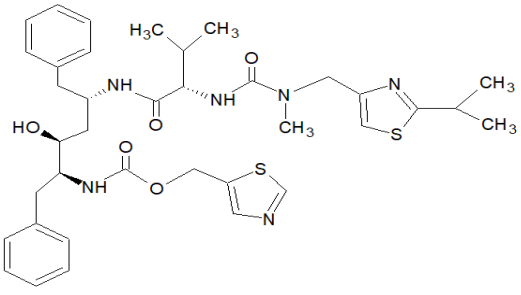

Figure 2: Structure of Ritonavir

2. EXPERIMENTAL WORK[11-16]:

2.1 Instrumentation: High Performance Liquid Chromatography (LC Prominence, Shimadzu), manual sampler, software Win chrome and detector (UV-visible), Column C-18 (Shim-pack) 250 x 4.6 mm, particle size 5 µm.

2.2 Preparation of stock solutions, working solutions and calibration standards

Standard Emtricitabine 100 mg was weighed and transferred to a 100 ml volumetric flask and dissolved in methanol. The flask was shaken and sonicate for 10 minute and volume was made up to the mark with solvent.10 ml of solution was pipetted out from this and transferred to 100 ml volumetric flask and the volume was made up to 100 ml with methanol. The conc. of prepared stock solution was 100 µg/ml.

Standard Ritonavir 100 mg was weighed and transferred to a 100 ml volumetric flask and dissolved in methanol. The flask was shaken and sonicate for 10 minute and volume was made up to the mark with solvent 10 ml of solution was pipetted out from this and transferred to 100 ml volumetric flask and the volume was made up to 100 ml with methanol. The conc. of prepared stock solution was 100 µg/ml.

2.3 Selection of wavelength

10 mcg concentration of Emtricitabine and methanol spectra were recorded in the UV- Visible spectrophotometer. The overlain spectra showed iso-absorptive point at 251 nm.

2.4 Selection of chromatographic condition

Chromatographic separation was achieved at ambient temperature on a reversed phase isocratic high performance liquid chromatography using a mobile phase consisting of Methanol and water (80:20).Flow rate was 1.0 mL/min. The detector wavelength was set at 251 nm and run time was 15 minutes.

To optimize the chromatographic conditions, the effect of chromatographic variables such as mobile phase pH and flow rate were studied. The resulting chromatograms were recorded and the chromatographic responses were measured.

3. RESULT AND DISCUSSION

3.1 Optimized Method development[17-19].

The selection of the composition of mobile phase were studied and optimized. Separation was found to be satisfactory with Methanol and water in the ratio of 80:20 %, v/v. UV detection was carried out at 251 nm where both the drugs exhibit maximum absorption. Isocratic mode was chosen as the retention for both the drugs were less than 8 min at a flow rate of 1ml/min. Retention time for Emtricitabine and Ritonavir were found to be 3.25 and 7.77 min respectively. The chromatogram of optimized trial is shown in Fig 3.

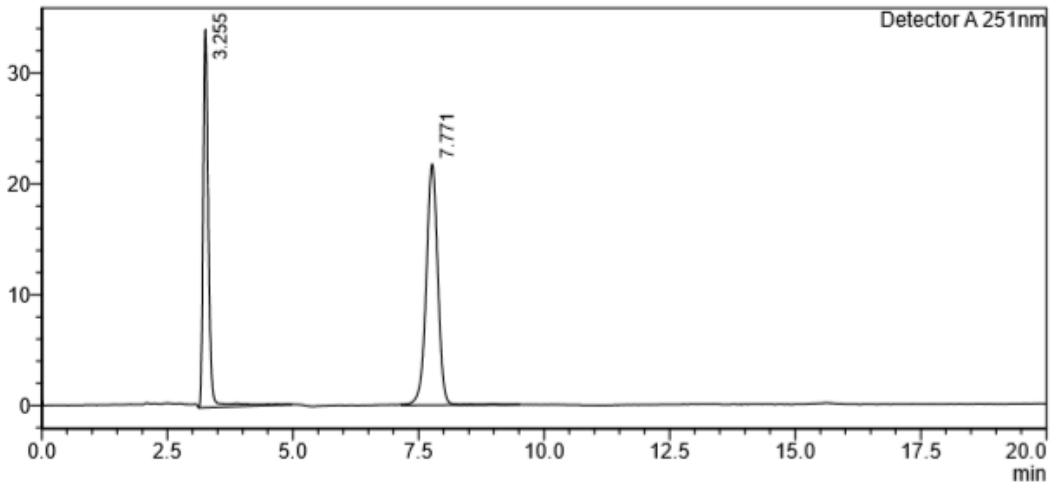

Figure 3 Optimized chromatogram of standard drug at Ratio 80:20 of Meth:Water containing 10 µg/mL Emtricitabine and 60 µg/mL Ritonavir at wavelength 251 nm.

3.2Validation of the developed method

3.2.1 Linearity curve for the Emtricitabine[20-22]:

From the standard stock solution of Emtricitabine (100µg/mL) 1, 2, 3, 4 and 5 were pipetted out and transferred to separate 10 ml of volumetric flasks and the volume was made up to 10 ml with the help of mobile phase. These concentrations were of 10, 20, 30, 40 and 50 µg/ml respectively. The injection was given at time interval of 10 minutes with run time of 15 minutes. The linearity was obtained in selected conc. ranges. Linearity of Emtricitabine is shown in Table 1 and calibration plot in Fig 4.

Table 1: Linearity curve of Emtricitabine at wavelength 251 nm.

Sr. No	Conc. (µg/mL)	Area (µ volt sec.)
1	10	368524
2	20	673457
3	30	1023101
4	40	1329665
5	50	1612351

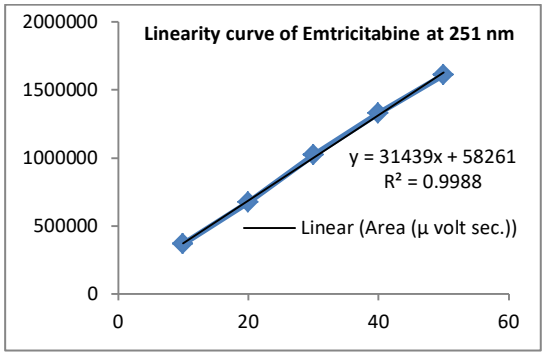

Figure 4: Calibration plot of Emtricitabine at 251nm

3.2.2 Linearity curve for the Ritonavir:

For Ritonavir from standard stock solution (100 µg/mL) 6, 7, 8,9 and 10 ml were pipetted out and transferred to separate 10 ml of volumetric flasks and the volume was made up to mark respectively with solvent. These concentrations were of 60, 70, 80, 90 and 100 µg/ml respectively. The linearity was obtained in selected conc. ranges. Linearity of Emtricitabine is shown in Table 2 and calibration plot in Fig 5.

Table 2: linearity curve of Ritonavir at wavelength 251 nm

Sr. No	Conc. (µg/mL)	Area (µ volt sec.)
1	60	807565
2	70	943472
3	80	1075238
4	90	1191207
5	100	1328949

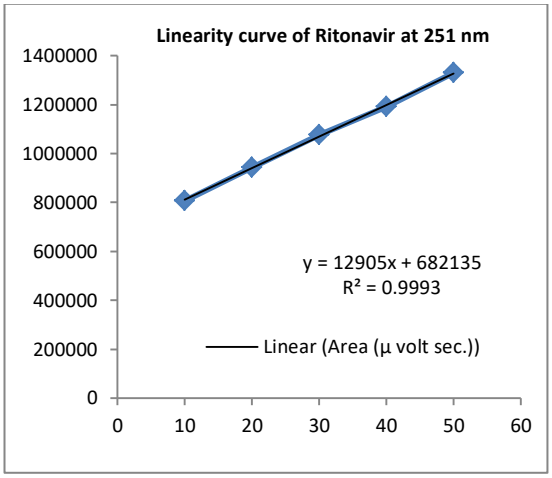

Figure 5: Linearity of Ritonavir at 251nm

3.2.3 Accuracy[23-26]

Accuracy is the closeness of the test results obtained by the method to the true value. Accuracy may often express in terms of percent recovery of assay of known amount of analyte added. Recovery studies were carried out by addition of standard drug to the sample at 3 different levels of spiking i.e. 80%, 100% and 120% of the actual amount taking into consideration percentage purity of added bulk drug sample. Accuracy of Emtricitabine and Ritonavir are shown in table 3 & 4 respectively.

Table 3: Accuracy of Emtricitabine at wavelength 251 nm.

Sr. No.	Amt. of sample Taken (µg/mL)	Amt. of drug added (µg/mL)	Level of addition (%)	Amount Recovered µg/mL			Mean	S.D.	% RSD	% Drug recovery
				1	2	3				
1	40	30	80%	69.73	69.75	69.77	69.75	0.020	0.028	99.79
2	40	40	100%	79.82	79.76	79.95	79.84	0.097	0.121	99.87
3	40	50	120%	89.74	89.80	89.83	89.79	0.045	0.051	99.88
Mean of % RSD									0.067	99.42
Mean of % Drug Recovery										

Table 4 : Accuracy of Ritonavir at wavelength 251 nm.

Sr. No.	Amt. of sample Taken (µg/mL)	Amt. of drug added (µg/mL)	Level of addition (%)	Amount Recovered µg/mL			Mean	S.D	% RSD	% Drug recovery
				1	2	3				
1	70	60	80%	129.70	129.85	129.74	129.76	0.077	0.059	99.55
2	70	70	100%	139.77	139.91	139.68	139.78	0.115	0.082	99.63
3	70	80	120%	149.89	149.80	149.75	149.81	0.070	0.047	99.71
Mean of % RSD									0.063	99.63
Mean of % Drug Recovery										

3.2.4Precision[27-29]

The precision of an analytical method is the degree closeness of agreement between a series of measurements obtained from the multiple sampling of the same sample. Precision include repeatability, inter and intraday precision and reproducibility.

3.2.4 Interday & intraday precision

Interday & intraday precision of conc. 20, 30, 40 µg/mL was prepared and data was obtained for Emtricitabine. 3 Replicates were prepared for 3 days. Intraday and Inter day precision of Emtricitabine are shown in table 5 & 7 respectively.

Interday & intraday precision of conc. 60, 70, 90 µg/mL was prepared and data was obtained for Ritonavir. 3 replicates were prepared for 3 days. The absorbance for intraday were measured in 2 hours of interval table 6 & 8 respectively.

Table 5: Intraday precision of Emtricitabine at wavelength 251 nm.

Conc.	20µg/mL	30 µg/mL	40 µg/mL
Area (µ volt sec.)	673457	1023101	1329665
	673455	1023112	1329660
	673452	1023115	1329662
Mean	673454.7	1023109	1329662
S D	2.516611	7.371115	2.516611
% RSD	0.000374	0.00072	0.000189

Table 6: Intraday precision of Ritonavir at wavelength 251 nm.

Conc.	70µg/mL	80 µg/mL	90 µg/mL
Area (µ volt sec.)	943472	1023118	1191212
	943474	1023115	1191207
	943475	1023117	1191212
Mean	943473.7	1023117	1191210
S D	1.527525	1.527525	2.886751
% RSD	0.000162	0.000149	0.000242

Table 7: Interday precision of Emtricitabine at wavelength 251nm

Conc.	20 µg/mL	30 µg/mL	40 µg/mL
Absorbance	673469	1023119	1329665
	673465	1023120	1329669
	673463	1023115	1329663
Mean	673465.7	1023118	1329666
S D	3.05505	2.645751	3.05505
% RSD	0.000454	0.000259	0.00023

Table 8: Interday precision of Ritonavir at wavelength 251 nm.

Conc.	70µg/mL	80 µg/mL	90 µg/mL
Area (µ volt sec.)	943472	1075239	1191207
	943474	1075241	1191214
	943475	1075242	1191212
Mean	943473.7	1075241	1191211
S D	1.527525	1.527525	2.886752
% RSD	0.000162	0.000142	0.000243

3.2.5 Repeatability[30]

For repeatability minimum of 6 determinants were prepared of 30 µg/mL and 80 µg/mL conc. Of Emtricitabine and Ritonavir, respectively The chromatogram responses were obtained by injecting one by one. The standard deviation & relative standard deviation was calculated for each type of precision. Repeatability of Emtricitabine and Ritonavir are shown in table 9 & 10 respectively.

Table 9: Repeatability of Emtricitabine at wavelength 251nm

Sr. No	Area (µ volt sec.)
1	1023119
2	1023120
3	1023115
4	1023118
5	1023115
6	1023117
Mean	1023117
S D	2.065591
%RSD	0.000202

Table 10: Repeatability of Ritonavir at wavelength 251nm

Sr. No	Area (µ volt sec.)
1	1075237
2	1075232
3	1075235
4	1075239
5	1075241
6	1075242
Mean	1075238
S D	3.777124
%RSD	0.000351

3.2.6Limit of detection (LOD)[31-32]

LOD is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. LOD was calculated by the standard deviation of the response and the slope.

$$LOD = \frac{3.3 \times \sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The slope and standard deviations were calculated from the linearity curve obtained for conc. ranges of 10-50 µg/mL for Emtricitabine and 50-100 µg/mL for Ritonavir

3.2.7 Limit of quantitation (LOQ) [33-35]

It is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOQ was calculated by the standard deviation of the response and the slope. The data was obtained from linearity curve and the LOQ was calculated.

$$LOQ = \frac{10 \times \sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The slope and standard deviations were calculated from the linearity curve obtained for conc. ranges of 15-50 µg/mL for Emtricitabine and 50-100 µg/mL for Ritonavir.

LOD & LOQ for Emtricitabine and Ritonavir are shown in table 11 & 12 respectively.

Table 11: LOD & LOQ of Emtricitabine at 251 nm wavelength

Conc.	Abs.	Amount Recovered µg/mL	% Drug recovery
10	368524	9.868729921	98.68729921
20	673457	19.56792519	97.83962594
30	1023101	30.68927129	102.297571
40	1329665	40.44034479	101.100862
50	1612351	49.43191577	98.86383155
Mean			99.75783793
SD			1.862712756
SE of Intercept			20816.33453
SD Of Intercept			46546.73905
LOD			4.885786407
LOQ			14.80541335

Table 12: LOD & LOQ of Ritonavir at 251 nm wavelength

Conc.	Abs.	Amount Recovered µg/mL	% Drug recovery
60	807565	59.71956606	99.5326101
70	943472	70.2509105	100.3584436
80	1075238	80.46137156	100.5767145
90	1191207	89.44773344	99.38637049
100	1328949	100.1212708	100.1212708
Mean			99.99508189
SD			0.517363878
SE of Intercept			15716.41021
SD Of Intercept			35142.96158
LOD			8.986576771
LOQ			27.23205082

3.2.8 Robustness [36-39]

Robustness is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameter. For HPLC robustness was carried out by changing wavelength and flow rate. Robustness of a method was done by change in wavelength, or change in flow rate of a mobile phase. Injection of 20 µg/mL was prepared from the stock solution and the recorded. Robustness data for Emtricitabine and Ritonavir are shown in Table 13 and 14.

Change in Wavelength

Table 13: Robustness of Emtricitabine and Ritonavir at wavelength 251±2 nm.

Wavelength	Difference	R_t of Emtricitabine (min.)	R_t of Ritonavir (min.)
249	-2	3.272	7.826
251	0	3.275	7.827
253	+2	3.278	7.829

Table 14: Robustness of Emtricitabine and Ritonavir at wavelength 251nm by changing the flow rate.

Flow rate (ml/min.)	Difference	Emtricitabine (min.)	Ritonavir (min.)
0.9	-0.1	3.278	7.829
1	0	3.278	7.828
1.1	+0.1	3.278	7.827

The overall validation parameter data are shown in Table 15. It complied with ICH guidelines.

Table 15: Summary of validation parameters of RP-HPLC at 251nm wavelength.

Parameter	Emtricitabine	Ritonavir
Linear range in (µg/mL)	10-50	60-100
Regression coefficient (R^2)	0.998	0.999
%Accuracy	99.42	99.63
Repeatability (n=6)	%RSD NMT 2	%RSD NMT 2
Precision Interday precision Intraday precision	%RSD NMT 2	%RSD NMT 2
LOD	4.885786407	8.986576771
LOQ	14.80541335	27.23205082

DISCUSSION

Reversed phase-high performance liquid chromatography (RP-HPLC)

The method was developed for the simultaneous estimation of Emtricitabine and Ritonavir using Reverse Phase – High Performance Liquid Chromatography. All the parameters were validated according to the ICH guidelines and meet all the limits. Various trials were taken for the Emtricitabine and Ritonavir at certain conditions. The linearity for method was obtained at 251 nm for Emtricitabine and Ritonavir. The R^2 values were found 0.9988 and 0.9993 for Emtricitabine and Ritonavir respectively. The R^2 value was within the limits of 0.995- 0.999, and has good linearity. For accuracy of method the % drug recovery was calculated for both drugs at wavelength 251 nm at conc. of 80, 100, 120% and average recovery was found 99.42 % for Emtricitabine and 99.63 % for Ritonavir. As the % drug recovery should be considered within limits i.e. 100 ± 2%. Interday precision was calculated by preparing dilutions of 20, 30 and 40 µg/ml conc. for Emtricitabine and 70, 80, 90 µg/ml concentrations for Ritonavir & responses were obtained at wavelength 251nm. Interday precision for Emtricitabine for 1st day the %RSD was found 0.0003, 0.0007 and 0.0001 at wavelength 251. For 2nd day the %RSD was found 0.0004, 0.0002 and 0.0002 at wavelength 251 nm. For 3rd day the %RSD was found 0.0003, 0.0001 and 0.0001% at wavelength 251 nm. The concentration 70, 80 and 90 µg/ml was used in triplicate at different days. Interday precision for Ritonavir for 1st day the %RSD was found 0.0001, 0.0001 and0.0002 at wavelength 251nm. For 2nd day the %RSD was found 0.0001,0.0001 and0.0002 at wavelength 251 nm. For 3rd day the %RSD was found 0.0002, 0.0001 and0.0002 at wavelength 251 nm. The concentration 20, 30, 40 µg/ml of Emtricitabine was used in triplicate at different days. For repeatability the % RSD was found 0.0001 % at wavelength 251 nm for Emtricitabine. The concentration 70, 80, 90 µg/ml of Ritonavir was used in triplicate at different days For Ritonavir the % RSD was found 0.0002% at wavelength 251nm i.e. within the limit. It shows that the method qualifies the criteria of repeatability. LOD & LOQ was calculated from the linearity curve at wavelength 251nm and the LOD was found 1.1350 µg/ml and LOQ was found 3.4395 µg/ml for Emtricitabine. For Ritonavir LOD was found 4.5240 µg/ml and LOQ was found 13.7091 µg/ml at wavelength 251 nm. Robustness of Emtricitabine and Ritonavir at 251 nm

The method was found robust as there was change in wavelength to ± 2 mL and change in flow rate of ± 0.1 mL/min.

4. CONCLUSION:

RP-HPLC method was developed for Emtricitabine and Ritonavir. The chromatographic condition for optimized method was found to consisting of Column C-18 (Shim-pack) 250 x 4.6 mm, particle size 5 µm , mobile phase methanol and water in the ratio 80:20. The retention time were found to be 3.25 and 7.8 min and average percentage recovery 99.42% and 99.63% for Emtricitabine and Ritonavir respectively. The proposed methods were found to comply with ICH guidelines. These methods can be further employed in future for the routine determination of Emtricitabine and Ritonavir in bulk drug.

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