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HPLC METHOD DEVELOPMENT AND VALIDATION FOR DETERMINATION OF RALTEGRAVIR IN BLOOD PLASMA

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ABSTRACT

A simple, precise and specific reverse phase high performance liquid chromatographic method has been developed and validated for the determination of raltegravir in blood. The HPLC separation was carried out by reverse phase chromatography on Shimadzu HPLC system consisted of Welchrom 5µ C_{18} column (250 X 4.6 mm), SPD 10A UV detector and LC 10AD Pumps. Rheodyne injector with 20 µl loop with a mobile phase composed of Acetonitrile : Water pH 2.5 (70:30) at a flow rate 1.0 mL/min. Naproxen was used as internal standard in the determination. The retention time of raltegravir and internal standard was found to be 5.7 and 10.2 respectively. The detection was monitored at 251 nm. The calibration curve for raltegravir was linear from 0.1-10µg/mL with correlation coefficient of 0.999. The interday and intraday precision was found to be within limits. The method was validated as per the guidelines.

KEYWORDS: **Raltegravir, HPLC Method, Reversephase chromatography, Validation,**

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INTRODUCTION

Raltegravir (RG *N*-(2-(4-(4-fluorobenzylcarbamoyl)-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl)propan-2-yl).
Raltegravir (RGV, Fig 1) antiretroviral drug used to treat HIV infection. It targets integrase, an HIV enzyme that integrates the viral genetic material into human chromosomes, a critical step in the pathogenesis of HIV[1]. According to the literature survey it was found that few methods have been reported for the determination of raltegravir in pure drug and in pharmaceutical dosage forms using HPLC (Rezk NL. et al., 2008; Poirier JM et al., 2008; Takahashi M et al., 2008; Notari S. et al., 2009; Talameh JA et al., 2010)[2-6] and LC-MS (Acosta EP et al., 2008; Decosterd LA et al., 2009; Heine RT et al., 2009; Wang LZ et al., 2011)[7-10] either in single or in combined forms**.** The proposed method was found to be simple, precise, accurate and rapid for determination of raltegravir. The mobile phase is simple to prepare and economical. Hence, this method can be easily and conveniently adopted for routine analysis of raltegravir in pure form and its dosage forms from blood plasma

MATERIALS AND METHODS

Raltegravir was obtained as a gift sample from Lupin lab, pune and Naproxen from Dr Reddys Lab, Hyderabad. Water and Acetonitrile used were of HPLC grade while Glacial acetic acid AR Grade.

EXPERIMENTAL

Instrumentation

Chromatographic separation was performed on Shimadzu HPLC system consisted of welchrom ODS 5µ C_{18} column (250 X 4.6 mm), SPD – 10 UV detector and LC 10 ADVP Pumps, Rheodyne injector with 20µL capacity. The mobile phase comprised of Acetonitrile : water, pH 2.5 (70:30) at flow rate 1.0 mL/min. The mobile phase was filtered through a 0.45 µ membrane filter and degassed for 30 min. Analysis was performed at ambient temperature. The detection was monitored at 251 nm.

Preparation of water pH 2.5

The HPLC grade water was maintained at a pH 2.5 using glacial acetic acid

Preparation of mobile phase

The mobile phase was prepared by mixing Acetronitile and water (pH 2.5) in the ratio of 70:30 v/v.

Study of beer- lamberts law

Raltigravir and Naproxen (Internal Standard, IS) stock solution was prepared using acetonitrile having a concentration of 1mg/mL. IS was further diluted to obtain a concentration of 10 µg/mL and Raltegravir standard stock was diluted to obtain 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, 7.5 and 10 µg/mL after extraction. To 0.1 mL of plasma, 20 µL of IS, raltigravir at different concentration were added to get the resultant concentrations as stated above, vortex for 5 minutes and proteins precipitated by adding 0.8 mL of acetonitrile and vertex for 15 minutes and centrifuged for 15 min at 4000 RPM. The supernatant was collected, evaporated to dryness and the residue was dissolved in 0.1 mL of mobile phase and after filtration 20 µL of the sample injected into HPLC. The peak area of drug and internal standard were noted and a graph of ratio of area vs concentration was plotted and correlation coefficient was determined. The representative chromatogram is shown in Fig 2a. The observations are shown in Table I and plot is shown in Fig 2b

Table I
Study of Linearity test

Drug(µg/mL)	Drug Area (mV)	Internal Std Area (mV)	PAR
0.1	3612.65775	185264.5	0.020
0.25	4866.322212	180234.156	0.027
0.5	9289.226853	182141.703	0.051
0.75	13331.59951	173137.656	0.077
1	16615.73625	171296.25	0.097
2.5	36592.78539	174251.359	0.210
5	71101.50474	169289.297	0.420
7.5	108990.08	170297	0.640
10	146937.225	177675	0.827

Table II
Observation of system suitability parameters

Sr. No.	Standard Weight + IS Taken	A.U.C of Raltegravir	A.U.C of Naproxen
1	~10.0 + 10.0 mg	4113671.875	5668412.750
2		4113558.472	5668452.253
3		4113559.962	5668473.125
4		4113562.486	5668476.324
5		4113567.247	5668462.157
Mean		4113584.008	5668455.322
±S.D.		44.034	22.920
Theoretical plate/column		5667	3956
Retention time		5.72	10.25
Asymmetry		1.362	1.254
Resolution		-	11.31

Table III
Results of recovery studies (accuracy)

Sr. No.	Amt. of Pure Drug Added (mg) + IS	Amount Recovered(mg)	Mean amt. of Drug Estimated (mg)	% Recovery
1	1.0 + 10.0	0.974	0.9747	97.47
		0.983		
		0.967		
2	3.0 + 10.0	2.932	2.965	98.83
		2.975		
		2.988		
3	30.0 + 10.0	29.11	29.38	97.93
		29.32		
		29.70		
			Mean	98.077
			± Overall SD	0.565

Table IV
Results of Precision study

Drug	Intraday Study		Inter-day Study	
Raltegravir	**% Estimated***	**% RSD**	**% Estimated***	**% RSD**
Level 1	99.13	0.210	98.23	0.385
Level 2	99.43	0.149	----	----

Each is a mean of three replicates

Table V
Results of stability studies

3 freeze and Thaw	**Conc. (µg/ml)**	**Trail 1**	**Trail 2**	**Trail 3**	**Mean**	**SD**
	0.3	0.25	0.24	0.25	0.247	0.006
	3	2.95	2.92	2.96	2.943	0.021
	30	29.93	29.95	29.97	29.950	0.020
24 hr incubation	**Conc. (µg/ml)**	**Trail 1**	**Trail 2**	**Trail 3**	**Mean**	**SD**
	0.3	0.24	0.25	0.250	0.26	0.010
	3	2.93	2.97	2.950	2.95	0.020
	30	29.96	29.93	29.947	29.95	0.015
1 month incubation	**Conc. (µg/ml)**	**Trail 1**	**Trail 2**	**Trail 3**	**Mean**	**SD**
	0.3	0.24	0.23	0.243	0.26	0.015
	3	2.95	2.93	2.947	2.96	0.015
	30	29.95	29.97	29.950	29.93	0.020

Table VI
Observation of Robustness study

Sr. No.	Deliberate Changes	A.U.C. (Sample)	R.T.	Asymmetry
1.	Standard Condition	4113562.486	5.721	1.303
2.	Change in Flow Rate (1.1 mL/min)	3856162.625	5.145	1.215
3.	Change in Flow Rate (0.9 mL/min)	4369257.750	6.286	1.388
4.	Wavelength change (256 nm)	4437991.545	5.718	1.264
5.	Wavelength Change (246 nm)	3780794.500	5.724	1.229
6.	Change in Mobile Phase Conc. (73:27)	2635966.25	6.023	1.225
7.	Change in Mobile Phase Conc. (67:33)	5563831.00	6.248	1.304
8.	Change in pH (2.3)	4020038.803	6.220	1.556
9.	Change in pH (2.7)	4226704.578	6.313	1.425

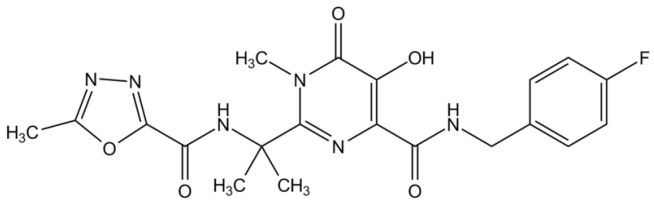

Figure 1
Structure of raltegravir

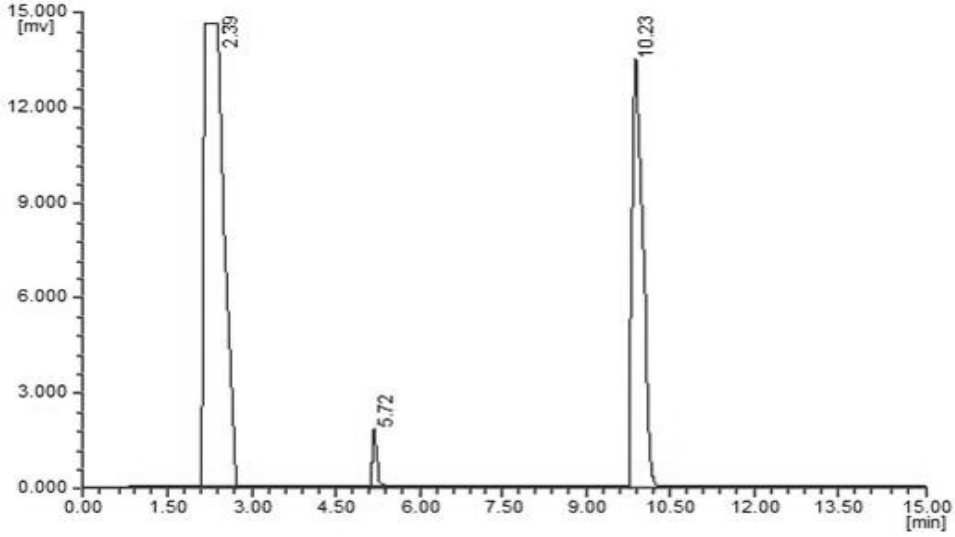

Figure 2a
Representative chromatogram of 0.75 µg/ml of Raltegravir + 10 µg/ml of IS

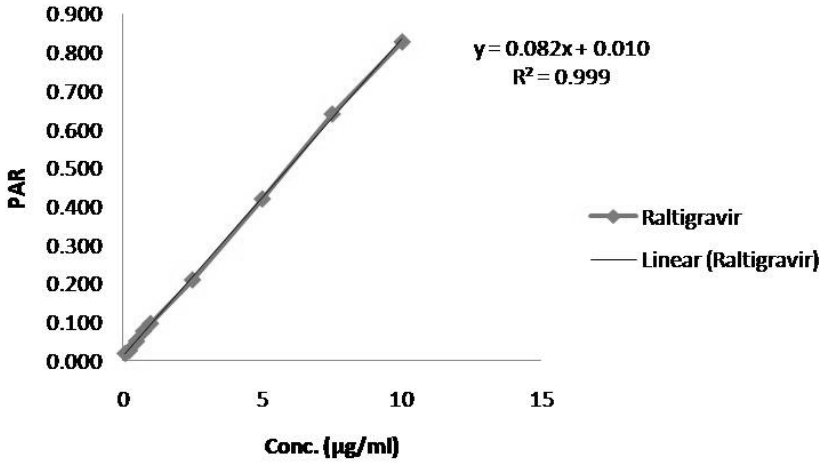

Figure2b
Plot of linearity study

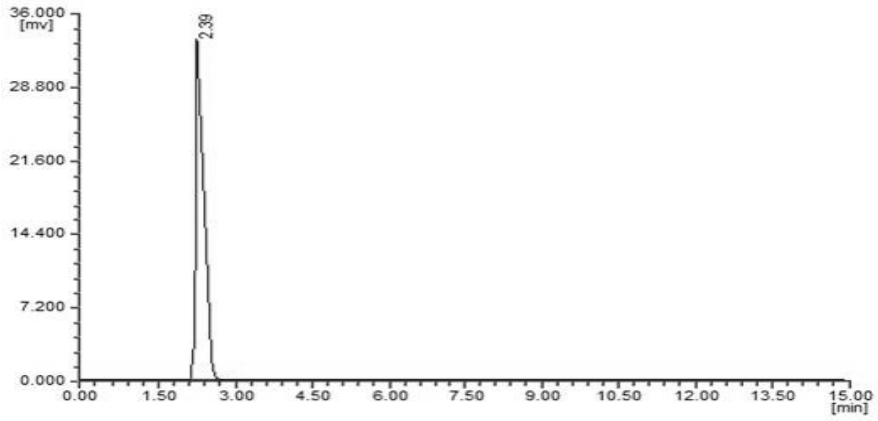

Figure 2a
Chromatogram of blank

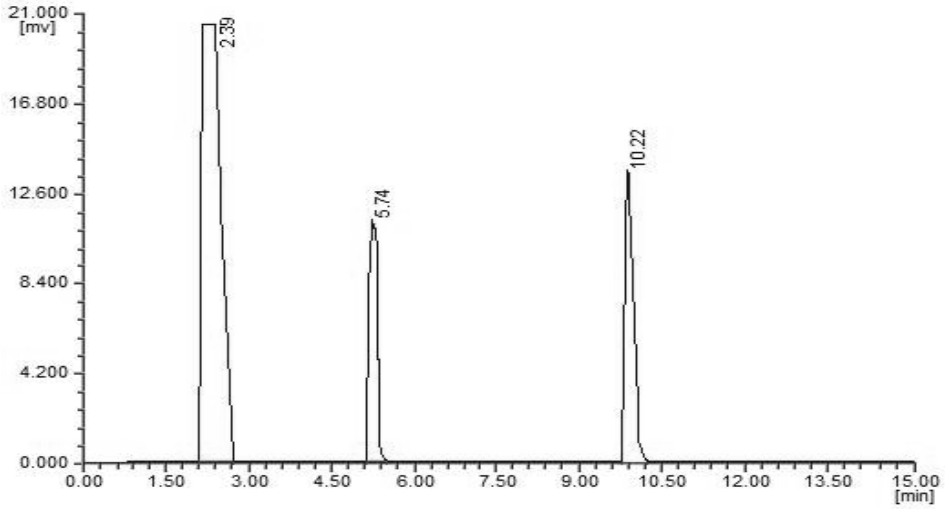

Figure 2b
Chromatogram of bulk drug and internal standard (sample)

RESULTS AND DISCUSSION

Method Validation

The method was validated as per the guidelines in terms of parameters like, precision, accuracy (recovery studies), system suitability parameters, linearity and range etc.

Study of system suitability parameters

System suitability parameters can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validation have been completed. The USP (2000) defines parameters that can be used to determine system suitability prior to analysis. The results are shown in Table II

Preparation of sample

Mix standard solution of raltegravir and internal standard naproxen having a concentration of 10µg/mL each was prepared in mobile Phase. A 20 µL solution was injected through manual injector and chromatographed. The chromatogram blank and sample so obtained are shown in Fig 3a and 3b.

Specificity

The specificity of the method was used to determine any interference in the chromatogram during the retention time of both Raltigravir and Naproxen.

Accuracy

It was calculated by comparing the measured values and the true values and expressed as SD. Three concentration levels were selected at low (1.0µg/mL), medium (3µg/mL) and high (30µg/mL) and three replicates of each level were injected into the system and SD was calculated. The SD value was found to be within the range. Results are shown in Table III

Precision

Precision of proposed method was ascertained by replicate analysis of homogenous samples. The solution was prepared in the same manner as described in the beer lamberts law. Percentage relative standard deviation (% RSD) was found to be 0.256 which is less than 2%, proves that method is precise.

Intraday and Interday precision

In order to assess the intra- and inter-day precision and accuracy, Raltigravir concentrations were prepared at two levels. The intra-day precision of the assay was assessed by calculating the coefficient of variation (CV) for the analysis of samples in three replicates and twice in a day. And inter-day precision was determined by the analysis of samples on three consecutive days. The summary of results of the studies are shown in Table IV

Stability

To access the stability of the drug during storage conditions The Drug was evaluated for stability after 3 freezing and thawing cycles, after 24 hours and 1 month storage at -20^0c. The stability is determined by comparing the concentration before and after the storage conditions. The results are shown in Table V

Robustness

The robustness of an analytical procedure measures its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The system suitability parameter was evaluated for each varied condition. Observations are shown in Table VI.

a) Change in Flow Rate
b) Change in pH of Mobile Phase Buffer
c) Change in Mobile Phase Concentration
d) Change in wavelength

LOD & LOQ Determination

Lower limit of detection and lower limit of quantification was determined by using the formula

$$LOD = \frac{3.3 * Std\ Dev}{Slope}$$

$$LOQ = \frac{10 * Std\ Dev}{Slope}$$

The LOD and LOQ were found to be 0.259 and 0.786 3µg/mL respectively.

DISCUSSION AND CONCLUSION

From the optical characteristics of the proposed method it was found that the drug obeys linearity range within the concentration of 0.1-10µg/mL. The results found reveals that percent RSD is less than 2%, which indicates that the method has good reproducibility (precision) and accuracy determined was found to be 98.62, indicating that the method is accurate. The system suitability parameters are within the specified limits. The stability study reveals that the sample was found to be stable and percent RSD was found to be below 2. The method was found to be accurate, precise and robust. Hence, this method can be easily and conveniently adopted for routine analysis of raltegravir in pure form and its dosage forms.

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