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Method development and validation of raltegravir by RP-HPLC method

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ABSTRACT

Objective

The objective of the present research work was to develop an innovative, simple, and economic method for estimation of Raltegravir in bulk and dosage form by RP-HPLC.

Methods

The chromatographic conditions were performed on Symmetry Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d. as stationary phase and mobile phase was prepared with a mixture of Phosphate buffer (pH=3.0) : Methanol with 30:70, flow 1.0 ml/min, with Injection Volume 10µl, at detection wavelength 246 nm and run time at 5.0 min.

Results

The analytical method is valid for estimation of Raltegravir over a range of 20 µg/ml–70 µg/ml. The results of system suitability test, linearity, precision and accuracy, robustness, specificity, LOD and LOQ and stabilities presented in this report are within the acceptance range.

Conclusion

A specific, sensitive, economic method estimation of Raltegravir has been developed based on ICH Guidelines with bulk and dosage forms.

Keywords: Raltegravir, HPLC, Method Development, ICH, Validation, Accuracy, Precision.

INTRODUCTION

Raltegravir (RAL), sold under the brand name Isentress, is an antiretroviral medication used, together with other medication, to treat HIV/AIDS. [1] It may also be used, as part of post exposure prophylaxis, to prevent HIV infection following potential exposure. It is taken by mouth. [2] Common side effects include trouble sleeping, feeling tired, nausea, high blood sugar, and headaches. [3] Severe side effects may

include allergic reactions including Stevens-Johnson syndrome, muscle breakdown, and liver problems. It is unclear if use during pregnancy or breastfeeding is safe. [4] Raltegravir is an HIV integrase strand transfer inhibitor which blocks the functioning of HIV integrase which is needed for viral replication. As an integrase inhibitor, raltegravir targets integrase, an HIV enzyme that integrates the viral genetic material into human chromosomes, a critical step in the pathogenesis of HIV. The drug is metabolized away via glucuronidation. [5-7] Raltegravir significantly alters HIV viral dynamics and decay and further research in this area is ongoing. In clinical trials patients taking raltegravir achieved viral loads less than 50 copies per millitre sooner than those taking similarly potent non-nucleoside reverse transcriptase inhibitors or protease inhibitors. [8] This statistically significant difference in viral load reduction has caused some HIV researchers to begin questioning long held paradigms about HIV viral dynamics and decay.[9] Research into raltegravir's ability to affect latent viral reservoirs and possibly aid in the eradication of HIV is currently ongoing. [10-13] The major mechanism of clearance of raltegravir in humans is glucuronidation mediated by UGT1A1, the renal clearance of unchanged drug is a minor pathway of elimination of raltegravir (9% of total dose). Raltegravir inhibits HIV integrase to prevent the viral genome being incorporated into the human genome. Raltegravir is primarily metabolized by glucuronidation. [14-16]

The IUPAC Name of Raltegravir is N-[(4-fluorophenyl)methyl]-5-hydroxy-1-methyl-2-{2-[(5-methyl-1,3,4-oxadiazol-2-yl)formamido]propan-2-yl}-6-oxo-1,6-dihydropyrimidine-4-carboxamide. [17]

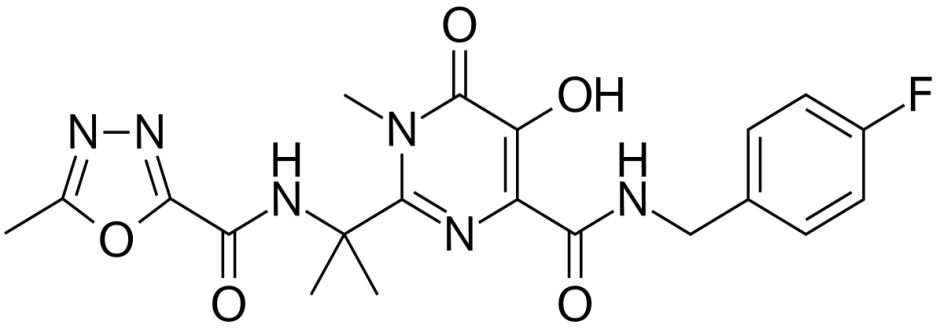


Fig-1: Structure of Raltegravir

A survey of literature reveals that good analytical methods are not available for Raltegravir.[18] The present research manuscript describes innovative, simple, economical, accurate, specific, robust, rugged and rapid RP-HPLC method developed in selected solvent system (Mobile Phase) and validated in accordance with International Conference on Harmonization (ICH) Guidelines Q2 (R1), for the estimation of Raltegravir in bulk drug and in its dosage forms. [19-20]

EXPERIMENTAL

Materials and Methods

Pharmaceutical grade working standard Raltegravir were obtained from Syncorp Pvt. Laboratories, Hyderabad, India. All chemicals and reagents were HPLC grade and were purchased from S D Fine-Chem Limited & Loba Chemie Pvt Ltd, Mumbai, India.

Instrumentation

The analysis was performed using HPLC (Waters-717 series) with PDA detector and data handling system EMPOWER2 software, UV-Visible double beam spectrophotometer (T60-LAB INDIA), analytical balance 0.1mg Sensitivity (SHIMADZU), pH meter (Labindia), ultra sonicator. The column used is Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d. (as Stationary phase) with the flow rate 1.0ml/min (isocratic).

Sample & Standard Preparation for the Analysis

25 mg of Raltegravir standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with solvent. Further dilution was done

by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with solvent.

Selection of wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis. It is scanned in the UV spectrum in the range of 200 to 400nm. While scanning the Raltegravir solution we observed the maxima at 246 nm.

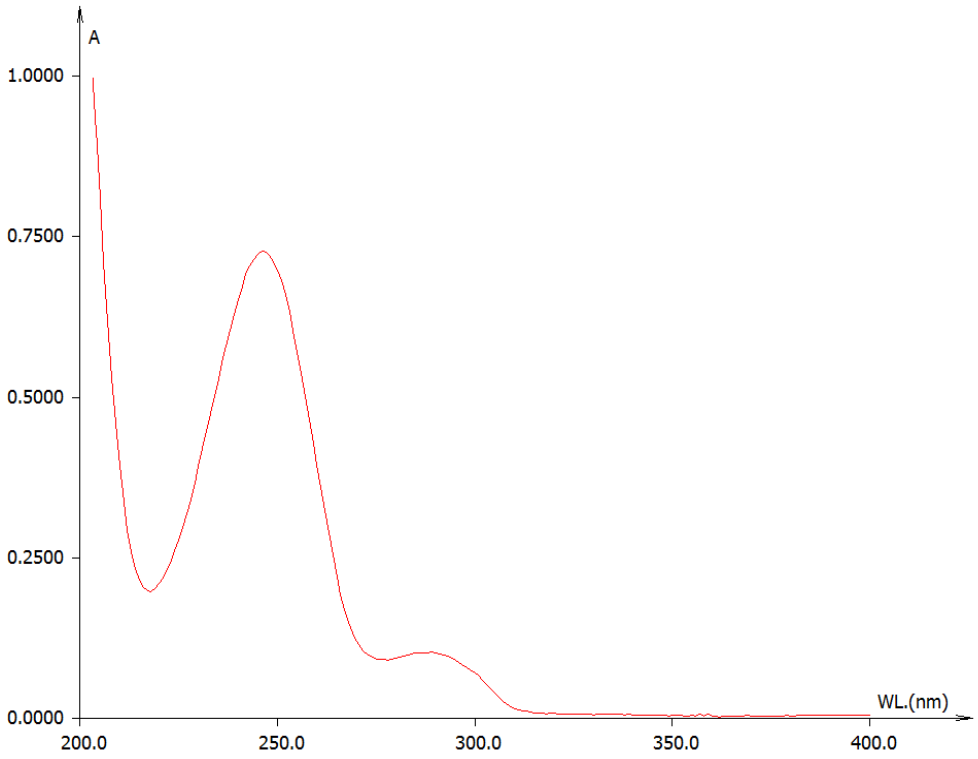


Fig-2: UV Spectrum for Raltegravir

METHOD DEVELOPMENT

Preparation of Mobile Phase

The mobile phase used in this analysis consists of a mixture of Phosphate Buffer (pH 3.0) and Methanol in a ratio of 30:70. 300 ml of this buffer solution was added and properly mixed with 700 ml of Methanol and a homogenous solution is achieved. This mobile phase was filled and sonicated for 15 minutes before using in the experiment.

Summary of Optimized Chromatographic Conditions

The Optimum Chromatographic conditions obtained from experiments can be summarized as below:

Table-1: Summary of Optimized Chromatographic Conditions

Column	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.
Mobile phase	Phosphate buffer (pH=3.0) : Methanol= 30:70
Wavelength	246nm
Flow rate	1.0 ml/ min.
Column temperature	Ambient
Injection Volume	10 µl
Run time	5 mins

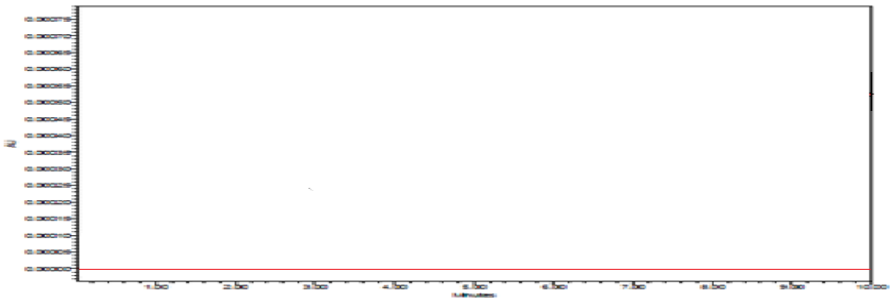
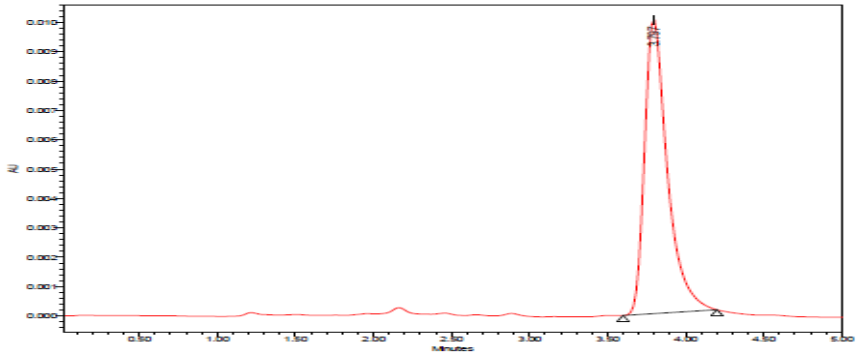


Fig-3: Chromatogram for Blank Preparation



Fig–4: Chromatogram of Raltegravir in Optimized Condition

METHOD VALIDATION

Linearity & Range

Calibration standards at five levels were prepared by appropriately mixed and further diluted standard stock solutions in the concentration ranges from 20-70µg/ml for Raltegravir. Samples in triple injections were made for each prepared concentration. Peak areas were plotted against the corresponding concentration to obtain the linearity graphs. Chromatograms of each solution were recorded.

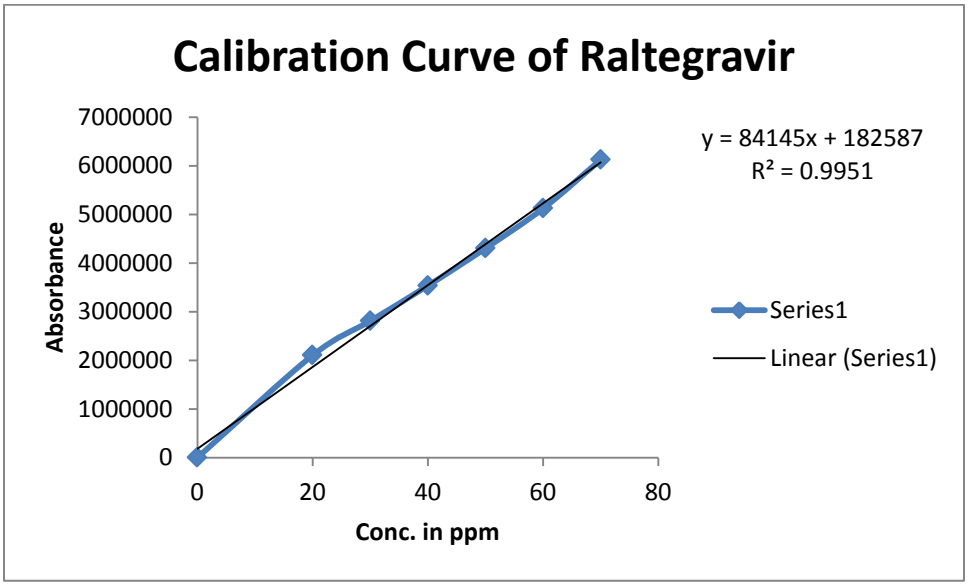


Fig-5: Calibration Curve of Raltegravir (API)

Table-2: Concentration of Raltegravir

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
20	2103282
30	2809668
40	3535360
50	4302725
60	5122592
70	6123521

Accuracy

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Raltegravir were taken and added to the pre-analyzed formulation of concentration 50µg/ml. From that percentage recovery values were calculated. The results were shown in table-3.

Table-3: Accuracy Readings of Raltegravir

S. No.	Pure drug	Peak Area	Conc. Found	% Recovery of Pure drug	Statistical analysis
S_1 : 80 %	40	3423142	40.46	101.15	Mean= 101.0917
S_2 : 80 %	40	3424651	40.48	101.20	S.D. = 0.146487
S_3 : 80 %	40	3415214	40.37	100.925	R.S.D.= 0.144905%
S_4 : 100 %	50	4251284	50.30	100.60	Mean= 100.32
S_5 : 100 %	50	4223124	49.97	99.94	S.D. = 0.341174
S_6 : 100 %	50	4243414	50.21	100.42	R.S.D.= 0.340086%
S_7 : 120 %	60	5124752	60.68	101.133	Mean= 100.5217
S_8 : 120 %	60	5123654	60.67	101.116	S.D. = 1.044173
S_9 : 120 %	60	5032564	59.59	99.316	R.S.D. = 1.038754%

Precision

Repeatability

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Raltegravir (API). The percent relative standard deviation was calculated for Raltegravir are presented in the Table-4.

Table-4: Repeatability Readings of Raltegravir

HPLC Injection Replicates of Raltegravir	Retention Time	Area
Replicate – 1	3.797	10613428
Replicate – 2	3.799	10576247
Replicate – 3	3.801	10353604
Replicate – 4	3.802	10576247
Replicate – 5	3.805	10176752
Replicate – 6	3.803	10325641
Average	**3.801166667**	**10436986.5**
Standard Deviation	**0.002857738**	**177195.3912**
% RSD	**0.0751875743**	**1.697763921**

Intermediate precision

The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations

for Raltegravir revealed that the proposed method is precise.

Table-5: Results of Intra-Assay & Inter-Assay

Conc. Of Raltegravir (API) (µg/ml)	Observed Conc. Of Raltegravir (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
40	40.41	0.47	40.29	0.29
50	50.32	0.38	50.13	0.30
60	60.31	0.57	60.19	0.48

Method Robustness

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Wavelength of detection (±2nm) & organic phase content in mobile phase (±5%) studied to determine the robustness of the method are also in favour of (Table-6, % RSD < 2%) the developed RP-HPLC method for the analysis of Raltegravir (API).

Table-6: Results of Method Robustness Test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.38
Flow (0.9 ml/min)	0.32
More Organic	0.08
Less Organic	0.04
Wavelength of Detection (248 nm)	0.21
Wavelength of detection (244 nm)	0.19

LOD & LOQ

- The detection limit (LOD) and quantitation limit (LOQ) may be expressed as:
- L.O.D. = 3.3(SD/S).
- L.O.Q. = 10(SD/S)
- Where, SD = Standard deviation of the response
- S = Slope of the calibration curve
- The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.06 & 0.36 µg/ml respectively.

System Suitability Parameter

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-7.

Table-7: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs > 2	9.81
2	Asymmetry	T ≤ 2	Raltegravir=0.13
3	Theoretical plate	N > 2000	Raltegravir=3355
4	Tailing Factor	T<2	Raltegravir=1.22

Estimation of Raltegravir in Tablet Dosage Form

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 100 mg of drugs were transferred to 100 ml volumetric flask, and 70 ml of HPLC grade

methanol was added and solution was sonicated for 15 minutes, there after volume was made up to 100 ml with same solvent. Then 10 ml of the above solution was diluted to 100 ml with HPLC grade methanol. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. From this stock solution (3.5 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system. The solution prepared was injected in five replicates into the HPLC system and the observations were recorded. A duplicate injection of the standard solution was also injected into the HPLC system and the peak areas were recorded.

Table-8: Assay of RALTEGRAVIR Tablets

Brand name of Raltegravir	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Isentress Tablets (400 mg) (Apple Pharmaceuticals, Mumbai, Maharastra)	400mg	397.21 (± 0.426)	99.302 (± 0.363)

RESULTS

The optimized chromatographic conditions were Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d. as stationary phase and mobile phase was prepared with a mixture of Phosphate buffer (pH=3.0) : Methanol with 30:70, flow 1.0 ml/min, with Injection Volume 10µl, at detection wavelength 246 nm and run time at 5.0 min. In these chromatographic conditions the peak was pure, sharp, symmetric and found a greater number of theoretical plates.

The results obtained in method validation were

Linearity & Range: The calibration curve showed good linearity in the range of 20-70 µg/ml, for Raltegravir (API) with correlation coefficient (r^2) of 0.995 (Fig-5). A typical calibration curve has the regression equation of y = 84145x + 18258. for Raltegravir.

Accuracy

The mean recoveries were found to be 101.0917, 100.32 and 100.5217 % for Raltegravir. The limit for mean % recovery is 98-102% and as both the values are within the limit, hence it can be said that the proposed method was accurate.

Repeatability

The repeatability study which was conducted on the solution having the concentration of about 50 µg/ml for Raltegravir (n =6) showed %RSD of 1.697763921%. It was concluded that the analytical technique showed good repeatability.

LOD & LOQ

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.06 & 0.36 µg/ml respectively.

Assay: The assay in Isentress Tablets containing Raltegravir was found to be 99.302 %.

DISCUSSION:

To develop a precise, linear, specific RP-HPLC method for analysis of Raltegravir, different chromatographic conditions were applied & the results observed were compared with the methods available in literatures.

R. Ravi Chandra Babu, et al.[21] achieved separation by using ammonium formate : acetonitrile (20:80, v/v) as mobile phase. A. Lakshmana Rao, et al [22] developed method by using a mobile phase in combination of phosphate buffer pH 2.5 and acetonitrile in the ratio of (40:60 v/v) but we have used Phosphate buffer (pH=3.0) : Methanol with (30:70). As per T.Sudha, et al. [23] used Symmetry C18 (4.6 x 150mm, 5 µm XTerra) column. Rambabu Kuchi, et al.[24] used kromosil C18 column (250 x 4.6mm, 5 µm). Khagga Bhavyasri, et al. used Hypersil BDS, C18, 100 x 4.6 mm, 5 m column and mobile phase used is Buffer and acetonitrile taken in the ratio in the ratio of 60:40(v/v) used as stationary phase.

The result shows the developed method is yet another suitable method for assay which can help in the analysis of Raltegravir in formulations.

CONCLUSION

A sensitive & selective stability indicting RP-HPLC method has been developed & validated for the analysis of Raltegravir API. Based on peak purity results, obtained from the analysis of samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of Raltegravir indicated that the developed method is specific for the estimation of Raltegravir. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.

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