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Original Article

Quantitative Reverse-Phase High-Performance Liquid Chromatographic method for the quantification of Raltegravir Potassium in bulk and dosage forms

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ABSTRACT

Objectives: The aim of this present study is to develop an accurate, precise and linear Reverse-phase High-performance Liquid Chromatographic (RP-HPLC) method for the estimation of raltegravir potassium in the bulk and pharmaceutical dosage form. **Methods:** The chromatographic system employs a reverse phase shim-pack C_{18} column, (150 x 4.6 mm; 5 µ) using the mobile phase acetonitrile: (0.05 M) ammonium acetate buffer, (pH -4 adjusted with glacial acetic acid) in the proportion of 50:50 v/v, delivered at a flow rate of 0.8 ml/min with the detection wavelength of 271 nm. **Results:** The developed method resulted in the retention of raltegravir at 4.31 min. Raltegravir potassium exhibited linear relationship (r^2> 0.9999) over the analytical range 10-50 µg/ml. The precision was exemplified by a relative standard deviation of 1.60 %. The percentage recovery was found to be in the range of 100-102 %, during accuracy studies. The Limit of Detection (LOD) and Limit of Quantitation (LOQ) was found to be 0.104 µg/ml and 0.315 µg/ml, respectively. **Conclusion:** An accurate, precise and linear RP-HPLC method was developed and validated for the quantitative estimation of raltegravir potassium in (20 mg, 50 mg) tablet as per ICH guidelines and hence it can be used for the routine analysis in various pharmaceutical industries.

Key words: RP-HPLC, Method Development, Method optimization, Validation, Raltegravir potassium.

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INTRODUCTION

Highly Active Antiretroviral Therapy (HAART) has brought new hope for those people who are suffering from HIV/AIDS by decreasing the death rate among people infected with HIV. HAART enhanced the quality of life among the people who have HIV/AIDS.

Raltegravir potassium is an HIV integrase strand transfer inhibitor, which prevents the insertion of viral DNA into the genome of the host cell and consequently viral replication. Raltegravir potassium, chemically is a potassium salt of 4-[(4-fluorobenzyl)carbamoyl]-1-methyl-2-(1-methyl-1-{[(5-methyl-1,3,4-oxadiazol-2-yl)carbonyl]amino}ethyl]-6-oxo-1,6-dihydropyrimidine-5-olate. (Figure 1)[1] A thorough review of the literature was carried out to enumerate the reported analytical methods for the quantification of raltegravir potassium individually and incombination with other drugs using various analytical techniques.

Girija B and co-worker have reported a simple spectrophotometric method for estimation of raltegravir potassium in bulk and tablet formulation. The method was validated as per ICH guidelines.[2] Balaji M and co-workers had reported an RP-HPLC method for estimation of related compounds (imp-I, imp-II, imp-III and imp-IV) in raltegravir potassium by using purosphere star RP C_{18} column. The separation was performed in gradient elution mode using 0.1% perchloric acid and acetonitrile at 30°C and the eluents were detected at 300 nm with a flow rate 1 ml/min.[3] Antonio DA and co-workers had reported an accurate, sensitive, HPLC coupled with photodiode array detection method for the quantification of human immunodeficiency syndrome integrase inhibitor Raltegravir (RGV), Etravirine (ETV) and 11 other antiretroviral agents in human plasma.[4] Ajay G and co-workers had reported a novel fast and simple method for the quantification of raltegravir with RP-HPLC-MS method. The chromatographic separation was achieved on a Chromolith RP-18e end capped (100 x 4.6 mm; 5 µm) column with a shorter runtime of 2.0 min. The mobile phase was ammonium formate buffer (pH 4.0): acetonitrile (30:70) in isocratic mode.[5] Pardhi SS and co-workers, in their review article, stated that Highly Active Antiretroviral Therapy (HAART), a combination drug therapy is a topic of current interest in the treatment of HIV and AIDS. Techniques for the analysis and the quality control of antiretroviral drugs, particularly in the drug combinations are vital in achieving the quality of these drugs and the treatments involved. Integrase inhibitors are a class of antiretroviral drug designed to block the action of integrase, a viral enzyme that inserts a viral genome into the DNA of the host cell. Since integration is a vital step in retroviral replication, blocking it can halt further spread of the virus. Integrase inhibitor was initially developed for the treatment of HIV infection. The HPLC, UV and HPTLC methods available for the analysis of raltegravir and Elvitegravir, the recently used drug for HIV and AIDS are reviewed in this article.[6] Khagga BS and co-workers had reported a validated simple, shorter and effective HPLC method with UV detection (213 nm) for the quantification of raltegravir amidst its degradation products using an Hypersil BDS, C_{18} (100 x 4.6 mm, 5 µm) column and water: methanol (20:80) as a mobile phase. The characterization of degradants were carried by LC-MS/MS.[7] Juan PV and co-workers worked on an analytical methodology based on micellar liquid chromatography for the quantification of abacavir, lamivudine and raltegravir in plasma. In the experimental procedure, the samples were diluted in micellar media and the analytes were resolved in less than 30 min, through

a C_{18} column (125 x 4.6 mm; 5 µm).[8] Rambabu K and co-worker had reported a RP-HPLC method for the analysis of raltegravir. Chromatographic separation of raltegravir was performed by using a kromosil C_{18} column, a mobile phase comprising of 0.01 M Ammonium dihydrogen phosphate (pH 3.4): acetonitrile (50:50 v/v) at a flow rate of 1.0 ml/min and UV detection at 253 nm.[9] Rami RBV and co-worker had reported a Reverse-phase Ultra Performance Liquid Chromatographic (RP-UPLC) method for the assay of raltegravir Potassium. The chromatographic separation was achieved on UPLC with BEH Shield 100 x 2.1 mm, 1.7 µm column. The mobile phase employed for the assay was a mixture of Sodium perchlorate (pH 2.5±0.05 with perchloric acid) and acetonitrile in the ratio of 65:35 (v/v) and the detection wavelength was 240 nm.[10] From the literature survey, it was evident that most of the methods were utilizing phosphate buffer for the quantification of raltegravir. Thus the present study aims to develop and validate a simple, rapid, precise, accurate, highly sensitive and selective RP HPLC method which is compatible for LC-MS/MS and can be routinely used for quality control studies. The method showed a recovery of 100-102 % with a simple mobile phase composition of ammonium acetate buffer (pH 4) and acetonitrile (50:50 v/v) in isocratic mode with shim pack C_{18} column. This developed method is robust enough to transfer into LC-MS/MS and HR/MS characterization for the determination of raltegravir potassium in bulk and pharmaceutical formulations.

This developed RP-HPLC method was validated as per the ICH guideline.[11,12]

MATERIALS AND METHODS

Chemicals

Acetonitrile HPLC grade, methanol and ammonium acetate AR grade were procured from Merck (Mumbai, India). Milli Q water purification system (Bangalore, India) was used to generate Water HPLC grade. Reference substance of raltegravir potassium (API) was obtained from Mylan laboratory, Hyderabad, India as a gift sample. The raltegravir formulation was procured from the local market.

Instrumentation

HPLC experiments were performed on a Shimadzu LC-2010A Autosampler (Shimadzu Corporation, Kyoto, Japan). The system was equipped with a Liquid Chromatograph system comprising LC-2010A Quaternary low pressure mixing pump, Shimadzu SPD-20A UV-VISIBLE detector, a shim pack RP-C_{18} column (150 X 4.6 mm; 5 µ) and an autosampler with 20 µL aliquots sample loop volume. The software for data collection and analysis in the HPLC system used was Class VP data station. The UV spectra were recorded using Shimadzu 1700 (E) spectrometer.

Selection of Wavelength

A standard solution of 1 mg/ml raltegravir potassium was prepared in methanol. From the above stock solution, 10 µg/ml solution was prepared with the mobile phase,and the UV spectra were recorded by scanning the standard solution between 200-400 nm. Raltegravir showed maximum absorbance at 271 nm (Figure 2). Thus it was selected for monitoring the chromatographic eluents using a UV detector.

Standard and sample solutions Preparation

Primary standards of raltegravir potassium was prepared by dissolving 10 mg of raltegravir potassium with mobile phase in a 10 ml volumetric flask (1 mg/ml). From the above standard stock solution, series of dilutions viz., 100 µg/ml, 10 µg/ml and 1 µg/ml were prepared in the mobile phase and was used as 100 % target concentration.

Assay of the marketed formulations

10 tablets of raltegravir potassium were weighed and the average weight was calculated and powdered. The tablet powder equivalent to 10 mg of raltegravir potassium was transferred to 10 ml volumetric flask. About 7 ml of mobile phase was added and sonicated to dissolve it completely. The volume was made up to the mark with the mobile phase. Then it was mixed well and filtered through 0.45 µm filter (1000 µg/ml). The above solution was suitably diluted with mobile phase to obtain the final concentration (10 µg/ml). This was analyzed in triplicate using the optimized chromatographic condition. The chromatograms were recorded and the amount present, standard deviation and % RSD were calculated and reported.

Method validation

The developed RP-HPLC method for the quantification of raltegravir potassium was validated as per the ICH Guidelines for the following parameters: specificity, linearity, precision, accuracy, detection limit, quantification limits, Robustness and Ruggedness.

Specificity

Specificity is the ability of the method to measure the response of the analyte in the presence of other excipients and potential impurities. The specificity was demonstrated by comparing the chromatogram of drug extracted from the tablet with that of standard solution for the presence of excipients, potential impurities and other degradants. No interference was observed in the sample solution at the retention time of raltegravir potassium.

Linearity and range

The linearity of an analytical method indicates the response obtained, which is linearly proportional to the concentration of the analytes in a definite range. The linearity of the proposed method was evaluated over a range of 10-50 µg/ml. These working standards were prepared in mo-

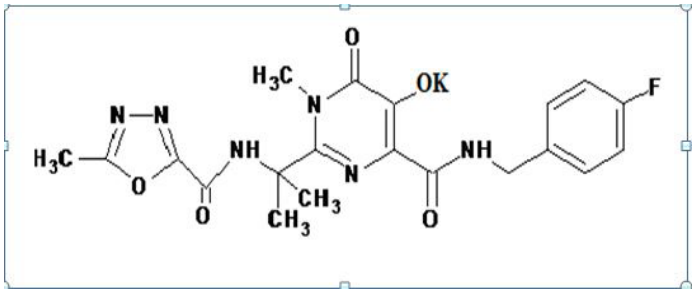

Figure 1: Structure of Raltegravir potassium.

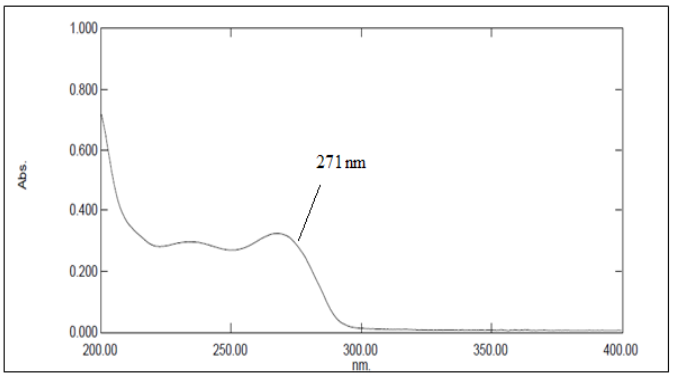

Figure 2: Typical UV spectra of Raltegravir potassium.

bile phase from 1 mg/ml standard stock solution. The working standards for the linearity were injected in triplicate under the optimized chromatographic conditions and the chromatograms were recorded. The linearity was established based on the correlation coefficient obtained by plotting a graph with concentration (µg/ml) in X-axis and Area of raltegravir potassium in Y-axis.

Precision and accuracy Studies

Interday and intraday precision studies evaluated the method precision. Six independent injections of three different concentrations viz., 10, 30, 50 µg/ml (LQC, MQC and HQC level) were used to study the method precision. Intraday precision/Repeatability was carried out by injecting the above samples on the same day and inter-day precision was carried out by injecting the same samples on three different days. The mean and values of % relative standard deviation were calculated.

The accuracy of the method was reported as recovery studies. It was carried out by standard addition method, i.e., by addition of known concentration of standard drug to the real sample and analysis by the optimized chromatographic condition. The recovery experiments were studied at three different levels (10, 30, 50 µg/ml) and the % mean recovery, standard deviation and % relative standard deviation were calculated.

Limit of detection and limit of quantification

A method is considered sensitive when it is capable of detecting a low concentration of the analyte. The Detection Limit (LOD) and Quantification Limit (LOQ) was determined by analyzing a low concentration of the standard solution using the developed RP-HPLC method. The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio 3). The LOQ is the smallest concentration of the analyte, which gives a response that can be accurately quantified (signal to noise ratio 10). The following formula can measure LOD and LOQ value:

LOD = 3.3 σ/S and LOQ = 10 σ/S;

Where σ = Standard deviation of the response; S = Slope of the deviation curve.

Robustness and Ruggedness

The ruggedness and robustness of the methods were studied by making slight changes in the experimental conditions (Analyst, Reagent source and columns of various brands) and optimized chromatographic conditions (pH, mobile phase composition, mobile phase ratio and flow rate).

System suitability

System suitability parameters are considered as an essential part in analytical method development and validation. They ensure the optimal performance of the system. Chromatographic parameters *Viz*., Number of theoretical plates (*N*) Retention time (*Rt*), Resolution (Rs) and peak asymmetric factor (A) were monitored after six replicate injections of the standard raltegravir at a concentration of 100 µg/ml.

RESULTS AND DISCUSSION

Method development

The chromatographic conditions for the present method were optimized based on various trial experiments carried out by modifying the mobile phase composition, mobile phase ratio, pH and column utilized to achieve symmetric analyte peak and short run time. Acetonitrile was used as an organic modifier in the mobile phase.[13,14] Initial separations were carried out using acetonitrile and water as mobile phase at different ratios, which showed peak asymmetry. Then the aqueous phase was replaced with LC-MS/MS compatible buffers. Finally, a symmetrical analyte peak with short run time was achieved with acetonitrile and 0.05 M Ammonium acetate buffer (4 pH adjusted with glacial acetic acid) with a mobile phase ratio of 50: 50 v/v at a flow rate of 0.8 ml/min. The stationary phase used was shim pack C_{18} column, (150 x 4.6 mm; 5 µ) and the eluents were monitored at 271 nm. The raltegravir potassium eluted at 4.31±0.05 min. The solvents used for the preparation of the mobile phase were filtered using 0.45 µ PTFE membrane filter (Poly tetra fluoro ethylene) before delivering into the HPLC system. The chromatograms were recorded and processed using the class VP data station.

Method validation

Specificity/ selectivity

The method specificity was demonstrated by injecting the diluents, standard solution of raltegravir potassium and the sample solution extracted from the tablet formulation for any coeluting peaks at the retention time of the drug (4.31 ± 0.05 min). There were no coeluting peaks; the peak shape was symmetric and sharp, indicating the specificity of the optimized chromatographic method. The chromatograms of the sample solutions and standard are depicted in Figure 3 and 4.

Accuracy and Precision

The Accuracy of the method was expressed as % mean recovery for three different concentration levels (10, 30, 50 µg/ml) by the standard addition

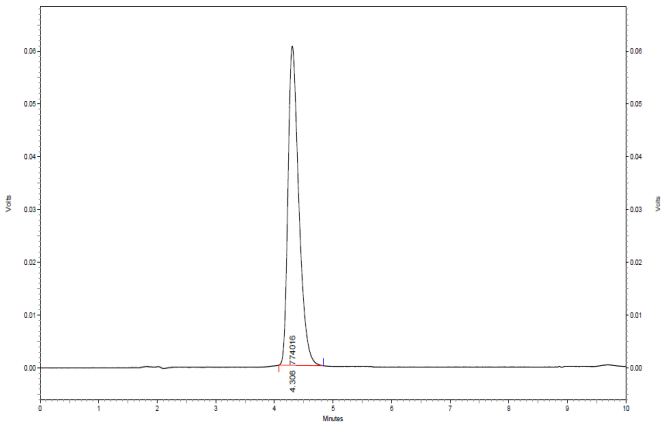

Figure 3: A typical chromatogram of the standard solution (Raltegravir potassium – 10 µg/ml).

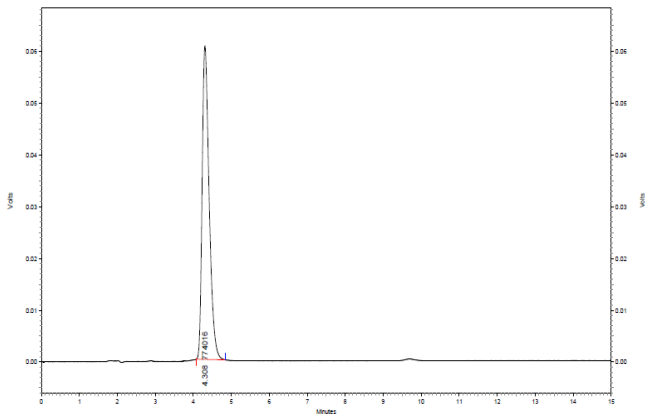

Figure 4: Typical chromatogram of the sample solution extracted from the tablet dosage form.

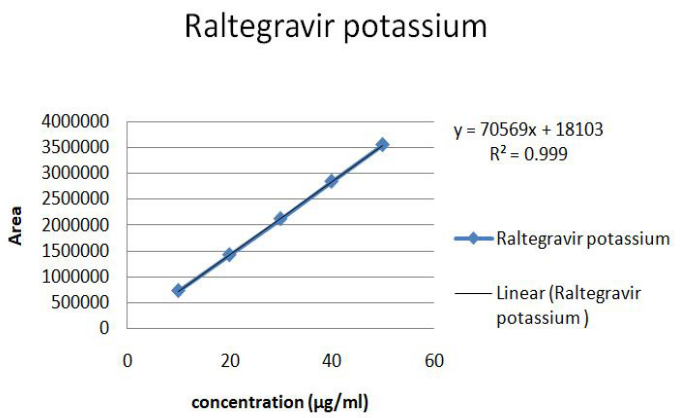

Figure 5: Linearity graph of raltegravir potassium.

Table 1: **Accuracy studies raltegravir potassium.**

S.No	Actual concentration (µg/ml)	Recovered concentration (µg/ml) ± SD; %RSD (n=3)	Percentage recovered
1	10	8.96±0.0264; 0.2656	100.8%
2	30	29.01±0.0964; 0.3224	101.4%
3	50	48.96±0.1850; 0.3709	99.6%

Table 2: **Precision studies of raltegravir potassium.**

S.No	Concentration (µg/ml)	Intraday Mean ±SD; %RSD(n=6)	Interday Mean ±SD; %RSD (n=6)
1	10 (LQC)	7292034±5911.19; 0.81	7293961±6190.84; 0.84
2	30 (MQC)	2140986±34358.62;1.60	2142041±24819.97; 1.15
3	50 (HQC)	3521151±49120.96;1.39	4390415±44609.61; 1.27

Table 3: **System Suitability parameters of raltegravir potassium.**

Sr.no	Parameters	Raltegravir potassium
1.	Retention time (min)	4.31min
2.	Theoretical plates (*N*)	2524
3	Tailing Factor	1.6
4	Asymmetry factor (A)	1
5	Regression coefficient (R^2)	0.9999
6	Regression equation	Y=70568x-68758
7	Linearity and Range	10-50µg/ml
8	Limit of Detection (LOD)	0.104 µg/ml
9	Limit of Quantitation (LOQ)	0.315 µg/ml

method. Triplicate analysis was performed at each level. Percent mean recovery was calculated. The accuracy of the developed method was between 100–102 % (Table 1). The developed method was also used for the assay of the marketed raltegravir potassium tablet formulation. The % RSD for the recovery studies carried out in the real samples were less than 2 %, indicating the accuracy of the method (Table 5).

Table 4: **Robustness Results of raltegravir potassium.**

Sr. no.	Parameters		Retention time (min)
1.	Mobile phase ratio (Acetonitrile: buffer)	60:40	4.31 ± 0.2
		50:50	4.4 ± 0.2
		65:45	6.2 ± 0.2
2.	pH of buffer	3.5	4.7 ± 0.2
		4	4.62 ± 0.2
		4.5	5.1 ± 0.2
3.	Flow rate (ml/min)	0.8	4.6 ± 0.2
		1	4.8 ± 0.2
		1.2	4.92 ± 0.2

Table 5: **Recovery studies of Raltegravir Potassium.**

Sr. No	Sample	Label claim	Amount present(mg/vial)±SD; %RSD
1	Formulation –I	20 mg	19.89 ± 0.1520; 1.9832
2	Formulation –II	50 mg	59.89 ± 0.1482; 1.6871

Linearity

Sr.no	Concentration µg/ml	Area
1	10	732615
2	20	1425124
3	30	2122337
4	40	2844280
5	50	3551477

The intraday and inter day precision studies carried out showed a % RSD value less than 2 indicating the precision of the method. (Table 2)

Linearity

The calibration curve was plotted for five different concentrations of drug Vs. corresponding peak area. Excellent correlation between the concentrations and peak area were observed within the concentration range of (10-50 µg/ml) for the drug (Table 6). The correlation coefficient of raltegravir potassium is more significant with a value ≥0.9999, the slope and the intercept was found to be 70569 and 18103, respectively. The linearity graph is represented in Figure 5.

Detection Limit and Quantification Limit

The Detection and Quantification limit represents the sensitivity of the proposed method. The LOD and LOQ were found to be 0.104 and 0.315 µg/ml, respectively indicating the sensitivity of the method.

System suitability

The system suitability was carried out by performing the experiments and observing the changes in separation, retention time and asymmetry of the peaks with six replicate injections of the standard at the working

concentration. The results of system suitability were found to be within the limits which are summarized in (Table 3).

Robustness and Ruggedness

Robustness of the method was carried out by altering the flow rate (±0.1 ml/min), pH (± 0.2), column temperature (± 2ºC) and organic phase composition (± 2 %) variations (Table 4).

No significant changes in the chromatographic parameters were observed with change on the experimental conditions proving that the developed method was robust (Table 4).

A rapid, simple and sensitive RP-HPLC method was developed for the quantification of raltegravir potassium utilizing LC-MS/MS compatible solvents with a short run time of 4.31 min.

CONCLUSION

A rapid, simple, sensitive, precise, accurate RP-HPLC method was developed and validation was carried out as per ICH guidelines. The relative standard deviation was 0.709 %, indicating the method precision. Raltegravir showed excellent linearity in the concentration range of 10 and 50 µg/ml. Recovery studies expressed the accuracy of the method. The mean recovery for the present validated method ranged between 100 – 102 %. Thus it may be concluded that an accurate, precise and rapid RP HPLC method was developed and validated for the routine quantification of raltegravir potassium in bulk and pharmaceutical formulations.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

RP-HPLC: Reverse-phase high-performance liquid chromatography, **LC-MS/MS**: Liquid chromatography tandem mass spectrometry; **ICH:** International Conference on Harmonization; **API:** Active Pharmaceutical Ingredient; **HAART:** Highly Active Antiretroviral Therapy; **HIV/AIDS**: Human Immunodeficiency Virus/Acquired Immune Deficiency.

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