Development and Validation of Forced Degradation Studies of Raltegravir using RP-HPLC and Characterization of Degradants by LC-MS/MS

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Abstract

Aim:
Develop and validate a simple, shorter and effective HPLC method with UV detection (213nm) and subsequent validation for forced degradation studies of Raltegravir using RP-HPLC and characterization of degradants by LC-MS/MS.

Materials and method:
The method uses isocratic the mobile phase mixture of Buffer and acetonitrile taken in the ratio in the ratio of 60:40(v/v) on Hypersil BDS, C18, 100 x 4.6 mm, 5μm column.

Results:
The RSD for five injections was observed to 0.2 percentage and linearity range of 25-150 percentage of label claims established with 1.0 correlation. The observed result shows that the method was rapid, precise, accurate and simple. The method was validated as per ICH guidelines.

Key Words-Raltegravir, Method development, LC-MS, Hypersil BDS.

INTRODUCTION

Raltegravir Molecular formula C20H21FN6O5. Molecular weight is 444.42gr/mol. IUPAC Name N-(2-(4-(4-flurobenzylcarbamoyl)-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl)propan-2-yl)-5-methyl-1,3,4-oxadiazole-2-carboxamide. 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. [1,2,3]. Literature survey revealed that a few analytical methods have been reported for the determination of raltegravir in pure drug and in pharmaceutical dosage forms using HPLC [4-8] and LC-MS [9-12] either in single or in combined forms. The aim of the present work is to develop and validate a simple, fast and reliable isocratic RP-HPLC method with UV detection for the determination of raltegravir in bulk and in tablet dosage forms. Confirmation of the applicability of the developed method was validated according to the International Conference on Harmonization (ICH) for the determination of raltegravir in bulk and in tablet dosage forms[13].

Figure: 1 Chemical structure of raltegravir

MATERIALS AND METHODS

Chemicals
Qualified standards and samples of raltegravir were obtained from local laboratories and were used without any further purification. The chemicals like Potassium dihydrogen Orthophosphate, triethylamine and Ortho phosphoric acid were purchased from Merck, Mumbai. Millipore water generated from TK water system. The analytical column used was Hypersil BDS, C18, 100 x 4.6 mm, 5μm.

Instruments
A Waters prominence HPLC system equipped with a quaternary UFLC LC-20AD pump, a DGU-20A degasser, a SPD-M20A diode array detector, a SIL-20AC auto sampler, a CTO-20AC column oven and CBM-20A communications bus module was used for method development and validation studies.
**Standard preparation**
Accurately weighed and transferred 10mg of raltegravir working standards into a 10 ml clean dry volumetric flask, add 0.4ml of water: methanol 20:80 v/v as diluent, sonicated for 30 minutes and make up to the final volume with diluents.

**Preparation of sample:**
5 tablets were weighed and calculate the average weight of each tablet. Then the weight equivalent to 5 tablets were transferred into a 500mL volumetric flask, 300mL of diluent added and sonicated for 30 minutes, further the volume made up with water and filtered. From the filtered solution 0.1ml pipette out into a 10ml volumetric flask and made up to 10ml with diluents.

**Chromatographic conditions**
The chromatographic column used was Hypersil BDS column with dimensions of 100 mm X 4.6 mm with 5µm particle size. The column temperature was maintained at 30°C and detection was monitored at a wavelength of 213nm. Injection volume was 10 µl and the mobile phase flow was set at 1.0mL/min. The water, methanol in the ratio 20:80 v/v was used as diluents for preparation of solutions.

**METHOD VALIDATION**
The developed method for determination of raltegravir was validated for system suitability along with method selectivity, specificity, linearity, range, precision, accuracy, range, ruggedness, robustness according to the ICH guidelines.

**Method validation parameters**
The system suitability was conducted using standard preparation and evaluated by injecting five replicate injections. Specificity is the ability of analytical method to assess unequivocally the analyte in the presence of component that may be expected to be present. Performed the specificity parameter of the method by injecting Diluent, placebo into the chromatographic system and evaluated by show any peak at the retention time of analyte. Performed the linearity with raltegravir in the range of 25 to 150% of specification limit. Recorded the area response for each level and calculated slope, intercept & correlation coefficient. Also performed precision at higher level by injecting six times into the chromatographic system.

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogeneous sample. The precision of analytical method is usually expressed as the standard deviation or relative standard deviation of series of measurements. The system precision was conducted using raltegravir and evaluated by making six replicate injections. The Accuracy of the method by recoveries of raltegravir sample solutions at different concentration levels ranging from 50 to 150%. The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

**RESULTS AND DISCUSSION:**

**Optimization of chromatographic conditions:**
Method development includes selection of appropriate chromatographic conditions/factors like detection wavelength, selection and optimization of stationary and mobile phases. The wavelength of 213 nm was selected due to it produces less noise, which minimizes problems that may exhibit around the active ingredient when attempting to quantify raltegravir. Preliminary development trials were performed with various ODS and BDS columns of different types and dimensions from different manufacturers were tested for the peak shape and the number of theoretical plates for specification concentrations. Finally by switching to Hypersil BDS, C18, 100 x 4.6 mm, 5µm column there a significant improvement in the peak shapes with 1.0 tailing factor.

**System suitability:**
The RSD from five replicate injections of standard preparation was 0.2 %, tailing factor for raltegravir peak was 1.0 and theoretical plates obtained 5050.

**Selectivity:**
Performed the specificity parameter of the method by injecting diluent, standard preparation sample preparation and placebo preparation into the chromatographic system and recorded the retention times. Specificity study of the method proved no peak observed at retention time of raltegravir. Specificity results of raltegravir given in the below Table-1. The selectivity chromatograms shown in the Figures 2 and 3.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Sample</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blank</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Placebo</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Standard</td>
<td>2.820</td>
</tr>
<tr>
<td>4</td>
<td>Sample</td>
<td>2.823</td>
</tr>
</tbody>
</table>

![Fig: 2 Chromatogram of raltegravir standard](image)

![Fig: 3 Chromatogram of raltegravir sample](image)
Linearity:
To demonstrate the linearity with raltegravir standard in the range of 25 to 150% of specification limit. Correlation coefficient of raltegravir was 0.999. The linearity results shown in the below Table-2

Table 2: Linearity results of raltegravir

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Concentration in ppm</th>
<th>Area response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>25</td>
<td>190265</td>
</tr>
<tr>
<td>2.</td>
<td>50</td>
<td>377693</td>
</tr>
<tr>
<td>3.</td>
<td>75</td>
<td>575376</td>
</tr>
<tr>
<td>4.</td>
<td>100</td>
<td>758196</td>
</tr>
<tr>
<td>5.</td>
<td>125</td>
<td>945056</td>
</tr>
<tr>
<td>6.</td>
<td>150</td>
<td>1126400</td>
</tr>
</tbody>
</table>

Oxidation:
To 1 ml of stock solution of raltegravir, 1 ml of 20% hydrogen peroxide (H$_2$O$_2$) was added separately. The solutions were kept for 30 min at 60°C. For HPLC study, the resultant solution was diluted to obtain 40µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation
To 1 ml of stock solution Raltegravir, 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60°C. The resultant solution was diluted to obtain 40µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies
To 1 ml of stock solution Raltegravir, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60°C. The resultant solution was diluted to obtain 40µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Thermal/ Dry Heat Degradation Studies:
The standard drug solution was placed in oven at 105°C for 6 hr to study dry heat degradation. For HPLC study, the resultant solution was diluted to obtain 40µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Photo Stability Studies:
The photochemical stability of the drug was also studied by exposing the 100µg/ml solution to UV light by keeping the beaker in UV chamber for 7 days or 200 Watt hours/m² in photo stability chamber For HPLC study, the resultant solution was diluted to obtain 40µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Mass spectral fragmentation:
The analysis of the degradation products was carried by LC and LC-MS. Raltegravir was subjected to LC-MS/MS with atmospheric pressure chemical ionization (APCI) to know the fragmentation pattern of drug. The MS² analysis of the precursor ion (m/z 704) of the drug given below with molecular structure and molecular weight.

CONCLUSION:
A validated stability indicating assay LC-PDA method was developed to study the degradation behavior of Raltegravir under hydrolysis (acid, base and neutral), oxidation, thermal and UV conditions. LC-MS/MS characterization of degradation products was carried out and pathways of decomposition were proposed. The drug was found to be degraded extensively in all conditions except oxidation due to presence of carbamate and urea linkage, which were susceptible to hydrolysis.

3.3. Degradation of Raltegravir
The degradation behavior of LV under various stress conditions was investigated by LC.
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REFERENCES


