DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR DETERMINATION OF RALTEGRAVIR AND ITS IMPURITIES IN BULK DRUG AND DOSAGE FORMS

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ABSTRACT
A simple, sensitive, rapid high performance liquid chromatography method has been developed for estimation of related compounds (imp-I, imp-II, imp-III and imp-IV) in Raltegravir potassium. The chromatographic separation was achieved with purosphere star RP 18 column using gradient elution using mobile phase A containing 0.1% perchloric acid and mobile phase B consists of acetonitrile at 30ºC and detected at 300nm with a flow rate 1ml/min. The resolution between raltegravir and four impurities is found to be greater than 2.5. The method was validated for parameters like precision, accuracy, linearity, LOD, LOQ, ruggedness as per ICH guidelines.

KEYWORDS: Raltegravir, development, validation, HPLC.

INTRODUCTION
Raltegravir is an antiretroviral drug used to treat HIV infection(1). It received approval by USFDA on October 2007, the first of a new class of HIV drugs, the integrase inhibitors, to receive such approval(2-3). Reltegravir potassium(N-[(4-Fluorophenyl) methyl]-1,6-dihydro-5hydroxy-1-methyl-2-[1-methyl-1-[[(5-methyl-1,3,4-oxadiazol-2-yl)carbonyl]amino]ethyl]-6-oxo-4 pyrimidine carboxamide monopotassium salt) is available in chewable form, older adolescents will use the adult formulation(4).

Several methods have been reported Raltegravir in human plasma by HPLC, LC-MS (5-14), assay by HPLC (15-17), degradation impurity by LC, LC-MS(18), assay by UPLC (19) and content by UV (20). The present work describes the quantification of four impurities in raltegravir potassium. To the best of our knowledge till today there was no method was reported for the estimation of Raltegravir potassium and its new impurities in bulk drug and pharmaceutical dosage form by RP-HPLC.
potassium 4-((4-fluorobenzyl)carbamoyl)-1-methyl-2-(2-(5-methyl-1,3,4-oxadiazole-2-carboxamido)propan-2-yl)-6-oxo-1,6-dihydropyrimidin-5-olate

**Fig. 1 Raltegravir potassium**

benzyl (2-(4-((4-fluorobenzyl)carbamoyl)-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl)propan-2-yl)carbamate

**Fig. 2 Imp-I**

2-(2-amino propan-2-yl)-N-(4-fluorobenzyl)-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxamide hydrobromide

**Fig. 3 Imp-II**
EXPERIMENTAL
Chemicals and Reagents
The HPLC grade acetonitrile and analytical grade per chloric acid were purchased from Merck, Mumbai. High purity water was prepared by using a Millipore Milli Q plus water purification system. High purity of all impurities namely IMP-I, IMP-II, IMP-III, IMP-IV and Raltegravir potassium as a gift.

Chromatographic conditions
Alliance e2695 separation module (Waters Corporation, Milford, MA) equipped with 2998 PDA detector with empower 2 software used for analysis. The method was developed using purosphere star RP18 (150x4.6) mm, a 5µm column with a mobile phase consist a gradient mixture of A and B. 0.1%perchloric acid was used as solvent A and acetonitrile was used as solvent B. The gradient program was set as (T/%B) 0/20,15/30,30/70,35/7035.1/20 and 40/20. The mobile phase flow rate was set as 1.0ml/min and the temperature was maintained as 30°C. The injection volume was 10µl and the wavelength was monitored at 300nm. Water: acetonitrile ratio (1:1) was used as diluent.
Preparation of standard and sample solutions

A working standard of raltegravir and its related impurities were prepared by appropriate weighing and respective dilution of impurity and a reference standard in a mixture of water and acetonitrile in the ratio of 50:50 (%v/v) to yield a final concentration of raltegravir =1µg/ml, IMP-I=1µg/ml, IMP-II=1µg/ml, IMP-III=1µg/ml and IMP-IV=1µg/ml. A stock solution of Raltegravir potassium (1.0mg/ml) is prepared by dissolving the appropriate amount of Raltegravir solid in the diluent. The drug product equivalent to 100mg of sample is transferred to 100ml flask dissolved and diluent volume with diluent (1.0mg/ml), this solution then filtered through a 0.45µ nylon membrane filter.
RESULTS AND DISCUSSION

Method development and optimization

The main objective of the chromatographic method was to achieve separation of four impurities from Raltegravir and separate from each other. From UV profiling it was found the suitable wavelength for the raltegravir and its impurities is 300nm. Initial trials were done on Inertsil ODS 3V 250x4.6mm, a 5µ column with different mobile phase like potassium hydrogen phosphate. Ammoniumacetate with a combination of methanol as well as acetonitrile. Resolution between Imp-II and Imp-III are very less almost eluted as a single peak. The chromatographic separation achieved on Purosphere star RP18 150x4.6mm, 5µ column. The gradient method employs solution A 0.1% perchloric acid in water and solution B as acetonitrile used as mobile phases. The flow rate was 1.0ml/min. The HPLC gradient program was set as time/%B
0/20, 15/30, 30/70, 35/70, 35.1/20 and 40/20. The column temperature was maintained at 30°C, sample compartment temperature is maintained at 5°C. The injection volume 10µl. The peak shape of Raltegravir potassium was found to be symmetric and as well separated from its potential impurities. In the optimized condition Raltegravir potassium, Imp-I, Imp-II, Imp-III and Imp-IV were well separated with resolution greater than 2.5. System suitability results are shown in table-I.

Method validation

The propose method was validated as per the international conference on Harmonization (ICH) guidelines (21-22).

The method precision is evaluated by carrying out six independent preparations of a test sample of Raltegravir spiked to 0.1% each imp-I, imp-II, imp-III and imp-IV. The %RSD of the areas of impurities calculated. The intermediate precision of the method is evaluated using a different analyst, different column and different instrument; results are tabulated in table-I.

The LOD & LOQ were calculated for each compound as a signal-to-noise ratio (S/N) of approximately 3:1 & 10:1. It determined by the series of dilution of standard solution. The precision study is also carried out at the LOQ level by injecting six individual preparations of imp-I, imp-II, imp-III and imp-IV calculated the %RSD of areas. Results are shown in tabulated in table-I. The further linearity tests is prepared from a stock solution at six concentration levels from LOQ to 150% of Raltegravir potassium and impurity concentration. The linear calibration plot for the method is obtained over the tested calibration range and obtained correlation coefficient is greater than 0.999. The results revealed an excellent correlation between the peak area and analyte concentration. Results are tabulated in table-I. Similarly the accuracy is calculated for the recoveries of impurities by the method of standard additions. A known amount of impurities were added to a pre-quantified sample and amount of impurities were estimated. Accuracy of the method is evaluated in triplicated at four concentration levels LOQ levels, 50, 100 and 150%. Results are tabulated in table-I.

On the robustness of the method, experimental conditions were deliberately altered and the resolution between Raltegravir potassium and its impurities and tailing factor for Raltegravir and its impurities were recorded. The flow rate of the mobile phase was 1.0ml/min, to study the effect of flow rate on the resolution. Flow was changed by 0.2 units from 0.8 to 1.2ml/min. The effect of the column temperature on resolution studied at 25°C and 35°C and found that method is robust and similarly carried out solution stability of Raltegravir, and its impurities in the related substance by leaving the test solution and the solution spiking with the impurities at the
0.10 % level with respect to analyte concentration in a volumetric flask at room temperature for 48hrs. The solution was stable up to 48hrs.

Table-1 Validation results

<table>
<thead>
<tr>
<th>Validation parameter</th>
<th>Imp-I</th>
<th>Imp-II</th>
<th>Imp-III</th>
<th>Raltegravir</th>
<th>Imp-IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>System Precision</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%RSD of peak area</td>
<td>1.46</td>
<td>1.84</td>
<td>2.3</td>
<td>0.89</td>
<td>1.03</td>
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<tr>
<td>Resolution</td>
<td>23.5</td>
<td>2.6</td>
<td>12.2</td>
<td>25.9</td>
<td></td>
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<tr>
<td>Tailing factor</td>
<td>1.05</td>
<td>0.99</td>
<td>1.13</td>
<td>1.06</td>
<td>1.08</td>
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<tr>
<td>Column efficiency</td>
<td>20050</td>
<td>53082</td>
<td>63550</td>
<td>179067</td>
<td>272543</td>
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<tr>
<td>Linearity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>11839</td>
<td>10745</td>
<td>10103</td>
<td>9265</td>
<td>10218</td>
</tr>
<tr>
<td>Intercept</td>
<td>3.8</td>
<td>-2.7</td>
<td>-1.1</td>
<td>-1.6</td>
<td>-4.0</td>
</tr>
<tr>
<td>r2</td>
<td>0.9985</td>
<td>0.9992</td>
<td>0.9983</td>
<td>0.9984</td>
<td>0.9993</td>
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<tr>
<td>Quantitation limit(%)</td>
<td>0.025</td>
<td>0.028</td>
<td>0.028</td>
<td>0.032</td>
<td>0.03</td>
</tr>
<tr>
<td>Detection limit(%)</td>
<td>0.0077</td>
<td>0.0085</td>
<td>0.0085</td>
<td>0.0098</td>
<td>0.0091</td>
</tr>
<tr>
<td>Precision at QL</td>
<td>2.8</td>
<td>1.8</td>
<td>2.3</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Accuracy mean%recovery at</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QL</td>
<td>108.2</td>
<td>105.5</td>
<td>106.3</td>
<td>NA</td>
<td>98.4</td>
</tr>
<tr>
<td>50%</td>
<td>97.4</td>
<td>97.0</td>
<td>99.8</td>
<td>NA</td>
<td>99.0</td>
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<tr>
<td>100%</td>
<td>97.9</td>
<td>102.2</td>
<td>100.6</td>
<td>NA</td>
<td>99.7</td>
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<tr>
<td>150%</td>
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<td>101.0</td>
<td>99.3</td>
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<tr>
<td>Method precision</td>
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<td>2.5</td>
<td>1.6</td>
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<td>0.7</td>
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<tr>
<td>Intermediate precision</td>
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<td></td>
</tr>
<tr>
<td>%RSD</td>
<td>1.1</td>
<td>1.8</td>
<td>1.2</td>
<td>NA</td>
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CONCLUSIONS

The rapid gradient RP-HPLC method was developed and validated for the determination of raltegravir potassium and its impurities in bulk drug and pharmaceutical dosage form. The developed method is precise, accurate, linear, sensitive and robust. The method can be adapted for regular analysis.

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