CHAPTER 6

METHOD DEVELOPMENT AND VALIDATION OF RALTEGRAVIR POTASSIUM AND RILPIVIRINE HCL BY HPLC AND HPTLC METHODS
METHOD DEVELOPMENT AND VALIDATION OF RALTEGRAVIR POTASSIUM AND RILPIVIRINE HCL BY HPLC AND HPTLC METHODS

6.1 PHASE I

RALTEGRAVIR POTASSIUM

The analysis of Raltegravir potassium was done by the following methods

1. High performance liquid chromatographic method (HPLC)
2. High performance thin layer chromatographic method (HPTLC)

6.1.1 Preparation of Tablet (Raltegravir potassium 400 mg) formulation

Direct compression method (Leon Lachmen, et al., 1990)1

Required quantity of hydroxy propyl methyl cellulose was weighed and 16.00gms of drug was weighed, added and mixed well. Then microcrystalline cellulose and lactose were weighed, added and mixed well. Then required quantity of talc and magnesium stearate were weighed and added. The blended powder mixture was punched by using the single punching machine.

6.1.2 Evaluation test for Tablets (Indian Pharmacopeia)2

6.1.2.1 Weight variation test

Individually 20 tablets were taken at random, weighed and the average weight was determined. Not more than 2 of the individual weight deviated from the average weight. The percentage deviation was shown in the following table 4.

<table>
<thead>
<tr>
<th>Average weight of tablet</th>
<th>Percentage deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>130 mg or less</td>
<td>10</td>
</tr>
<tr>
<td>More than 130 mg but</td>
<td>7.5</td>
</tr>
<tr>
<td>less than 324 mg</td>
<td></td>
</tr>
<tr>
<td>324 mg or more</td>
<td>5</td>
</tr>
</tbody>
</table>

6.1.2.2 Hardness test

Tablet requires a certain amount of strength or hardness to withstand mechanical shocks of handling in manufacture, packing and shipping. Strength of the tablet was expressed as tensile strength (Kg / cm²). The tablet crushing load, which is the force required to break a tablet was determined using Monsanto hardness tester.

6.1.2.3 Friability test

The tablets were weighed accurately (W initial), and they were placed in the drum. The drum was rotated 100 times at the rate of 25 ± 2 rpm, and the tablets were taken. Any loose dust was removed from the tablets and weighed accurately (W final).

6.1.2.4 Disintegration test

The USP device to test disintegration uses 6 glass tubes that are 3 inches long, open at the top, and held against a 10 mesh screen at the bottom end of the basket rack assembly. To test for disintegration time, one tablet was placed in each tube, and the basket rack was positioned in a one litre beaker of water, simulated gastric fluid or simulated intestinal fluid, at 37 ° C, such that the tablets remain 2.5cm from the bottom of the beaker. A standard motor driven device was used to move the basket assembly containing the tablets up and down through a distance of 5 - 6 cm at a frequency of 28 - 32 cycles per minute.

A single tablet was placed in a small wire mesh basket fastened at the bottom of the shaft connected to a variable speed motor. The basket was immersed in the dissolution medium contained in a 100 ml flask. The flask was cylindric with a hemispherical bottom. The flask was maintained at 37 ° C by a constant temperature bath, the motor was adjusted to turn at the specified speed, and the samples of the fluid are withdrawn at interval to determine the amount of drug in solution.

6.1.2.5 Assay (content estimation)

10 tablets of Raltegravir potassium were weighed and the average weight was calculated and powdered. The tablet powder equivalent to 10 mg of Raltegravir 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 (9 µg / ml). The standard solution was prepared in the same manner. By using the following formula the percentage purity of RAL was calculated.

$$\text{Assay\%} = \frac{\text{AT} \times \text{WS} \times \text{DT} \times \text{P} \times \text{Avg. Wt}}{\text{AS} \times \text{DS} \times \text{WT} \times 100} \times 100$$

6.1.3 High Performance liquid chromatographic method

6.1.3.1 Selection of chromatographic method

Proper selection of the methods depends upon the nature of the sample, molecular weight and solubility. Reverse phase chromatographic technique was selected for initial separations by considering the properties of the compound. C18 column was chosen as the stationary phase and a mixture of methanol and phosphate buffer was used as mobile phase in the ratio of 55:45 % v/v.

6.1.3.2 Selection of wavelength

A solution of Raltegravir (9 µg / ml) was scanned in the UV region using methanol: phosphate buffer (pH 3.0) in the ratio of 55:45% v/v. The absorbance was found at 218 nm. Raltegravir potassium in different mobile phase ratios, different pH and different flow rate value were also scanned in the UV region. There was no significant change in the absorbance and absorbance maximum. Hence 218 nm was selected as detection wavelength for the estimation of Raltegravir potassium by HPLC method.

6.1.3.3 Preparation of phosphate buffer

7.0 grams of potassium dihydrogen phosphate was weighed and was transferred into a 1000 ml standard flask. 700 ml of HPLC grade water was added and dissolved. Then it was diluted to volume with water. pH value 3.0 was adjusted with ortho phosphoric acid.
6.1.3.4 Preparation of Mobile phase:

450 ml of phosphate buffer (45 %) (pH 3.0) and 550 ml of methanol HPLC grade were mixed and degassed in ultrasonic water both for 5 minutes. Then it was filtered through 0.45 µ filter under vacuum.

6.1.3.5 Initial separation conditions

The following chromatographic conditions were fixed initially to improve the separation of Raltegravir potassium

- Mode of operation: Isocratic
- Stationary phase: C₁₈ column (150mm X 4.6mm i.d., 5µ)
- Mobile phase: Methanol: phosphate buffer pH 2.5
- Ratio: 45:55% v/v
- Detection wavelength: 218 nm
- Flow rate: 0.5 ml / min
- Temperature: ambient
- Sample volume: 20 µl
- Quantification method: External standard calibration method

The mobile phase was allowed to run for 5 minutes to record a steady baseline. Raltegravir potassium drug solution was injected and chromatogram was recorded. It was observed that the drug was eluted at 6.176 min. Hence the different ratios of mobile phase were tried to reduce the retention time of Raltegravir potassium.

6.1.3.6 Effect of Composition and ratio of mobile phase

The different mobile phase compositions like methanol, phosphate buffer and acetonitrile were used in this study. Phosphate buffer was tried with methanol and acetonitrile. The different ratios of mobile phase tried were 50:50, 55:45, 45:55, 60:40 and 30:70 % v/v. By changing the composition of mobile phase, these ratios were studied. Phosphate buffer with acetonitrile showed poor baseline. Phosphate buffer
with methanol in the ratio of 55:45 %v/v showed good base line and sharp peak was obtained.

6.1.3.7 Effect of pH of mobile phase

The different pH were tried i.e. 2.5, 3.0 with methanol and phosphate buffer in the ratio of 55:45% and the chromatograms were recorded. When comparing the chromatograms obtained for the different pH solutions, the peak at pH 3.0 was better than the peak at pH solution 2.5.

6.1.3.8 Effect of flow rate on separation

Different flow rates like 0.5 ml / min and 0.6 ml / min were tried with methanol and phosphate buffer. In flow rate 0.6 ml / min the chromatogram showed better separation than in flow rate 0.5 ml/min. Hence methanol and phosphate buffer (pH 3.0) in the ratio of 55:45%v/v with 0.6 ml/min flow rate was used for further studies.

6.1.4 Optimized chromatographic conditions

The following parameters were used for RP-HPLC analysis of Raltegravir potassium

Mode of operation : Isocratic
Stationary phase : C18 column (150mm X 4.6mm i.d., 5µ)
Mobile phase : Methanol: phosphate buffer pH 3.0
Ratio : 55:45 % v/v
Detection wavelength : 218 nm
Flow rate : 0.6 ml / min
Temperature : ambient
Sample volume : 20 µl
Quantification method : External standard calibration method
6.1.4.1 Preparation of standard stock solution

10 mg of Raltegravir potassium was accurately weighed and transferred into a 10 ml volumetric flask. 7 ml of mobile phase was added and sonicated to dissolve it completely and made up to the volume with mobile phase (1000 µg / ml).

6.1.4.2 Preparation of working sample solution

10 tablets of Raltegravir potassium were weighed and the average weight was calculated. The tablets were powdered and the tablet powder equivalent to 10 mg of Raltegravir potassium was transferred into a 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 Whatmann filter paper no 41(1000 µg / ml).

6.1.4.3 System Suitability studies

System suitability tests are an integral part of any chromatographic analysis method which is used to verify reproducibility of the chromatographic system. To ascertain its effectiveness, certain system suitability test parameters were checked by repeatedly injecting the drug solution at the concentrations of 30 µg / ml. 20 µl standard solutions were injected and chromatograms were recorded.

6.1.4.4 Preparation of calibration graph

In this method, the aliquots of stock solution of Raltegravir potassium (0.1 - 0.5 ml of 1000 µg / ml) were transferred into five 10 ml volumetric flasks and made up to the mark with mobile phase. The solutions containing 10 - 50 µg / ml of Raltegravir potassium in mobile phase were injected and the chromatograms were recorded at 218 nm. It was found that the above concentration range was linear. The peak area was plotted against concentration and the calibration curve was constructed.

6.1.4.5 Limit of detection and limit of Quantification

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact
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value. The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

The detection limit (LOD) for the proposed method was calculated using the following equation

$$\text{LOD} = 3.3 \frac{S}{K}$$

where ‘S’ is the standard deviation of replicate determination values under the same conditions as for sample analysis in absence of the analyte and ‘K’ is the sensitivity namely the slope of the calibration graph. The limit of quantification (LOQ) was defined as

$$\text{LOQ} = 10 \frac{S}{K}.$$

6.1.4.6 Quantification of formulation (Assay)

30 µg/ml of standard and sample solutions were prepared individually. 20 µl of standard and sample solutions were injected into the chromatographic system separately and the peak area for the Raltegravir potassium was measured. The percentage purity was calculated by using the following formula.

$$\text{Assay\%} = \frac{\text{AT}}{\text{AS}} \times \frac{\text{WS}}{\text{DS}} \times \frac{\text{DT}}{\text{WT}} \times \frac{\text{P}}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

where:

- AT = Peak Area of Raltegravir potassium obtained with test preparation
- AS = Peak Area of Raltegravir potassium obtained with standard preparation
- WS = Weight of working standard taken in mg
- WT = Weight of sample taken in mg
- DS = Dilution of Standard solution
- DT = Dilution of sample solution
- P = Percentage purity of working standard
6.1.4.7 Precision and intermediate precision

Precision is the degree of repeatability of an analytical method under normal operational conditions. The precision of assay was determined by repeatability (intraday) and intermediate precision (interday) and reported as % RSD for a statistically significant number of replicate measurements. Repeatability and intermediate precision of the method were determined by analyzing six times standard solutions prepared in the concentration of 30 µg/ml. 20 µl of the above said concentration were injected into the chromatographic system six times separately and the peak area was recorded each time.

6.1.4.8 Accuracy

The recovery experiment was done by adding known concentration of Raltegravir raw material to preanalysed formulation. The tablet powder equivalent to 10 mg of Raltegravir was weighed and transferred into a series of three 100 ml standard flask. To that, 5 mg, 10 mg, and 15 mg of raw material were added (50%, 100%, 150%) and dissolved with mobile phase and made up to the volume with the same. Then it was sonicated for 20 minutes. After, sonication the solution was filtered through Whatmann filter paper No 41. From the clear solution, 3ml of each test solution was pipetted out and made into six replicate for 10 ml volumetric flask. 20 µl solutions of each concentration were injected and the chromatograms were recorded. Each concentration was repeated for three times.

6.1.4.9 Robustness

For demonstrating the robustness study of the developed method, experimental conditions were purposely altered and evaluated. The method must be robust enough to withstand such changes and allow routine analysis of the sample. In the present study, the flow rate was varied from 0.5 to 0.7 ml / min and organic composition in the mobile phase was varied from 50 to 60%. 30 µg / ml concentrations of standard and sample solutions were prepared separately and injected into chromatographic system to save the value of peak area.
6.1.4.10 Ruggedness

The degree of reproducibility of test results by the proposed method of Raltegravir was detected by analyzing the drug sample under following variety of test conditions. 1. Different analyst 2. Different instruments.

6.1.4.11 Specificity

The method specificity was assessed by comparing the chromatograms obtained from the drug and the most commonly used excipients mixture with those obtained from blank (excipients solution in water without drug). The excipients chosen were the one used commonly in tablet formulation, which included lactose, talc, microcrystalline cellulose, magnesium stearate and hydroxyl propyl cellulose

6.1.5 High performance thin layer chromatography

6.1.5.1 Preparation of mobile phase

60 ml of toluene, 30 ml of ethyl acetate, 7 ml of methanol and 3 ml of glacial acetic acid were taken and transferred into a 100 ml volumetric flask. All reagents used were in HPLC grade.

6.1.5.2 Initial separation conditions

The following chromatographic conditions were fixed initially to improve the separation of Raltegravir potassium

Stationary phase : 5mm band length in the 8 x 10 Silica gel 60F_{254} TLC plate
Mobile phase : Toluene: ethyl acetate: methanol: glacial acetic acid Ratio : 6:3:0.7:0.3
Scanner : Camag-TLC Scanner-2
Detection wavelength : 218 nm
Quantification method : External standard calibration method
6.1.5.3 Effect of mobile phase ratios

The different ratios of mobile phase were tried (1) 6:3:0.7:0.3 (2) 5: 4: 0.3: 0.7, (3) 7: 2: 0.5 (4) 4: 5: 0.6: 0.4 % v/v/v/v. For all the above ratios chromatograms were recorded. First three ratios of the chromatograms showed poor base line. Fourth ratio of (4: 5: 0.6: 0.4 %v/v/v/v) chromatogram showed good base line with sharp peak. Hence 4: 5: 0.6: 0.4 %v/v/v/v mobile phase ratio was selected for the further analysis.

6.1.6.1 Optimized chromatographic conditions

The following parameters were used for HPTLC analysis of Raltegravir potassium:

- **Stationary phase**: 5 mm band length in the 8 x 10 Silica gel 60F254 TLC plate
- **Mobile phase**: Toluene: ethyl acetate: methanol: glacial acetic acid
- **Ratio**: 4: 5: 0.6: 0.4 %v/v/v/v
- **Scanner**: Camag-TLC Scanner-2
- **Detection wavelength**: 218 nm
- **Quantification method**: External standard calibration method

6.1.6.2 Preparation of standard stock solution

About 10 mg of working standard of Raltegravir potassium was accurately weighed and transferred into a 100 ml volumetric flask. About 25 ml of methanol was added and sonicated for about 20 min. Finally the volume was made up to 100 ml with methanol to obtain the concentration of about 100 μg /ml. 0.1 ml was taken from this stock solution and the volume made up to 100 ml to get a concentration of about 100 ng / μl.

6.1.6.3 Preparation of sample solution

Ten tablets were weighed and finely powdered. The powder equivalent to 10 mg of the Raltegravir was weighed, mixed with 25 ml of methanol and sonicated for
15 min. The solution of tablet was filtered through Whatman filter paper No. 41 and the residue was thoroughly washed with methanol. The filtrate and washings were combined in a 100 ml volumetric flask and diluted to the mark with methanol to get the final concentration of 100 μg / ml of Raltegravir. 0.1 ml was taken from this stock solution and the volume made up to 100 ml to get a concentration of about 100 ng / μl.

6.1.6.4 Linearity (calibration Graph)

Aliquots (2, 4, 6, 8 and 10 μl) of standard solution of Raltegravir potassium were spotted on pre coated TLC plates using linomat IV sample spotter under nitrogen stream. The plate was dried in air and developed up to 90 mm at constant temperature with a mixture of Toluene : ethyl acetate: methanol : glacial acetic acid 4: 5: 0.6: 0.4 % v/v/v/v as mobile phase in a CAMAG twin trough chamber which was previously saturated with mobile phase for about 15 min. The plate was removed from the chamber and dried in air. Photometric measurements were performed at 219 nm in absorbance/reflectance mode with the CAMAG TLC scanner 2 using CATS 4 software incorporating track optimizing option. The standard plot of Raltegravir potassium was established by plotting the peak area vs. concentration (ng / ml) corresponding to each spot.

6.1.6.5 Limit of detection and limit of Quantification

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

The detection limit (LOD) for the proposed method was calculated using the following equation

\[ \text{LOD} = 3.3 \frac{S}{K} \]

where ‘S’ is the standard deviation of replicate determination values under the same conditions as for sample analysis in absence of the analyte and ‘K’ is the sensitivity namely the slope of the calibration graph. The limit of quantification (LOQ) was defined as \( \text{LOQ} = 10 \frac{S}{K} \).
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6.1.6.6 Quantification of formulation (Assay)

Six micro liters of sample solution was applied on a TLC plate under a nitrogen stream using a linomat IV sample spotter. The amount of Raltegravir potassium present in the sample solution was determined by fitting the area values of peaks corresponding to Raltegravir potassium into the equation of the line representing the calibration curve of Raltegravir. All determinations were performed in triplicate.

6.1.6.7 System Precision

The precision study was conducted for Raltegravir standard stock solutions. 6 µl concentration solutions were prepared and assessed by spotting six times on a TLC plate, followed by development of plate and recording the peak area of 6 spots. The % RSD for peak area was calculated.

6.1.6.8 Method Precision

The method reproducibility (The intra-day precision) was determined by analyzing standard solutions in the concentration of 600 ng /spot of drug for 3 times on the same day and inter-day precision was determined by analyzing corresponding standards daily for 3 days over a period of one week. The intra-day and interday related standard deviation was found to be 0.84 % and 1.84 % respectively. The smaller values of intraday and interday variation in the analysis indicate that the method was precise.

6.1.6.9 Accuracy

Accuracy of the method was evaluated by calculating recovery studies of addition of standard drug Raltegravir solution to the prepared sample solution at different concentration levels (40%, 60% and 80%) (n = 3) within the range of linearity of the drug. The results of recovery studies were obtained by method validation by statistical evaluation.
6.1.6.10 Ruggedness

The degree of reproducibility of test results by the proposed method of Raltegravir was detected by analyzing the drug sample under following variety of test conditions. 1. Different analyst 2. Different instruments.

6.1.6.11 Specificity

The method specificity was assessed by comparing the chromatograms obtained from the drug and most commonly used excipients mixture with those obtained from blank (excipients solution in water without drugs). It was observed that there was no interference of the peak obtained for the chromatogram of blank and placebo with that of Raltegravir peaks obtained for the chromatograms of sample and standard.

6.1.7 Results and Discussions

Two methods are developed for the determination of Raltegravir potassium in pure form and in tablet dosage from. The methods employed for the analysis of Raltegravir potassium were,

1. High performance liquid chromatographic method (HPLC)
2. High performance thin layer chromatographic method (HPTLC)

6.1.7.1 Quality control tests for tablets

40 tablets were prepared and evaluation tests were performed for those tablets. In weight variation test 20 tablets were used. The individual and average weight of the tablet was calculated. 5 % deviation was allowed as per IP. All the tablets were within the limit and it passed the test. In hardness test using Monsanto tester, 5 tablets were tested and the average hardness was found to be 4.5 kg/cm$^3$. In friability test, 10 tablets were used. Tablets were weighed and measured before and after the test. The deviation was found to be 0.4291 % (Limit up to 1%). In disintegration test, 5 tablets were used and the average disintegration time was calculated. The average disintegration time was found to be 2.18 minutes and it was within the limit. In dissolution test, 6 tablets were used and the percentage release was found to be 96 %
after 8 hrs. The percentage purity was found to be 100.62 % in HPTLC and 99.85 % in HPLC method. The results of quality control test for tablet were shown in the table 5.

<table>
<thead>
<tr>
<th>No</th>
<th>Parameters</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Weight variation test</td>
<td>Within the limit</td>
</tr>
<tr>
<td>2</td>
<td>Hardness test</td>
<td>4.5 kg/cm²</td>
</tr>
<tr>
<td>3</td>
<td>Friability test</td>
<td>0.4291%</td>
</tr>
<tr>
<td>4</td>
<td>Disintegration test</td>
<td>2.18 minutes</td>
</tr>
<tr>
<td>5</td>
<td>Dissolution test</td>
<td>96% release in 8 hrs</td>
</tr>
<tr>
<td>6</td>
<td>Assay</td>
<td>HPLC- 99.85%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPTLC- 100.62%</td>
</tr>
</tbody>
</table>

### 6.1.8 High performance liquid chromatography

The solution of 9 µg/ml of Raltegravir potassium in mobile phase (Methanol and Phosphate buffer pH 3.0 in the ratio of 55:45% v/v) was prepared and the solution was scanned in the UV region. At 218 nm the drug showed maximum absorbance. Hence this was selected as a detection wavelength. The spectrum was shown in figure 10.
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The optimization was done by changing the ratio of mobile phase, changing pH value of phosphate buffer and flow rate. Methanol, acetonitrile, Phosphate buffer were tried in the method. Blank was run with the help of Methanol and Phosphate buffer in the ratio of 55:45% v/v. The separation was first tried with acetonitrile and phosphate buffer. By keeping pH (3.0) and flow rate (0.5 ml/min) as constant, and by changing the mobile phase ratio like 50:50, 60:40, 30:70% v/v three trails were performed. They showed poor base line and high retention time. Then the separation was tried with methanol and phosphate buffer. By keeping pH (2.5) and flow rate 0.5 ml / min as constant three trails were performed and they showed poor baseline and high retention time. Then the separation was tried with the same by changing flow rate from 0.5 to 0.6 ml / min and by changing the mobile phase composition. It showed poor base line. Then the mobile phase composition was again changed. With methanol and phosphate buffer in the ratio 55:45 % v/v, it showed good separation. The chromatograms recorded in the trail were shown in the figure 11. The optimized chromatogram was shown in the figure 12. By considering all the above parameters, Methanol and phosphate buffer (pH 3.0) was used as the mobile phase in this study. The retention time in optimized chromatogram was found to be 4.350 minutes.
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Fig. 11 Trial Chromatograms for Raltegravir by HPLC method

Fig. 12 Optimized Chromatogram for Raltegravir by HPLC method
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6.1.8.1 Method validation (ICH Q2A, Q2B Guidelines)\(^1,4\)

The system suitability studies were carried out as specified in ICH and USP\(^5\). The parameters like tailing factor, asymmetry factor, number of theoretical plate capacity factor were calculated. The results were shown in table 6.

Table 6 System Suitability Parameters report

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Experimental Value</th>
<th>Limit As Per USP</th>
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</thead>
<tbody>
<tr>
<td>Tailing factor</td>
<td>1.6</td>
<td>Less than 2</td>
</tr>
<tr>
<td>Asymmetric factor</td>
<td>1.0</td>
<td>Less than 2</td>
</tr>
<tr>
<td>No. of theoretical plates</td>
<td>2524</td>
<td>More than 2000</td>
</tr>
<tr>
<td>Capacity factor</td>
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<td>2-10</td>
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<tr>
<td>HETP</td>
<td>0.059</td>
<td>-</td>
</tr>
<tr>
<td>Theoretical plate per unit length</td>
<td>16.82</td>
<td>-</td>
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</tbody>
</table>

With the optimized chromatographic conditions, stock solution of Raltegravir potassium was prepared by using mobile phase (phosphate buffer pH 3.0 and methanol in the ratio 45:55% v/v) and various concentrations were prepared in the range of 10 - 50 µg / ml and injected individually. The chromatograms were recorded at 218 nm. The chromatograms were shown in the figures 13. The values obtained in linearity study were shown in table 7.

Table 7 Linearity Study – Raltegravir Potassium by RP-HPLC Method

<table>
<thead>
<tr>
<th>S.No</th>
<th>Concentration (µg/ml)</th>
<th>Average Peak Area</th>
<th>Correlation Coefficient</th>
<th>LOD (µg/ml)</th>
<th>LOQ (µg/ml)</th>
<th>Slope</th>
<th>Intercept</th>
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<td>732615</td>
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<td>0.09</td>
<td>70827</td>
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<td>3</td>
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<td>3551477</td>
<td></td>
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</tbody>
</table>

\(^1\)International Conference on Harmonization ICH. (1994) Validation of Analytical : Definition and Terminology Q2A.Switzerland, p 1-4


The calibration curve was plotted using concentration against peak area. The correlation coefficient was found to be 0.999 indicated that the concentration of Raltegravir potassium had good linearity. The calibration curve was shown in figure 14.
The limit of detection and the limit of quantification were determined based on the signal to noise ratio. The limit of detection was found to be 0.027 µg / ml and the limit of quantification was found to be 0.09 µg /ml. The chromatograms for LOD and LOQ were shown in the figure 15 respectively.

Assay was performed to determine the purity of the Raltegravir potassium. 30 µg / ml solutions were prepared by using pure drug and sample. They were injected and the areas of injection were recorded in HPLC. The percentage purity of the Raltegravir potassium was found to be 99.85%. The chromatograms were shown in the figure 16. The assay reports were shown in table 8.
Table 8 Analysis of Raltegravir tablet formulation by the Proposed 
RP-HPLC Method

<table>
<thead>
<tr>
<th>S.No</th>
<th>Standard peak area</th>
<th>Sample peak area</th>
<th>Mean %</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>2113245</td>
<td>99.85</td>
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</tr>
</tbody>
</table>

Fig. 16 Standard and Sample Chromatograms for Raltegravir potassium

Precision study was done with the Raltegravir potassium standard. 30 μg/ml solution of Raltegravir potassium was prepared from the stock solution and injected five times and the areas of five injections were recorded in HPLC. The % RSD (Gupta
SP, 1991)\(^6\) was found to be 1.43. The % RSD for the area of five replicate injections was found to be within the specified limit. It showed that the drug was having good precision and it was shown in table 9 and the chromatograms were shown in figure 17.

Table 9 Precision study – Raltegravir Potassium by RP-HPLC Method

<table>
<thead>
<tr>
<th>S.No</th>
<th>Peak Area</th>
<th>Average</th>
<th>SD</th>
<th>(%) RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15234.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>15729.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>15456.3</td>
<td>15545.5</td>
<td>222.614</td>
<td>1.43</td>
</tr>
<tr>
<td>4</td>
<td>15518.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>15788.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Chromatograms](image)

**Fig. 17 Precision Chromatograms for Raltegravir potassium**

Intermediate precision (intraday) and repeatability (interday) study was done with the Raltegravir potassium standard. 30 µg/ml solution of Raltegravir potassium was prepared from the stock solution and injected five times and the areas of five injections were recorded in HPLC. The % RSD was found to be 0.26. The % RSD for the area of five replicate injections was found to be within the specified limit. It showed that the intermediate precision was within the specified limit and it was shown in the table 10 and the chromatograms were shown in the figure 18 & 19.

Table 10 Intermediate Precision & Repeatability Study – Raltegravir Potassium by RP-HPLC Method

<table>
<thead>
<tr>
<th>S.No</th>
<th>Intermediate precision Peak area</th>
<th>Repeatability Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2132155</td>
<td>2134621</td>
</tr>
<tr>
<td>2</td>
<td>2133191</td>
<td>2130124</td>
</tr>
<tr>
<td>3</td>
<td>2127358</td>
<td>2128498</td>
</tr>
<tr>
<td>4</td>
<td>2123351</td>
<td>2131678</td>
</tr>
<tr>
<td>5</td>
<td>2120112</td>
<td>2129123</td>
</tr>
<tr>
<td>Average</td>
<td>2127233</td>
<td>2130809</td>
</tr>
<tr>
<td>SD</td>
<td>5601.7</td>
<td>2446.3</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.56</td>
<td>1.72</td>
</tr>
</tbody>
</table>

Fig.18  Interday Precision Chromatogram of Raltegravir Potassium (30 µg/ml) by HPLC method (Repeatability)
Accuracy was confirmed by recovery studies by adding known amount of pure drug to the previously analysed formulation and the mixture was analysed by the proposed method and chromatograms were shown in the figures 20. The percentage recovery of Raltegravir potassium present in formulation was found to be 101.6%. The values were shown in the table 11.
METHOD DEVELOPMENT AND VALIDATION OF RALTEGRAVIR POTASSIUM AND RILPIVIRINE HCL BY HPLC AND HPTLC METHODS

Table 11 Accuracy Study for Raltegravir Potassium by HPLC Method

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Area</th>
<th>Amount Added(mg)</th>
<th>Amount Found(mg)</th>
<th>Recovery (%)</th>
<th>Mean Recovery (%)</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1084150</td>
<td>5.36</td>
<td>5.46</td>
<td>101.8</td>
<td>101.6</td>
<td>0.2</td>
<td>0.19</td>
</tr>
<tr>
<td>100</td>
<td>2113360</td>
<td>10.5</td>
<td>10.6</td>
<td>101.4</td>
<td>101.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>3226684</td>
<td>16.0</td>
<td>16.2</td>
<td>101.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chromatogram for Recovery of Raltegravir Potassium (50%) by HPLC Method

Chromatogram for Recovery of Raltegravir Potassium (100%) by HPLC Method

Chromatogram for Recovery of Raltegravir Potassium (150%) by HPLC Method

Fig. 20 Chromatograms for Accuracy studies
METHOD DEVELOPMENT AND VALIDATION OF RALTEGRAVIR POTASSIUM AND RILPIVIRINE HCL BY HPLC AND HPTLC METHODS

Robustness was performed by changing the flow rate and by changing the organic composition of the mobile phase. The chromatograms for robustness studies were shown in the figure 21. The results were shown in the table 12. It showed that there was no change in the values even after making deliberate change in the analytical procedure.

Table 12 Reports for Robustness study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>USP plate count</th>
<th>USP tailing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variation in flow rate (ml / min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actual 0.5 ml / min</td>
<td>2451</td>
<td>1.7</td>
</tr>
<tr>
<td>0.6 ml / min</td>
<td>2669</td>
<td>1.6</td>
</tr>
<tr>
<td>0.7 ml / min</td>
<td>2773</td>
<td>1.5</td>
</tr>
<tr>
<td>Change in organic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 % less</td>
<td>2973</td>
<td>1.5</td>
</tr>
<tr>
<td>Composition of mobile phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actual</td>
<td>2669</td>
<td>1.6</td>
</tr>
<tr>
<td>10 % more</td>
<td>2145</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Fig. 21 Robustness Study- Raltegravir Potassium by RP-HPLC Method
Table 13 Ruggedness study for Raltegravir potassium by RP-HPLC method

<table>
<thead>
<tr>
<th>Different Conditions</th>
<th>Percentage obtained 1</th>
<th>Percentage obtained 1</th>
<th>Percentage obtained 1</th>
<th>Average Percentage</th>
<th>SD</th>
<th>(%) RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyst-I</td>
<td>100.00</td>
<td>98.12</td>
<td>99.88</td>
<td>99.33</td>
<td>1.052</td>
<td>1.059</td>
</tr>
<tr>
<td>Analyst-II</td>
<td>98.03</td>
<td>99.17</td>
<td>100.69</td>
<td>99.29</td>
<td>1.334</td>
<td>0.148</td>
</tr>
</tbody>
</table>

Ruggedness refers to the precision of a lab over multiple days which may include multiple analysts, multiple instruments and different sources of the reagents. The developed method was validated for ruggedness. It was confirmed by using different analysts. The percentage RSD values were found to be less than 2% for Raltegravir. Hence the precision was further confirmed. The results were shown in table 13.

6.1.9 High performance thin layer liquid chromatography

In the present work, HPTLC method was developed for estimation of Raltegravir pure powder and its pharmaceutical formulation. HPTLC method is cost effective and less time consuming. Raltegravir was soluble in methanol; therefore methanol was selected as solvent. The formulation was dissolved in methanol with sonication for 10 min to assure complete release of drug from the formulation matrix.

6.1.9.1 Method optimization

For optimization, different mobile phases and composition were employed to achieve the good separation. The method development was initiated with using a mobile phase of Toluene–ethyl acetate in various proportions. In the above conditions, elution was very broad for Raltegravir. Introduction of methanol in the above mobile phase gave sharp peaks, but poor separation and band broadening was observed. Early elution with a little separation was observed with the mobile phase consisting of Toluene: ethyl acetate: methanol (6:3:1). In the same mobile phase change of proportion of Toluene: ethyl acetate: methanol (6:2:2) gave reasonable Rf but not sharp band. Therefore further optimization was needed on the other hand. Addition of acetic acid solution to the mobile phase helped in sharpening of the peak.
Finally, the mobile phase consisting of the mixture of Toluene: ethyl acetate: methanol: Acetic acid (6:3:0.7:0.3) giving poor base line was obtained. So trying different ratios in these combinations viz (5:4:0.3:0.7), (7:2:0.5:0.5) the same problem was obtained. The trial chromatograms were shown in figure 22. Finally, the mobile phase consisting of the mixture of Toluene: ethyl acetate: methanol: Acetic acid (4:5:0.6:0.4) resolved Raltegravir spot with better peak shape. Therefore the combination of Toluene: ethyl acetate: methanol: Acetic acid (4:5:0.6:0.4% v/v) offered optimum migration \( (R_f = 0.12 \pm 0.03) \) and resolution of RAL from other components of formulation matrix. Even saturation of TLC chamber with the mobile phase for 15 min assured better reproducibility and better resolution. The typical densitogram of Raltegravir was shown in figure 23. Raltegravir showed significant UV absorbance at wavelength 254 nm. Hence this wavelength has been chosen for detection in the analysis of Raltegravir.

**Fig. 22 Trial chromatograms for Raltegravir**
Fig. 23 Optimized chromatogram for Raltegravir by HPTLC method

6.1.10 Method validation

A representative calibration curve of Raltegravir was obtained by plotting the mean peak area of Raltegravir against the concentration over the range of 2 – 10 µl / spot. The corresponding chromatograms were shown in figure 24. Calibration curve was shown in figure 25.
A correlation coefficient was found to be 0.9998. This indicated that the concentrations of Raltegravir had good linearity. The average linear regression equation was represented as $Y = 2545.84X + 159.461$ where $X = \text{concentration of Raltegravir}$ and $Y = \text{peak area}$. The limit of detection and limit of quantification were determined based on the signal to noise ratio. The limit of detection and limit of quantification for Raltegravir were found to be 0.037 $\mu g / ml$ and 0.0012 $\mu g / ml$ respectively. The reports of analysis were shown in table 14.
Table 14 Linearity Study – Raltegravir Potassium by HPTLC Method

<table>
<thead>
<tr>
<th>S.No</th>
<th>Concentration (µl/spot)</th>
<th>Average Peak Area</th>
<th>Correlation Coefficient</th>
<th>LOD (µg/ml)</th>
<th>LOD (µg/ml)</th>
<th>Slope</th>
<th>Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>5611.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>10569.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>15234.5</td>
<td>0.9998</td>
<td>0.0037</td>
<td>0.0012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>20533.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>25693.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The nominal concentration from calibration curve was selected and quantification of Raltegravir in formulation was performed. The in-house prepared tablet was selected for the analysis and the amount present was found to be 100.89%. The results were shown in table 15.

Table 15 Analysis of Raltegravir tablet formulation by the Proposed HPTLC Method

<table>
<thead>
<tr>
<th>S.No</th>
<th>Standard peak area</th>
<th>Sample peak area</th>
<th>Mean (%)</th>
<th>SD</th>
<th>(%) RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15417.0</td>
<td>15545.1</td>
<td>100.89</td>
<td>0.4529</td>
<td>0.4489</td>
</tr>
<tr>
<td>2</td>
<td>15254.1</td>
<td>15464.2</td>
<td></td>
<td>0.4529</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>15664.4</td>
<td>15739.1</td>
<td></td>
<td>0.4529</td>
<td></td>
</tr>
</tbody>
</table>

System precision of the instrument was checked by repeatedly scanning of the same spot (600 ng / spot). The % RSD for measurement of peak area was found to be 1.43%. The % RSD for measurement of peak area and sample applications (less than 2%), ensured proper functioning of the HPTLC system. The chromatograms were shown in figure 26 and the report of analysis was shown in table 16.
Table 16: Precision Study – Raltegravir Potassium by HPTLC Method

<table>
<thead>
<tr>
<th>S.No</th>
<th>Peak Area</th>
<th>Average</th>
<th>SD</th>
<th>(%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15234.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>15729.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>15456.3</td>
<td>15545.5</td>
<td>222.614</td>
<td>1.43</td>
</tr>
<tr>
<td>4</td>
<td>15518.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>15788.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 26 Precision Chromatograms for Raltegravir by HPTLC method
The method reproducibility (intra-day precision) was determined by analyzing standard solutions in the concentration of 600 ng/spot of drug for 3 times on the same day and inter-day precision was determined by analyzing corresponding standards daily for 3 days over a period of one week. The intra-day and interday related standard deviations were found to be 0.84 % and 1.84 % respectively. The smaller values of intraday and interday variation in the analysis indicated that the method was precise. The corresponding chromatograms were shown in figure 27 & 28. The results were shown in table 17.

**Table17 Intraday & Interday Precision Study for Raltegravir Potassium by HPTLC Method**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Intraday precision Peak area</th>
<th>Interday Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15416.4</td>
<td>15421.3</td>
</tr>
<tr>
<td>2</td>
<td>15945.7</td>
<td>15842.1</td>
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<td>3</td>
<td>15336.9</td>
<td>15222.0</td>
</tr>
<tr>
<td>4</td>
<td>15147.3</td>
<td>15468.3</td>
</tr>
<tr>
<td>5</td>
<td>15789.6</td>
<td>15611.2</td>
</tr>
<tr>
<td>Average</td>
<td>15227.18</td>
<td>15512.98</td>
</tr>
<tr>
<td>SD</td>
<td>330.458</td>
<td>230.780</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.84</td>
<td>1.84</td>
</tr>
</tbody>
</table>
Fig. 27  Intraday Precision Chromatograms for Raltegravir by HPTLC method
Fig. 28 Interday Precision Chromatograms for Raltegravir by HPTLC method

Accuracy of method was evaluated by calculating recovery of drug by standard addition method at 3 levels of the calibration curve (n = 3). The percentage recovery was found to be in the range from 98.33 to 100.27 % ensuring that the method was accurate. The results indicated that the recovery of added sample was between 40 - 80 %. This clearly indicated that the method was accurate and precise. The chromatograms were shown in figure 29 and the data were shown in table 18.
Table 18 Accuracy Study for Raltegravir Potassium by HPTLC Method

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Area</th>
<th>Amount Added (mg)</th>
<th>Amount Found (mg)</th>
<th>Recovery (%)</th>
<th>Mean Recovery (%)</th>
<th>SD</th>
<th>(%) RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>16454.5</td>
<td>2.4</td>
<td>2.36</td>
<td>98.33</td>
<td>99.46</td>
<td>0.9152</td>
<td>0.9213</td>
</tr>
<tr>
<td>60</td>
<td>20948.6</td>
<td>3.6</td>
<td>3.61</td>
<td>100.27</td>
<td>99.46</td>
<td>0.9152</td>
<td>0.9213</td>
</tr>
<tr>
<td>80</td>
<td>24724.4</td>
<td>4.8</td>
<td>4.79</td>
<td>99.79</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Recovery Chromatogram for Raltegravir potassium by HPTLC method (40%)

Recovery Chromatogram for Raltegravir potassium by HPTLC method (60%)

Recovery Chromatogram for Raltegravir potassium by HPTLC method (80%)

Fig. 29 Accuracy chromatograms for Raltegravir potassium by HPTLC method
Ruggedness refers to the precision of a lab over multiple days which may include multiple analysts, multiple instruments and different sources of the reagents. The developed method was validated for ruggedness. It was confirmed by using different analysts. The percentage RSD values were found to be less than 2% for Raltegravir. Hence the precision was further confirmed. The results were shown in table 19.

Table 19 Ruggedness study for Raltegravir potassium by HPTLC Method

<table>
<thead>
<tr>
<th>Different Conditions</th>
<th>Percentage obtained 1</th>
<th>Percentage obtained 1</th>
<th>Percentage obtained 1</th>
<th>Average Percentage</th>
<th>SD</th>
<th>(%) RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyst-I</td>
<td>100.12</td>
<td>99.56</td>
<td>99.00</td>
<td>99.56</td>
<td>0.159</td>
<td>0.161</td>
</tr>
<tr>
<td>Analyst-II</td>
<td>99.52</td>
<td>100.23</td>
<td>101.23</td>
<td>100.33</td>
<td>0.221</td>
<td>0.220</td>
</tr>
</tbody>
</table>

The method was found to be specific for Raltegravir. The purity of the peak was determined by comparing the chromatograms at three different levels i.e. at peak start (S), peak apex (M) and peak end (E). Correlation between the three chromatograms indicated the purity of peak (correlation, r(S, M) = 0.9999, r(M, E) = 0.9996, fig. 23). The chromatogram extracted from tablet was also compared with the chromatogram of standard, which showed correlation 0.9998. It was observed that the excipients present in formulation did not interfere with the peak of Raltegravir.

6.2 PHASE II
RILPIVIRINE

The analysis of Rilpivirine was done by the following methods
1. High performance liquid chromatographic method (HPLC)
2. High performance thin layer chromatographic method (HPTLC)
6.2.1 Method development and validation of HPLC method

6.2.1.1 Selection of chromatographic method

The choice of chromatographic method is based on the nature of the sample (ionic or neutral molecule), its molecular weight and solubility. As the nature of the drug was polar, the reverse phase chromatographic technique was selected for the present work.

6.2.1.2 Selection of wave length

The sensitivity of the HPLC method depends upon the proper selection of the detection wavelength. An ideal wavelength is one that gives good response to be detected. A solution of Rilpivirine (10 µg / ml) was scanned in the UV region using mixed phosphate buffer pH (6.8): Acetonitrile in the ratio of 55:45% v/v. The absorbance was found at 272 nm. Rilpivirine in different mobile phase ratios, different pH and different flow rate values were also scanned in the UV region. There was no significant change in the absorbance and absorbance maximum. Hence 272nm was selected as detection wavelength for the estimation of Rilpivirine by HPLC method.

6.2.1.3 Preparation of Mixed phosphate buffer

2.954 grams of potassium dihydrogen phosphate and 0.545 grams of Dipotassium hydrogen phosphate were weighed and transferred into a 1000 ml standard flask and diluted to 1000 ml with the HPLC grade water. The pH was adjusted to 6.8 with ortho phosphoric acid.

6.2.1.4 Preparation of mobile phase

Mixed phosphate buffer 550 ml (55%) and Acetonitrile 450 ml (45%) HPLC grade were transferred into a 1000 ml standard flask and mixed well. After that the mobile phase was degassed in sonicator for 10 min. The mobile phase was filtered by using the membrane filtration method under the application of vacuum.
6.2.1.5 Initial separation conditions

The following chromatographic conditions were fixed initially to improve the separation of Rilpivirine:

- **Mode of operation**: Isocratic
- **Stationary phase**: YMC-C\textsubscript{18} short column
- **Mobile phase**: Mixed phosphate buffer pH 6.8: Acetonitrile
- **Ratio**: 55:45% v/v
- **Detection wavelength**: 272 nm
- **Flow rate**: 1.2 ml/min
- **Temperature**: Ambient
- **Sample volume**: 20 µl
- **Quantification method**: External standard calibration method

6.2.1.6 Effect of Composition and ratio of mobile phase

Different compositions of mobile phases and ratios were tried. The mobile phases like acetonitrile: water (50:50 % v / v), mixed phosphate buffer: acetonitrile (pH 6.8) (55:45 % v / v), Ammonium phosphate buffer: acetonitrile (pH3.0) (40:60%v/v), methanol: water (50:50 % v / v) were tried. The efficiency was found to be 656 by using the mobile phase acetonitrile: water (50:50 % v / v) with YMC C\textsubscript{18} column. To increase the efficiency value for Rilpivirine with mobile phase containing mixed phosphate buffer: acetonitrile (pH 6.8) (55:45 % v / v) with YMC C\textsubscript{18} column and ammonium phosphate buffer: acetonitrile (pH3.0) (40:60 % v / v) with BDS Hypersil C\textsubscript{18} column was used. The tailing factor value was not found within the limit. So the mobile phase consisting of methanol: water (50:50 % v / v) with YMC C\textsubscript{18} column was tried. The retention time was found to be above 9.0 minutes. Finally the mobile phase containing mixed phosphate buffer: acetonitrile (pH 6.8) (60:40 % v / v) with BDS Hypersil C\textsubscript{18} column was used. The retention time was found to be 4.350 min. Hence mixed phosphate buffer: acetonitrile (pH 6.8) (60:40 % v / v) was used for the further analysis.
6.2.1.7 Effect of flow rate on separation

Different flow rates like 1.0 ml / min and 1.2 ml / min were tried with above said mobile phase composition. When 1.0 ml / min flow rate was used, the chromatograms showed better separation than when 1.2 ml / min flow rate was used. Hence mixed phosphate buffer (pH 6.8): acetonitrile in the ratio of (60:40%v/v) with 1.0 ml / min flow rate was used for further studies.

6.2.2 Optimized chromatographic conditions

The following parameters were used for RP-HPLC analysis of Rilpivirine

Mode of operation : Isocratic
Stationary phase : BDS Hypersil C_{18} column, (250 mm x 4.6 mm i.d., 5µ)
Mobile phase : Mixed phosphate buffer (pH 6.8): acetonitrile
Ratio : 60:40% v/v
Detection wavelength : 272nm
Flow rate : 1.0ml/min
Temperature : ambient
Sample volume : 20 µl
Quantification method : External standard calibration method

6.2.2.1 Preparation of standard stock solution:

10 mg of Rilpivirine pure was accurately weighed and transferred into 100ml volumetric flask. About 7ml of mobile phase was added and sonicated to dissolve it completely and the volume was made up to the mark with the same mobile phase to obtain a concentration of working standard solution (100 µg / ml).

6.2.2.2 Preparation of working sample solution

10 tablets of Edurant (containing 25 mg of Rilpivirine) were weighed and powdered. The tablet powder equivalent to 10 mg of Rilpivirine was transferred into a 100ml standard flask and 50 ml of mobile phase was added. The solution was sonicated for 15 minutes and it was filtered through 0.45µTeflon membrane filter.
Then the final volume was made with the same to obtain the concentration (100 µg / ml).

6.2.2.3 System Suitability

System suitability tests are an integral part of any chromatographic analysis method which is used to verify reproducibility of the chromatographic system. To ascertain its effectiveness, certain system suitability test parameters were checked by repeatedly injecting the drug solution at the concentrations of 5 µg / ml. 20 µL standard solutions were injected and chromatograms were recorded.

6.2.2.4 Preparation of calibration graph

Calibration curve was constructed by plotting peak area concentration of Rilpivirine solutions. Aliquots of standard stock solution of Rilpivirine (0.1 to 1.0 ml of 100 µg / ml) were transferred into 10 ml standard flask and made up to the volume with mobile phase to get a concentration range of (1 to 10 µg / ml). The solutions were injected and the chromatograms were recorded at 272 nm. It was found that the above concentration range was linear with the concentration range of 1 - 10 µg / ml.

6.2.2.5 Limit of detection and limit of Quantification

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

The detection limit (LOD) for the proposed method was calculated using the following equation

\[ \text{LOD} = 3.3 \frac{S}{K} \]

where ‘S’ is the standard deviation of replicate determination values under the same conditions as for sample analysis in absence of the analyte and ‘K’ is the sensitivity namely the slope of the calibration graph. The limit of quantification (LOQ) was defined as \( \text{LOQ} = 10 \frac{S}{K} \).
6.2.2.6 Quantification of formulation (Assay)

5 µg / ml of standard and sample solution were prepared separately and 20 µl of standard and sample solutions were injected into the chromatographic system separately and the peak area for the Rilpivirine was measured and the percentage purity was calculated by using the following formula.

\[
\text{Assay} \% = \frac{\text{AT} \times \text{WS} \times \text{DT} \times \text{P} \times \text{Avg. Wt}}{\text{AS} \times \text{DS} \times \text{WT} \times 100 \times \text{Label Claim}} \times 100
\]

6.2.2.7 Precision and intermediate precision

Precision is the degree of repeatability of an analytical method under normal operational conditions. The precision of assay was determined by repeatability (intraday) and intermediate precision (interday) and reported as % RSD for a statistically significant number of replicate measurements. Repeatability and intermediate precision of the method were determined by analyzing six sample solutions and standard solutions prepared in the concentration of 5 µg / ml. 20 µl of sample and standard solutions were injected into the chromatographic system and the peak area was recorded.

6.2.2.8 Accuracy

The recovery experiment was done by adding known concentration of Rilpivirine raw material to preanalyzed formulation. The tablet powder equivalent to 10 mg of Rilpivirine was weighed and transferred into a series of three 100ml standard flask. To that, 3 mg, 6 mg and 9 mg of raw material were added (30 %, 60 %, 90 %) and dissolved with mobile phase and made up to the volume with the same. Then it was sonicated for 15 minutes. After sonication the solution was filtered through 0.45µ Teflon membrane filter. From the clear solution, 0.5 ml of each test solution was pipetted out made into six replicate for 10ml volumetric flask. Each concentration 20 µl solutions were injected and the chromatograms were recorded. Each concentration was repeated for three times.
6.2.2.9 Robustness

For demonstrating the robustness study of the developed method, experimental conditions were purposely altered and evaluated. The method must be robust enough to withstand such changes and allow routine analysis of the sample. In the present study, the flow rate was varied from 0.8 to 1.2 ml / min and organic composition in the mobile phase was varied for more than 10 % and less than 10 %. 5 µg / ml concentrations of standard and sample solutions were prepared separately and injected into chromatographic system to save the value of peak area.

6.2.2.10 Ruggedness

The degree of reproducibility of test results by the proposed method of Rilpivirine was detected by analyzing the drug sample under following variety of test conditions. 1. Different analyst 2. Different instruments

6.2.2.11 Specificity

The method specificity was assessed by comparing the chromatograms obtained from the drug and the most commonly used excipients mixture with those obtained from blank (excipients solution in water without drug). It was observed that there was no interference of the peak obtained for the chromatogram of blank and placebo with that of peaks obtained for the chromatograms of sample and standard.

6.2.3 High performance thin layer chromatographic method (HPTLC)

6.2.3.1 Preparation of mobile phase

80 ml of ethyl acetate, 10 ml of methanol and 10 ml of chloroform were taken and transferred into a 100 ml volumetric flask. All reagents used were in HPLC grade.

6.2.3.2 Initial separation conditions

The following chromatographic conditions were fixed initially to improve the separation of Rilpivirine
METHOD DEVELOPMENT AND VALIDATION OF RALTEGRAVIR POTASSIUM AND RILPIVIRINE HCL BY HPLC AND HPTLC METHODS

Stationary phase : 4 mm band length in the 20 x 10 Silica gel 60F<sub>254</sub>

Mobile phase : Methanol: ethyl acetate

Ratio : 8:2 % v/v

Scanner : Camag-TLC Scanner-3

Detection wavelength : 254 nm

Quantification method : External standard calibration method

6.2.3.3 Effect of mobile phase composition and ratios

The different compositions and ratios of mobile phase were tried. First Ethyl acetate: methanol (8:2 % v/v) was used. The peak was not found symmetrical and tailing was observed. The tailing was reduced by addition of chloroform. Hence ethyl acetate: methanol: chloroform (8:1:1 % v/v/v) was used for the further analysis.

6.2.4 Optimized chromatographic conditions

The following parameters were used for HPTLC analysis of Rilpivirine

Stationary phase : 4mm band length in the 20 x 10 Silica gel 60F<sub>254</sub>

Mobile phase : Ethyl acetate: Methanol: chloroform

Ratio : 8:1:1%v/v/v

Scanner : Camag-TLC Scanner-3

Detection wavelength : 254 nm

Quantification method : External standard calibration method

6.2.4.1 Preparation of standard stock solution

About 10 mg of working standard of Rilpivirine was accurately weighed and transferred into 100 ml volumetric flask. About 25 ml of methanol was added and sonicated for about 20 min. Finally the volume was made up to 100 ml with methanol to obtain the concentration of about 100 μg / ml. 0.1 ml was taken from this stock
solution and the volume made up to 100 ml to get a concentration of about 100 ng / µl.

6.2.4.2 Preparation of sample solution

10 tablets of Edurant (containing 25mg of Rilpivirine) were weighed and powdered. The tablet powder equivalent to 10 mg of Rilpivirine was transferred into 100 ml standard flask and 50 ml of mobile phase was added. The solution was sonicated for 15 minutes and it was filtered through 0.45µ filter. Then the final volume was made with the same to obtain the concentration (100 µg / ml). 0.1 ml was taken from this stock solution and the volume made up to 100 ml to get a concentration of about 100 ng /µl

6.2.4.3 Linearity (calibration Graph)

Aliquots (5, 10, 15, 20, 25 and 30 µl) of standard solution of Rilpivirine were spotted on pre coated TLC plates using a semi-automatic spotter under nitrogen stream. The plate was dried in air and developed up to 90 mm at constant temperature with a mixture of Ethyl acetate: Methanol: chloroform (8: 1:1 % v / v / v) as mobile phase in a CAMAG twin trough chamber which was previously saturated with mobile phase for about 15 min. The plate was removed from the chamber and dried in air. Photometric measurements were performed at 254 nm in absorbance/reflectance mode with the CAMAG TLC scanner 3 using CATS 4 software incorporating track optimizing option. The standard plot of Rilpivirine was established by plotting the peak area Vs concentration (ng / ml) corresponding to each spot.

6.2.4.4 Limit of detection and limit of Quantification

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy.
METHOD DEVELOPMENT AND VALIDATION OF RALTEGRAVIR POTASSIUM AND RILPIVIRINE HCL BY HPLC AND HPTLC METHODS

The detection limit (LOD) for the proposed method was calculated using the following equation

\[
\text{LOD} = 3.3 \frac{S}{K}
\]

where ‘S’ is the standard deviation of replicate determination values under the same conditions as for sample analysis in absence of the analyte and ‘K’ is the sensitivity namely the slope of the calibration graph. The limit of quantification (LOQ) was defined as \( \text{LOQ} = 10 \frac{S}{K} \).

6.2.4.5 Quantification of formulation (Assay)

Fifteen micro liters of standard and sample solutions were applied on a TLC plate using a semi-automatic spotter under a nitrogen stream. The amount of Rilpivirine present in the sample solution was determined by fitting the area values of peaks corresponding to Rilpivirine into the equation of the line representing the calibration curve of RLP. All determinations were performed in triplicate.

6.2.4.6 Precision and intermediate precision

Precision is the degree of repeatability of an analytical method under normal operational conditions. The precision of assay was determined by repeatability (intraday) and intermediate precision (interday) and reported as %RSD for a statistically significant number of replicate measurements. Repeatability and intermediate precision of the method were determined by analyzing six sample solutions prepared in the concentration of 15µl. Fifteen micro liters of sample solutions were applied on a TLC plate using a semi-automatic spotter under a nitrogen stream and followed by development of plate and recording the peak area of 6 spots. The %RSD for peak area was calculated.

6.2.4.7 Accuracy

Accuracy was determined for standard quality samples (in addition to calibration standards) prepared in triplicates at different concentration levels (40%, 80% and 120 %) \( (n = 3) \) within the range of linearity of the drug. The results of recovery studies were obtained by the method validation by statistical evaluation.
6.2.4.8 Ruggedness

The degree of reproducibility of test results by the proposed method of Rilpivirine was detected by analyzing the drug sample under following variety of test conditions. 1. Different analyst 2. Different instruments.

6.2.4.9 Specificity

The method specificity was assessed by comparing the chromatograms obtained from the drug and most commonly used excipients mixture with those obtained from blank (excipients solution in water without drugs). It was observed that there was no interference of the peak obtained for the chromatogram of blank and placebo with that of Rilpivirine peaks obtained for the chromatograms of sample and standard.

6.2.5 Results and Discussions

6.2.5.1 High performance liquid chromatography

The optimization was done by changing the mobile phase, mobile phase ratios, flow rate and column. Different ratios of mixed phosphate buffer and acetonitrile (pH 6.8) were tried. The mobile phase was filtered through 0.45 µm Teflon membrane filter and degassed by sonication prior to use. The different flow rates (1.0 ml / min, 1.5 ml / min and 2.0 ml / min) were tried. The trial chromatograms were shown in figure 30. Finally stationary phase BDS hypersil C\textsubscript{18} column with mobile phase containing mixed phosphate buffer adjusted to pH 6.8 and acetonitrile in the ratio of 60:40 %v/v with flow rate 1.0 ml/min. The retention time was found to be 3.137 min. Using the above chromatographic conditions resulted in the development of an efficient reproducible method for the determination of Rilpivirine in bulk and tablet dosage form. The optimized chromatogram was shown in figure 31.
METHOD DEVELOPMENT AND VALIDATION OF RALTEGRAVIR POTASSIUM AND RILPIVIRINE HCL BY HPLC AND HPTLC METHODS

Fig 30 Trial chromatograms for Rilpivirine by HPLC method

Fig. 31 Optimized Chromatogram for Rilpivirine by HPLC method
6.2.6 Method validation

6.2.6.1 High performance liquid chromatography

When a method has been optimized it must be validated before practical use. By following the ICH guidelines for analytical method validation Q2 (R1), the validation characteristics were addressed (ICH Q2A and Q2B).

The system suitability parameters like tailing factor, number of theoretical plates, %RSD, HETP and capacity factor were calculated and these values were compared with the standards limit as per USP. The results were shown in table 20.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Experimental Value</th>
<th>Limit as Per USP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tailing factor</td>
<td>1.057</td>
<td>Less than 2</td>
</tr>
<tr>
<td>Asymmetric factor</td>
<td>0.654</td>
<td>Less than 2</td>
</tr>
<tr>
<td>No. of theoretical plates</td>
<td>2653</td>
<td>More than 2000</td>
</tr>
<tr>
<td>Capacity factor</td>
<td>1.45</td>
<td>1-10</td>
</tr>
<tr>
<td>HETP</td>
<td>0.4567</td>
<td>-</td>
</tr>
<tr>
<td>Theoretical plate per unit length</td>
<td>231.12</td>
<td>-</td>
</tr>
</tbody>
</table>

Calibration curve was constructed by plotting the peak area vs. concentration. For HPLC method the mean equation of calibration curve consisting of ten points Y = 14701.67X + 91351.25 where ‘Y’ represents the peak area and ‘X’ represents the concentration of Rilpivirine. Correlation coefficient 0.9990 confirmed that the calibration curve was linear over the concentration range of 1 – 10 µg / ml. The corresponding chromatograms were shown in figure 32. The calibration curve is shown in figure 33. The limit of detection and the limit of quantification were determined based on the signal to noise ratio. The LOD and LOQ values were found to be 0.0427 µg /ml and 0.724 µg / ml respectively. The chromatograms for LOD and LOQ were shown in the figure 34. The reports were shown in table 21.
### Table 21 Linearity Study – Rilpivirine by RP-HPLC Method

<table>
<thead>
<tr>
<th>NO</th>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Concentration range</td>
<td>1-10 µg/ml</td>
</tr>
<tr>
<td>2</td>
<td>Slope</td>
<td>91351.25</td>
</tr>
<tr>
<td>3</td>
<td>Intercept</td>
<td>14701.67</td>
</tr>
<tr>
<td>4</td>
<td>Correlation coefficient</td>
<td>0.9990</td>
</tr>
<tr>
<td>5</td>
<td>Regression equation</td>
<td>Y = 14701.67X + 91351.25</td>
</tr>
<tr>
<td>6</td>
<td>LOD (µg/ml)</td>
<td>0.0427</td>
</tr>
<tr>
<td>7</td>
<td>LOQ (µg/ml)</td>
<td>0.724</td>
</tr>
</tbody>
</table>

![Graphs showing 1 µg/ml, 2 µg/ml, 3 µg/ml, and 4 µg/ml.](image-url)
METHOD DEVELOPMENT AND VALIDATION OF RALTEGRAVIR POTASSIUM AND RILPIVIRINE HCL BY HPLC AND HPTLC METHODS

Fig. 32  Linearity chromatograms for Rilpivirine by HPLC method  (1-10 µg / ml)
Fig. 33 Calibration Curve for Rilpivirine by HPLC method

Fig. 34 LOD and LOQ Chromatograms for Rilpivirine by HPLC method

The tablet formulation of Edurant was selected for analysis and the percentage purity was found to be 99.36 %. The procedure was repeated for three times to validate the method. The % RSD was found to be less than 2% which indicated that the method had good precision. The reports of analysis were shown in table 22 and the chromatograms were shown in figure 35.

Table 22 Analysis of Rilpivirine formulation by the Proposed RP-HPLC Method

<table>
<thead>
<tr>
<th>S.no</th>
<th>Standard peak area</th>
<th>Sample peak area</th>
<th>Mean (%)</th>
<th>SD</th>
<th>(%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>425713</td>
<td>429005</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>426112</td>
<td>428937</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>425543</td>
<td>427705</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>425789.3</td>
<td>428549</td>
<td>99.36</td>
<td>0.9820</td>
<td>0.9883</td>
</tr>
</tbody>
</table>

\[ y = 92329x - 17343 \]
\[ R^2 = 0.9993 \]
Precision study was done with the Rilpivirine standard. 5 µg / ml solution of Rilpivirine was prepared from the stock solution and injected five times and the areas of five injections were recorded in HPLC. The % RSD was found to be 0.6845. The % RSD for the area of five replicate injections was found to be within the specified limit. It showed that the drug was having good precision and it was shown in table 23 and the chromatograms were shown in figure 36.
Table 23 Precision & Intermediate Precision Study – Rilpivirine by RP-HPLC Method

<table>
<thead>
<tr>
<th>S.no</th>
<th>Precision Peak area</th>
<th>Intermediate Precision Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>426526</td>
<td>431013</td>
</tr>
<tr>
<td>2</td>
<td>422003</td>
<td>431517</td>
</tr>
<tr>
<td>3</td>
<td>422028</td>
<td>431577</td>
</tr>
<tr>
<td>4</td>
<td>428139</td>
<td>431612</td>
</tr>
<tr>
<td>5</td>
<td>422393</td>
<td>438790</td>
</tr>
<tr>
<td>Average</td>
<td>424217.8</td>
<td>432901.8</td>
</tr>
<tr>
<td>SD</td>
<td>2904.05</td>
<td>3300.56</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.6845</td>
<td>0.7624</td>
</tr>
</tbody>
</table>

Fig. 36 Precision Chromatograms for Rilpivirine by HPLC method
Intermediate precision study was done with the Rilpivirine standard. 5 µg/ml solution of Rilpivirine was prepared from the standard stock solution and injected five times and the areas of five injections were recorded in HPLC. The % RSD was found to be 0.7624 %. The % RSD for the area of five replicate injections was found to be within the specified limit. It showed the intermediate precision was within the specified limit and the results were shown in the table 23 and the chromatograms were shown in the figure 37.

Fig. 37 Intermediate Precision Chromatograms for Rilpivirine by HPLC method
Accuracy of the method was evaluated by calculating the recovery of drug by standard addition method at 3 levels of the calibration curve (n = 3). The percentage mean recovery was found to be 100.53% ensuring that the method was accurate. The results indicated that the recovery of added sample was between 30 - 90 %. This clearly indicated that the method was accurate and precise. The chromatograms were shown in figure 38 and the results were shown in table 24.

**Table 24 Accuracy Study-Rilpivirine by RP-HPLC Method**

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Mean area</th>
<th>Amount added(µg/ml)</th>
<th>Amount found (µg/ml)</th>
<th>Mean Recovery (%)</th>
<th>SD</th>
<th>(%) RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>241070.3</td>
<td>1.56</td>
<td>1.52</td>
<td>100.53</td>
<td>0.6429</td>
<td>0.6397</td>
</tr>
<tr>
<td>60</td>
<td>492788.6</td>
<td>3.05</td>
<td>3.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>7363844.0</td>
<td>4.54</td>
<td>4.53</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Accuracy Chromatograms for Rilpivirine by HPLC method (30%)**

**Accuracy Chromatograms for Rilpivirine by HPLC method (60%)**
The robustness of the method was evaluated by analyzing the system suitability standards and evaluating system suitability parameter data after varying individually, the HPLC pump flow rate and organic composition of the mobile phase. The results were shown in table 25. The chromatograms for robustness studies were shown in the figure 39. The result showed the method was robust.

**Table 25 Reports for Robustness study**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>System suitability parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>USP plate count</td>
</tr>
<tr>
<td>Flow rate variation</td>
<td></td>
</tr>
<tr>
<td>0.8ml/min</td>
<td>2562.2</td>
</tr>
<tr>
<td>1.0ml/min (actual)</td>
<td>2653.3</td>
</tr>
<tr>
<td>1.2ml/min</td>
<td>2609.6</td>
</tr>
<tr>
<td>Organic phase composition</td>
<td></td>
</tr>
<tr>
<td>10% less</td>
<td>2975.1</td>
</tr>
<tr>
<td>Actual (60:40%v/v)</td>
<td>2653.3</td>
</tr>
<tr>
<td>10 % more</td>
<td>2145.0</td>
</tr>
</tbody>
</table>
Ruggedness refers to the precision of a lab over multiple days which may include multiple analysts, multiple instruments and different sources of the reagents. The developed method was validated for ruggedness. It was confirmed by using different analysts. The percentage RSD values were found to be less than 2% for Rilpivirine. Hence the precision was further confirmed. The results were shown in table 26.

**Table 26 Ruggedness study for Rilpivirine by HPLC Method**

<table>
<thead>
<tr>
<th>Different Conditions</th>
<th>Percentage obtained 1</th>
<th>Percentage obtained 1</th>
<th>Percentage obtained 1</th>
<th>Average Percentage</th>
<th>SD</th>
<th>(%) RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyst-I</td>
<td>99.35</td>
<td>99.67</td>
<td>98.87</td>
<td>99.29</td>
<td>0.402</td>
<td>0.405</td>
</tr>
<tr>
<td>Analyst-II</td>
<td>99.35</td>
<td>101.17</td>
<td>98.88</td>
<td>100.10</td>
<td>1.209</td>
<td>1.211</td>
</tr>
</tbody>
</table>
The method specificities was assessed by comparing the chromatograms (HPLC) obtained from the drug and the most commonly used excipients mixture with those obtained from blank (excipients solution in water without drugs). It was observed that there was no interference from the peaks obtained for the chromatogram of blank and placebo with that of Rilpivirine HCL peaks obtained for the chromatogram of sample and standard Rilpivirine HCL. Hence the proposed method was highly selective and specific.

### 6.2.6.2. High performance thin layer liquid chromatography

Initially plane solvents like methanol, toluene, chloroform and ethyl acetate were tired. The spots were developed with methanol and ethyl acetate but tailing was observed. Then methanol and ethyl acetate in the ratio (8:2 % v / v) were used but the distance traveled by the developed spots was high at solvent front and tailing was also observed. Then methanol and ethyl acetate in the ratio (2:8 % v / v) was tired. In this condition Rf values were reduced. The proportion of methanol was increased and the Rf value of drug was satisfactory but peak was not symmetrical and tailing was observed. The tailing was reduced by addition of chloroform. The symmetrical peak was observed. Ultimately mobile phase consists of ethyl acetate: methanol: chloroform (8:1:1 % v / v / v) which gave good peaks at 254 nm. Trial chromatograms were shown in figure 40 and optimized chromatogram was shown in figure 41.

![Fig. 40 Trial Chromatograms for Rilpivirine by HPTLC method](image)
Fig. 41 Optimized Chromatogram for Rilpivirine by HPTLC method

A calibration curve of Rilpivirine was obtained by plotting the mean peak area of Rilpivirine against the concentration over the range of 5 - 30 µl / spot. The corresponding chromatograms were shown in figure 42. Calibration curve was shown in figure 43. A correlation coefficient was found to be 0.9992 indicated that the concentrations of Rilpivirine had good linearity. The average linear regression equation was represented as \( Y = 27865.03X + 22950.3 \) where \( X = \) concentration of Rilpivirine and \( Y = \) peak area. The limit of detection and limit of quantification were determined based on the signal to noise ratio. The limit of detection and limit of quantification for Rilpivirine were found to be 0.317 µg / ml and 0.513 µg / ml respectively. The reports of analysis were shown in table 27.

Table 27 Linearity Study – Rilpivirine by HPTLC Method

<table>
<thead>
<tr>
<th>S.No</th>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Concentration range</td>
<td>5-30 µl / spot</td>
</tr>
<tr>
<td>2</td>
<td>Slope</td>
<td>22950.3</td>
</tr>
<tr>
<td>3</td>
<td>Intercept</td>
<td>27865.03</td>
</tr>
<tr>
<td>4</td>
<td>Correlation coefficient</td>
<td>0.9992</td>
</tr>
<tr>
<td>5</td>
<td>Regression equation</td>
<td>( y = 27865.03X + 22950.3 )</td>
</tr>
<tr>
<td>6</td>
<td>LOD</td>
<td>0.317</td>
</tr>
<tr>
<td>7</td>
<td>LOQ</td>
<td>0.513</td>
</tr>
</tbody>
</table>
METHOD DEVELOPMENT AND VALIDATION OF RALTEGRAVIR POTASSIUM AND RILPIVIRINE HCL BY HPLC AND HPTLC METHODS

5 μl / spot

10 μl / spot

15 μl / spot

20 μl / spot

25 μl / spot

30 μl / spot

Fig. 42 Linearity chromatograms for Rilpivirine by HPTLC method
The nominal concentration from calibration curve was selected and quantification of Rilpivirine in formulation was performed. The Edurant tablet formulation was selected for the analysis and the amount present was found to be 100.24%. The reports were shown in Table 28.

**Table 28 Analysis of Rilpivirine formulation by the Proposed HPTLC Method**

<table>
<thead>
<tr>
<th>S.no</th>
<th>Standard peak area</th>
<th>Sample peak area</th>
<th>Mean (%)</th>
<th>SD</th>
<th>(%) RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>394102</td>
<td>391245</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>390230</td>
<td>390231</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>389901</td>
<td>389843</td>
<td>100.24</td>
<td>0.4158</td>
<td>0.4148</td>
</tr>
<tr>
<td>Average</td>
<td>391411</td>
<td>390439.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

System precision of the instrument was checked by repeated scanning of the same spot (1500 ng / spot) and % RSD for measurement of peak area was found to be 0.5088 %. The % RSD for measurement of peak area and sample applications (less than 2%), ensured proper functioning of HPTLC system. Intermediate precision of the method was determined by analyzing six sample solutions prepared in the concentration of 15µl and applied on the TLC plate. The % RSD for peak area was...
calculated. The precision and intermediate precision were found to be 0.3044% and 0.5088% respectively. The smaller values of precision and intermediate precision variation in the analysis indicated that the method was precise. The chromatograms were shown in figure 44 & 45 and the results were shown in table 29.

Table 29 Precision & Intermediate Precision Study – Rilpivirine by HPTLC Method

<table>
<thead>
<tr>
<th>S.no</th>
<th>Precision Peak area</th>
<th>Intermediate precision Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>392147</td>
<td>381457</td>
</tr>
<tr>
<td>2</td>
<td>391521</td>
<td>382368</td>
</tr>
<tr>
<td>3</td>
<td>389421</td>
<td>380634</td>
</tr>
<tr>
<td>4</td>
<td>390132</td>
<td>384942</td>
</tr>
<tr>
<td>5</td>
<td>391915</td>
<td>379942</td>
</tr>
<tr>
<td>Average</td>
<td>391027.2</td>
<td>381868.4</td>
</tr>
<tr>
<td>SD</td>
<td>1190.298</td>
<td>1943.029</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.3044</td>
<td>0.5088</td>
</tr>
</tbody>
</table>
Fig. 44 Precision Chromatograms for Rilpirvine by HPTLC method

Fig. 45 Intermediate Precision chromatograms for Rilpirvine by HPTLC method
Accuracy of the method was evaluated by calculating recovery of drug by standard addition method at 3 levels of the calibration curve (n = 3). The percentage mean recovery was found to be 100.17 % ensuring that the method was accurate. The results indicated that the recovery of added sample was between 40 - 120 %. This clearly indicated that the method was accurate and precise. The chromatograms were shown in figure 46. The reports of analysis were shown in table 30.

Table 30 Accuracy Study-Rilpivirine by HPTLC Method

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Mean area</th>
<th>Amount Added (µg/ml)</th>
<th>Amount found (µg/ml)</th>
<th>Mean Recovery (%)</th>
<th>SD</th>
<th>(%) RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>344917.6</td>
<td>3.01</td>
<td>3.02</td>
<td>100.17</td>
<td>0.8711</td>
<td>0.8692</td>
</tr>
<tr>
<td>80</td>
<td>432001</td>
<td>6.32</td>
<td>6.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>603513</td>
<td>9.10</td>
<td>9.10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Accuracy Chromatograms for Rilpivirine by HPTLC method (40%)

Accuracy Chromatograms for Rilpivirine by HPTLC method (80%)
Ruggedness refers to the precision of a lab over multiple days which may include multiple analysts, multiple instruments and different sources of the reagents. The developed method was validated for ruggedness. It was confirmed by using different analysts. The percentage RSD values were found to be less than 2% for Rilpivirine. Hence the precision was further confirmed. The results were shown in table 31.

Table 31 Ruggedness study for Rilpivirine by HPTLC Method

<table>
<thead>
<tr>
<th>Different Conditions</th>
<th>Percentage obtained 1</th>
<th>Percentage obtained 1</th>
<th>Percentage obtained 1</th>
<th>Average Percentage</th>
<th>SD</th>
<th>(%) RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyst-I</td>
<td>98.56</td>
<td>99.00</td>
<td>99.23</td>
<td>98.93</td>
<td>1.117</td>
<td>1.130</td>
</tr>
<tr>
<td>Analyst-II</td>
<td>100.2</td>
<td>98.8</td>
<td>100.9</td>
<td>99.96</td>
<td>1.059</td>
<td>1.065</td>
</tr>
</tbody>
</table>

The method was found to be specific for Rilpivirine. The purity of the peak was determined by comparing the chromatograms at three different levels i.e. at peak start (S), peak apex (M) and peak end (E). Correlation between the three chromatograms indicated the purity of peak (correlation, r(S, M) = 0.9994, r (M, E) = 0.9991, fig. 41). The chromatogram extracted from the tablet was also compared with the chromatogram of standard, which showed correlation 0.9992. It was observed that the excipients present in formulation did not interfere with the peak of RLP.
6.3 Conclusion

6.3.1 Phase 1

The proposed RP-HPLC and HPTLC methods are simple, reliable and selective, providing satisfactory accuracy and precision with lower limit of detection and lower limit of quantitation. More over the shorter duration of analysis time of Raltegravir. The more suitable were the reported methods for routine quantitative analysis in pharmaceutical dosage form (in-house tablet). The recoveries achieved were good by both the methods.

6.3.2 Phase II

A new, accurate and selective isocratic RP-HPLC and HPTLC methods were proposed for the determination of Rilpivirine bulk and in tablet dosage form validated as per the ICH guidelines. The methods were found to be simple, selective, precise, accurate and robust. Therefore, these methods can be used as routine quality control analysis of Rilpivirine bulk and in tablet dosage. All statistical results (Percentage, Mean, RSD, Percentage difference and recovery %) were within the acceptance criteria.

6.4 REFERENCES


