DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE ESTIMATION OF SIROLIMUS IN FORMULATION AND SERUM

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ABSTRACT
A simple, selective, linear, precise and accurate RP-HPLC method was developed and validated for rapid assay of Sirolimus in tablet dosage form. Isocratic elution at a flow rate of 1.0ml/min was employed on a symmetry C18 (250x4.6mm, 5µm in particle size) column at ambient temperature. The mobile phase consisted of acetonitrile : methanol 20:80 (V/V). The UV detection wavelength was 272nm and 20µl sample was injected. The retention time for Sirolimus was 2.740 min. The percentage RSD for precision and accuracy of the method was found to be less than 2%. The method was validated as per the ICH guidelines. The method developed can be applied for routine analysis of Sirolimus in tablet dosage form and in serum.

Key Words: Sirolimus, RP-HPLC, UV detection, serum, recovery, precise.

INTRODUCTION

Sirolimus is \((3S,6R,7E,9R,10R,12R,14S,15E,17E,19E,21S,23S,26R,27R,34aS)-9,10,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-[(1R)-2-[(1S,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c][1,4]oxazacyclopentacontine-1,5,11,28,29(4H,6H,31H)-pentone.\) Sirolimus also known as Rapamune or rapamycin is one of the macrocyclic lactones produced by \(\textit{Streptomyces hygroscopicus}\). Sirolimus is a potent immunosuppressive agent shown by variety of animal transplant models and human clinical trials. Sirolimus is metabolized in humans by hepatic and intestinal cytochrome P450 3A4, primarily leading to demethylated and hydroxylated metabolites. Therapeutic drug monitoring (TDM) of sirolimus concentrations plays an important role in the selection of the optimum dose of sirolimus. Sirolimus binds to the immunophilin FK506 binding protein and is sequestered in red blood cells, leading to whole-blood/plasma ratios of ~38. TDM of sirolimus values provides a useful prediction of inadequate immunosuppression or potential adverse events. When sirolimus is given with full-dose cyclosporine, the recommended therapeutic range for sirolimus whole-blood trough concentrations is 5–15µg/L. For an analytical method to be suitable for the TDM of sirolimus, it should be simple, sensitive (lower limit of quantification, <5 µg/L), and rapid (turn-around time, <24 h). Several HPLC assays using ultraviolet (UV) detection (HPLC-UV) have been reported for the determination of sirolimus concentrations in whole blood. The assays are complicated by interfering peaks in the chromatograms, requiring tedious extraction procedures and long run times to resolve the peaks. The goal of the present study was to develop a simple RP-HPLC method for the determination of sirolimus that was precise and accurate at low concentrations and was capable of a high throughput. The validation was performed in accordance with regulatory guidelines. In summary, a RP-HPLC method for the quantification of sirolimus in human whole blood has been developed and validated. The data presented in this report demonstrate that the method provides rapid, sensitive, precise, and accurate measurements of sirolimus concentrations in human whole blood.
an immunosuppressant drug used to prevent rejection in organ transplantation; it is especially useful in kidney transplants.\textsuperscript{20,21}

**Chemical Structure of Sirolimus**

![Chemical Structure of Sirolimus](image)

**EXPERIMENTAL**

**Chemicals and reagents**

HPLC grade actonitrile and methanol was purchased from Merck Specialties Pvt. Ltd.

**Instrumentation and analytical conditions**

The analysis of drug was carried out on a PEAK HPLC system equipped with a reverse phase C18 column (250x4.6mm, 5\(\mu\)m in particle size), a LC-P7000 isocratic pump, a 20\(\mu\)l injection loop and a LC-UV7000 absorbance detector and running on PEAK Chromatographic Software version 1.06. Isocratic elution with acetonitrile : methanol 20:80 (V/V) (pH-5.5) was used at a flow rate of 1.0ml/min. The mobile phase was prepared freshly and degassed by sonicating for 5 min before use.

**Stock and Working standard solutions**

10mg of Sirolimus working standard was transferred into a 10ml volumetric flask and diluent was added and sonicated to dissolve it completely and the volume was made up to the mark with the same solvent. Further 1ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent and was filtered through 0.45\(\mu\)m nylon filter paper. From the above filtered sample 30\(\mu\)g/ml solution was prepared. The calibration curve was plotted with the five concentrations of the 5.0-30.0\(\mu\)g/ml working standard solutions. Calibration solutions were prepared daily and analyzed immediately after preparation.

**Assay of Sirolimus tablets**

Weigh 20mg of Sirolimus (Rapacan -150mg) tablets were weighed and the average weight was calculated. An Accurately weighed amount of 10 mg of Sirolimus was transferred into a 10ml volumetric flask. Diluent was added and sonicated to dissolve it completely and made up to the
mark with diluents. The constituents were mixed well and filtered through 0.45um filter. Further 1ml of the above stock solution was pipetted out into a 10ml volumetric flask and diluted up to mark with diluent. From the above filtered sample 30µg/ml solution was prepared. An aliquot of this solution was injected into HPLC system. Peak area of Sirolimus was measured for the determination. The results are furnished in Table 3.

![HPLC Report](image)

**Fig 2: Typical chromatogram of Sirolimus Formulation**

**Validation procedure**

The objective of the method validation is to demonstrate that the method is suitable for its intended purpose as it is stated in ICH guidelines. The method was validated for linearity, precision (repeatability and intermediate precision), accuracy, specificity, stability and system suitability. Standard plots were constructed with five concentrations in the range of 0.5.0µg/ml to 30.0µg/ml prepared in triplicates to test linearity. The peak area of Sirolimus was plotted against the concentration to obtain the calibration graph. The linearity was evaluated by linear regression analysis that was calculated by the least square regression method. The precision of the assay was studied with respect to both repeatability and intermediate precision. Repeatability was calculated from five replicate injections of freshly prepared Sirolimus test solution in the same equipment at a concentration value of 100% (30µg/ml) of the intended test concentration value on the same day. The experiment was repeated by assaying freshly prepared solution at the same concentration additionally on two consecutive days to determine intermediate precision. Peak area of the Sirolimus was determined and precision was reported as %RSD.

Method accuracy was tested (% recovery and %RSD of individual measurements) by analyzing sample of Sirolimus at three different levels in pure solutions using three preparations for each level. The results were expressed as the percentage of Sirolimus recovered in the samples. Sample solution short term stability was tested at ambient temperature (20±10°C) for three days. In order to confirm the stability of both standard solutions at 100% level and tablet sample
solutions, both solutions protected from light were re-injected after 24 and 48 hours at ambient temperature and compared with freshly prepared solutions.

RESULT AND DISCUSSION

Optimization of the chromatographic conditions

Proper selection of the stationary phase depends up on the nature of the sample, molecular weight and solubility. The drug Sirolimus is non-polar. Non-polar compounds preferably analyzed by reverse phase columns. Among C8 and C18, C18 column was selected. Non-polar compound is very attractive with reverse phase columns. So the elution of the compound from the column was influenced by polar mobile phase. Mixture of methanol and acetonitrile was selected as mobile phase and the effect of composition of mobile phase on the retention time of Sirolimus was thoroughly investigated. The concentrations of the methanol and acetonitrile were optimized to give symmetric peak with short run time (Fig.2).

![HPLC Report](image)

Fig 3: Typical chromatogram of Sirolimus

Analysis of Sirolimus in Serum

From a local hospital blood was collected and serum was separated. 1ml of this serum was taken in a test tube and added 0.1ml of 1M NaOH and 5ml of dichloromethane and mixed for about 20min in vortex mixer and centrifuged at 3000 rpm for 10min. From this centrifuged solution, 4ml of organic layer was separated and evaporated to dryness to get residue. To this residue 100µl of 1M acetic acid and 3ml of n-Hexane were added and mixed for 5 min by vortex mixer and the organic layer was evaporated and finally the remaining sample was injected into HPLC and chromatogram was recorded. The amount of drug present in the blood sample was calculated from linearity graph.
Validation of method

Linearity

Five points graph was constructed covering a concentration range 5-30µg/ml (Three independent determinations were performed at each concentration). Linear relationships between the peak area signal of Sirolimus and the corresponding drug concentration were observed. The standard deviation of the slope and intercept were low. The statistical analysis of calibration is shown in Table 1.

Precision

The validated method was applied for the assay of commercial tablets containing Sirolimus. Sample was analyzed for five times after extracting the drug as mentioned in assay sample preparation of the experimental section. The results presented good agreement with the labeled content. Low values of standard deviation denoted very good repeatability of the measurement. Thus it was shown that, the equipment used for the study was correct and hence the developed analytical method is highly repetitive. For the intermediate precision, a study carried out by the same analyst working on the same day on three consecutive days indicated a RSD of 1.245. This indicates good method precision.

Stability

The stability of Sirolimus in standard and sample solutions containing different solutions was determined by storing the solutions at ambient temperature (20±10°C). The solutions were checked in triplicate, after three successive days of storage and the data were compared with freshly prepared samples. In each case, it could be noticed that solutions were stable for 48 hrs,
as during this time the results did not decrease below 98%. This denotes that Sirolimus is stable and standard for at least 2 days at ambient temperature.

**System suitability**

The system suitability parameters, like capacity factor, asymmetry factor, tailing factor and number of theoretical plates were also calculated. It was observed that all the values are within the limits (Table 3). The statistical evaluation of the proposed method revealed its good linearity, reproducibility and its validation for different parameters and leads us to the conclusion that, it could be used for the rapid and reliable determination of Sirolimus in tablet formulation. The results are furnished in Table 2.

**CONCLUSION**

A validated RP-HPLC method has been developed for the determination of Sirolimus in tablet dosage form. The proposed method is simple, rapid, accurate, precise and specific. Its chromatographic run time of 10 min allows the analysis of a large number of samples in short period of time. Therefore, it is suitable for the routine analysis of Sirolimus in pharmaceutical dosage. From this developed method we found 3.344 µg/ml sirolimus drug in serum.

**Table 1: linearity of sirolimus**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Linearity Level</th>
<th>Concentrations</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>5µg/ml</td>
<td>50822.9</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>10µg/ml</td>
<td>89746.3</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>15 µg/ml</td>
<td>130149.2</td>
</tr>
<tr>
<td>4</td>
<td>IV</td>
<td>20µg/ml</td>
<td>168017.7</td>
</tr>
<tr>
<td>5</td>
<td>V</td>
<td>25µg/ml</td>
<td>211372.3</td>
</tr>
<tr>
<td>6</td>
<td>VI</td>
<td>30µg/ml</td>
<td>249504.1</td>
</tr>
</tbody>
</table>

Correlation coefficient: 0.999

**Table 2: System stability parameters**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>values</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda$ max (nm)</td>
<td>272</td>
</tr>
<tr>
<td>Beers law limit(µg/ml)</td>
<td>5 - 30</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.999</td>
</tr>
<tr>
<td>Retention time</td>
<td>2.740</td>
</tr>
<tr>
<td>Theoretical plates</td>
<td>2682.15</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>1.22</td>
</tr>
<tr>
<td>Limit of detection (µg/ml)</td>
<td>10</td>
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<tr>
<td>Limit of quantification</td>
<td>35</td>
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</table>

**Table 3: Assay**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Label claim(mg)</th>
<th>% amount found</th>
</tr>
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<tbody>
<tr>
<td>Repacan</td>
<td>150mg</td>
<td>36%</td>
</tr>
</tbody>
</table>
Table 4: Chromatographic condition

<table>
<thead>
<tr>
<th></th>
<th>Acetonitrile (20%)</th>
<th>Methanol (80%)</th>
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</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>UV detection</td>
<td>272nm</td>
<td></td>
</tr>
<tr>
<td>Analytical column</td>
<td>C18 (250 x 4.6mm)</td>
<td></td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0ml/min</td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>Ambient</td>
<td></td>
</tr>
<tr>
<td>Injection volume</td>
<td>20µl</td>
<td></td>
</tr>
<tr>
<td>Run time</td>
<td>6 min</td>
<td></td>
</tr>
<tr>
<td>Retention time</td>
<td>2.740</td>
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</table>

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