**METHOD DEVELOPMENT AND VALIDATION OF SIROLIMUS IN HUMAN WHOLE-BLOOD SAMPLES BY LC-MS/MS**

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**ABSTRACT**

Sirolimus is an immunosuppressive agent. It is a macrocyclic lactone produced by *Streptomyces hygroscopicus* and used in organ transplantation. Our aim was to develop a robust sensitive and selective liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for determination of sirolimus in human whole-blood samples. We used LC-MS/MS system and atmospheric pressure ionization tandem mass spectrometry in the multiple reaction monitoring (MRM) detection mode. The whole blood was collected in the K$_3$EDTA vacutainer taken from healthy human volunteers. Chromatographic conditions and other parameters were suitable for using this method. Total analytical run time was 1.6 min, and retention times of analyte and internal standard were 0.88 and 0.86 minutes respectively. This LC-MS/MS method can be preferable for quantification of sirolimus in whole blood sample. Sample preparation was short and simple. It determined sirolimus drug selectively in human whole blood samples.

**KEYWORDS:** Method development, validation, sirolimus and human whole blood.

Abbreviations: LC-MS/MS: Liquid chromatography-tandem mass spectrometry IS: Internal standard.

**1. INTRODUCTION**

Sirolimus is an immunosuppressive drug with a molecular mass of 913.6 Da [¹] used in organ transplantation. [²] especially useful in kidney transplants. It was approved by the US Food
and Drugs Administration (FDA) in the year of 1999, for renal transplants and also known as Rapamycin® (RAPA). In cells, sirolimus binds to the immunophilin, FK binding protein-12 (FKBP-12), to generate a complex, the SRL-FKBP-12 complex does not affect calcineurin activity and inhibits a protein kinase, designated mammalian target of rapamycin (mTOR), which is a key enzyme in cell cycle progression. Inhibition of mTOR blocks cell-cycle progression at the G1 → S phase transition\[^{3,4}\]. Sirolimus inhibits T-cell cycle progression by blocking interleukin-2 production\[^{5}\]. In general immunosuppressive drugs have narrow therapeutic ranges. They may cause numerous side effects including immunological, renal, hepatic, and neurological complications, requiring dose adjustment or discontinuation in a significant percentage of patients\[^{6,7}\]. Traditionally most of the laboratories use immunological methods for quantification of immunosuppressive drugs. Due to cross-reactions with metabolites, overestimation of the concentrations is a major problem in this drug by using immunological techniques\[^{8,9}\]. LC-MS/MS was used for its high sensitivity and exclusively specific for the parent compound. Relatively the cost of the method also decreased when it compared to other methods. This method is more specific and sensitive than other immunological methods for sirolimus drug. LC-MS/MS is generally accepted as the technique of choice to study and quantify sirolimus in whole-blood samples. The validation was performed in compliance with Good Laboratory Practice and regulatory guidelines\[^{10,11}\].

2. METHOD

EDTA-treated sirolimus blood samples of healthy human volunteers were analyzed by using the LC-MS/MS in the laboratory.

2.1. Chemicals

HPLC grade methanol, acetonitrile, ammonium acetate, and 0.1 M zinc sulfate and sodium bicarbonate and TBME AR grade were purchased locally. Ultrapure water was supplied by a Milli-Q water Purification System from Millipore. sirolimus and tacrolimus were obtained from Vivan Lifesciences. All reagents and solvents were of analytical grade. Whole-blood calibrators and quality control (QC) samples (three-level whole blood controls) were prepared at inhouse. They were used for calibration of sirolimus assay. Concentration ranges were between 0.790 to 99.739 ng/mL. All calibrators and quality control samples were aliquoted and immediately stored at −20°C until analysis. Before sample preparation, one batch of calibrators and quality control samples were thawed.
2.2 Sample Preparation

Spiked whole human blood samples were withdrawn from the deep freezer and thawed at room temperature. 500μL of aliquot of blood sample and 50 μL of internal standard Tacrolimus (60 ng/mL) were added into 5 mL RIA vial and vortexed for 10 seconds. 500 μL Milli Q water was added in it and vortexed for 10 seconds. After which 50 μL of 0.1M zinc sulphate was added and vortexed for 10 seconds. Sodium bicarbonate 100μL of 0.1M was added in the same RIA vial and vortexed for 10 seconds. 3mL of TBME was added in it and vortexed by using vibromax shaker at 2500 rpm for 10 minutes. Later the sample mixture was centrifuged at 4500 rpm for 10 minutes at 4°C and 2.7 mL of supernatant organic layer was separated and evaporated at 40°C for 10 minutes. The residue was reconstituted with 250 μL of mobile phase and 10 μL of reconstituted sample was injected in to the LC/MS-MS. The temperature of auto sampler was adjusted at 10°C during the analysis for providing standard experimental conditions.

2.3. Instrumentation

A Thermo TSQ quantum ultra (Thermo Finnegan) equipped with a temperature controlled autosampler and HPLC pumps (Finnegan) was used. Waters XTerra RP 18 column was used for the analysis.

2.4. LC-MS/MS

At the beginning of chromatography, 10μL sample was injected into the system. The flow rate was 0.8mL per minute. Multiple reactions monitoring (MRM) mode was used. The total run time was 1.6 minutes by LC-MS/MS detection, including internal standard. Mass spectrometric detection with a Turbo Ion Spray interface was used for detection of the Sirolimus drug. The capillary temperature was set at 275°C, and the ion spray voltage was adjusted to 5000V. Auxillary and sheath gas pressure was kept as 40 and 60 psi respectively.

3. RESULTS AND DISCUSSION

LC-MS/MS method was developed and validated for the assay of sirolimus in human whole blood samples. The method was evaluated for linearity, specificity, and precision. The calibration curves showed linear relationships between the peak-height ratios for the drugs and IS. The correlation coefficient (r) was >0.98. The lowest quantifiable concentration for sirolimus in human whole blood was 0.791ng/mL and the linearity range was from 0.790 to 99.739 ng/mL during method validation.
The matrix used for preparation of calibration curve standards and QC samples were screened for interfering endogenous substances and were found to be free from interfering substances. No endogenous peaks were noticed at the retention time of sirolimus and the internal standard [IS], tacrolimus. The calibration curve was established by using $1/x^2$ weighing method and the $r^2$ value of calibration curve was 0.98. The appropriateness of validated method during subject sample analysis was ensured by organizing the analytical batch comprising of subject samples along with appropriate number of quality control samples. The data on precision and accuracy for the quality control samples of within batch coefficients of variation ranged between 3.67 to 15.10% and the percentage of nominal concentration ranged from 93.29 to 105.19% for sirolimus. The data on precision and accuracy for the quality control samples of between batch coefficients of variation ranged between 6.42 to 10.95% and the percentage of nominal concentration ranged from 95.87 to 101.73% for sirolimus as shown in Table No. 1.

LC-MS/MS assay permits sensitive and selective quantification of sirolimus in human whole-blood samples. We used mobile phase containing methanol:10 mM ammonium acetate (95:5) v/v, and the total analysis time was 1.6 min for analyte and internal standard. The representative calibration curve of sirolimus is shown in Figure 1. Acquired chromatogram was obtained by using the computer based software supplied by Thermo LC Quan 2.5.6. For system performance evaluation, we determined the limits of detection (LOD), the lower limits of quantification (LOQ), the squared correlation coefficients ($r^2$) the recovery rates, the standard deviation values, and the accuracy values. LOD and LOQ were estimated by injecting whole blood samples spiked with the analytes at low concentrations. The LOQ of sirolimus was 0.805 ng/mL, the assay was linear over the range of 0.790 to 99.739 ng/mL and $r^2$ was 0.98.

The analytical recovery results for sirolimus at LQC, MQC and HQC concentrations were 35.05%, 36.8%, and 37.14%, respectively. The control whole blood samples that have three different concentrations of sirolimus and internal standard were measured three times in the same run for intraday precision and three times in consecutive days for interday precision. The result of these precision measurements is presented in Table No.1. Determination of mean accuracy of sirolimus measurement was studied at whole blood control samples, which was calculated to be 100.2%, 99%, 100.3% and 97.9%, respectively. Recovery of sirolimus and internal standard tacrolimus were evaluated by comparing the peak response of six replicates of extracted low, middle and high quality control samples to six replicates of
unextracted appropriately diluted standard solutions. Total mean recovery of sirolimus and internal standard were 36.35 and 46.36% respectively. We compared the stabilities of sirolimus and internal standard tacrolimus in whole-blood samples by measuring, in six replicate assays, of stored samples over a different time periods along with freshly prepared the stock dilutions of same concentrations and found to be stable for five days (Table No.2).

Validation of analytical method is an important criterion to optimize the analysis of drugs in the developed method and to assure the quality and reproducibility of it. A wide variety of analytical methods have been reported in the recent and past for the determination of sirolimus in biological fluids. The methods used different internal standards, reversed phase columns with different particle size, different internal diameters and different mobile phase compositions for the quantification of sirolimus by using HPLC [12]. Here we used LC-MS/MS for determination of sirolimus drug in human whole blood. In this method of study, the total procedure of LC-MS/MS method is very simple and has high specificity for determining drug concentrations. Interfering effects of the sample matrix are an important problem in quantification of immunosuppressive drug. In this study, better matrix component elimination was achieved by using LC-MS/MS.

![Representative Calibration Curve for Sirolimus](image)

### Table No.1

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<thead>
<tr>
<th>Interday precision and accuracy</th>
<th>Intraday Precision and accuracy</th>
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Table No.2

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<th>Area of analyte 0 day</th>
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<th>Area of IS 0 day</th>
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<td>Mean 5467517</td>
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4. CONCLUSION

Here, we present a LC-MS/MS method for determination of sirolimus in whole blood samples. This method had shown consistent reproducible recovery of both analyte and internal standard. In this method the sample preparation procedure was very simple, the cost of total analyses was very low, and the time needed to do the analysis was very short.

Conflict of Interests: Authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

5. REFERENCES


