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Quantitation of Sirolimus in Human Whole Blood by Ultra Performance Liquid Chromatography Tandem Mass Spectrometry for a Bioequivalence Study

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ABSTRACT

A sensitive and high throughput ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method has been developed for the determination of sirolimus in human whole blood. The method involved protein precipitation with ZnSO4, followed by solid phase extraction of sirolimus along with tacrolimus as the internal standard (IS) from 100 µL of human blood. The chromatographic analysis was achieved on a Waters Acquity UPLC BEH C18 (50 mm × 2.1 mm, 1.7 µm) analytical column using gradient mobile phase, consisting of 10 mM ammonium acetate, pH 5.00 adjusted with acetic acid and premixed methanol and acetonitrile (60:40, v/v), at a flow-rate of 0.400 mL/min. The precursor → product ion transition for sirolimus (m/z 931.6 → 864.4), and IS (m/z 821.5 → 768.3) were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring and positive ionization mode. The method is validated over a wide dynamic concentration range of 0.05-50.0 ng/mL. Matrix effect is assessed by post-column analyte infusion experiment and the mean extraction recovery was greater than 93.0 % for sirolimus and IS. The method is rugged and rapid with a total run time of 1.5 min and was used in a clinical study with 16 healthy subjects. The assay reproducibility was successfully demonstrated by reanalysis of 87 subject samples.

Keywords: Sirolimus; UPLC-MS/MS; solid phase extraction; bioequivalence; high throughput; sensitive

INTRODUCTION

Sirolimus (rapamycin), a macrocyclic fermentation product isolated from *Streptomyces hygroscopicus* is a potent non-calcineurin inhibiting immunosuppressant. It is generally used in combination with calcineurin inhibitors like tacrolimus and cyclosporine A or mycophenolate mofetil for the prophylaxis of organ rejection in organ transplant patients [1-3]. It possesses both immunosuppressants as well as antiproliferative properties, and has a unique mechanism of action which is different from the calcineurin inhibitors. Sirolimus was approved in the US in 1999 and in Canada and Europe in 2001 for the prevention of renal transplant rejection [2]. Although sirolimus and tacrolimus have structural similarity, their mechanism of action is quite different. Like tacrolimus, sirolimus binds with FKBP12 however; the sirolimus-FKBP12 complex has no effect on calcineurin phosphatase unlike tacrolimus. Instead it binds with one or more proteins known as mammalian targets of rapamycin (mTOR) and thus inhibits both DNA and protein synthesis, resulting in obstruction of the cell cycle in late G1 phase as it progresses to the S phase [4, 5]. Thus, sirolimus reduces T-cell activation at a later stage in the cell cycle than the calcineurin inhibitors by inhibiting cytokine-induced signal transduction pathways, resulting in the suppression of interleukin (IL)-2- and IL-4-driven T-cell proliferation [6].

Sirolimus is essentially distributed in the erythrocytes (~ 95 %), which is not temperature or concentration dependent. The sequestration of sirolimus in the red blood cells is due to their rich content of immunophilins. Sirolimus displays concentration dependent binding to lipoproteins (about 40 %), while in the remaining 60 % unbound fraction only 4 % is bound to plasma proteins. Therefore, whole blood is the preferred matrix for therapeutic drug monitoring. Sirolimus is mainly metabolized by the CYP-450 enzyme system to give several metabolites, mainly hydroxyl-, demethyl-, di-demethyl- and dihydroxy-sirolimus. However, the metabolites show less than 10 % of the parent compounds activity and their elimination is through faecal pathway. Further, sirolimus has a long elimination half-life of approximately 62 h [7, 8].

All the immunosuppressant drugs are characterized by a narrow therapeutic range and thus presence of elevated concentration can result in severe toxicity and under dosing can render them ineffective. Due to the narrow therapeutic indices, lack of a reliable correlation between dose and drug exposure, variable pharmacokinetics, and potential drug–drug interactions, several reports have recommended therapeutic drug monitoring (TDM) for sirolimus, both to prevent side effects (mainly thrombocytopenia and hypercholesterolemia) and to optimize efficacy and reduce organ rejection [9].

Literature presents two approaches for the estimation of sirolimus concentration in whole blood, namely immunoassays [10-13] and liquid chromatographic methods with UV [14-18] or mass spectrometric detection [9, 19-41]. Although immunoassays are highly selective, cross-reactivity of the drug with some metabolites results in overestimation of the concentrations and thus is a major problem with such methods. Amongst the chromatographic methods, sirolimus has been determined either as a single analyte [9, 14-17, 19-22] or in combination with other immunosuppressants [18, 23-41]. So far only two methods based on UPLC-MS/MS have been reported for sirolimus together with three other immunosuppressants [42, 43]. The sensitivity achieved was 1.0 ng/mL for sirolimus in both the methods.

In the present work an accurate, highly sensitive and rapid UPLC-MS/MS method has been developed and fully validated for reliable measurement of sirolimus in human blood samples especially for routine therapeutic drug monitoring. The method requires only 100 µL human blood sample for extraction and demonstrates excellent performance in terms of ruggedness and efficiency (1.5 min per sample). Interference due to matrix was ascertained by post column infusion technique. It was successfully applied to a bioequivalence study in 16 healthy human subjects.

EXPERIMENTAL

Chemicals and materials

Reference standards of sirolimus (99.6%) and tacrolimus (IS, 98.5%) were obtained from Toronto Research Chemicals (Toronto, Canada). HPLC grade acetonitrile and methanol were procured from Merck (Darmstadt, Germany). Acetic acid and zinc sulfate were purchased from Spectrochem Pvt. Ltd. (Mumbai, India) and SD Fine Chem. Ltd (Mumbai, India) respectively. Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Bangalore, India). Lichrosep Sequence SPE Cartridge (30 mg, 1 mL) was purchased from Merck. Blank human blood was obtained from in-house clinical department and was stored at –20 °C until use.

Liquid chromatographic and mass spectrometric conditions

A Waters Acquity UPLC system (MA, USA) consisting of binary solvent manager, sample manager and column manager was used for setting the reverse-phase liquid chromatographic conditions. The analysis of sirolimus and IS was performed on an analytical column maintained at 40°C in a column oven. For gradient elution, the mobile phase solvent consisted of (A) 10mM ammonium acetate, pH 5.00 adjusted with acetic acid and (B) premixed methanol and acetonitrile (60:40, *v/v*). A binary step gradient at a flow rate of 0.400 mL/min was employed. Up to 0.5 min, the ratio of A and B was kept at 98:2 (*v/v*) and from 0.5 min to 1.0 min the ratio was switched to 2:98 (*v/v*), before returning to the starting conditions [A:B, 98:2 (*v/v*)] up to 1.5 min.

Ionization and detection of sirolimus and IS was carried out on a triple quadrupole mass spectrometer from Waters – Micro Mass Technologies (MA, USA), equipped with turbo ion spray interface and operating in positive ionization mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor precursor → product ion transitions of *m/z* 931.6 → 864.4 for sirolimus and *m/z* 821.5 → 768.3 for IS. A qualifying transition of *m/z* 931.6 → 896.4 and *m/z* 821.5 → 786.7 was also measured for the analyte and IS respectively. For both the compounds, the optimized mass spectrometer parameters were as follows, capillary voltage 3.0 kV, desolvation temperature 300 °C, desolvation gas flow 700 L/h, cone gas flow 100 L/h, and source temperature 100 °C. The compound specific parameters like cone voltage and collision energy were set at 30 V and 19 eV for sirolimus and 28 V and 21 eV for IS respectively. Quadrupole 1 and 3 were maintained at unit mass resolution and the dwell time was set at 100 ms for both the drugs. Data collection, peak integration, and calculations were performed using Mass Lynx software version 4.1.

Standard stock, calibration standards and quality control samples

The standard stock solution of sirolimus (1000 µg/mL) was prepared by dissolving requisite amount in methanol. Calibration standards (CSs) and quality control (QC) samples were prepared by spiking (2% of total blood volume) blank blood with stock solution. Calibration curve standards were made at 0.050, 0.100, 0.300, 1.20, 4.00, 10.0, 25.0, 40.0, and 50.0 ng/mL concentrations respectively, while quality control samples were prepared at three levels, viz. 45.0 ng/mL (HQC, high quality control), 25.0/2.50 ng/mL (MQC-1/2, middle quality control 1/2), and 0.150 ng/mL (LQC, low quality control). Stock solution (0.2 mg/mL) of the internal standard was prepared by dissolving 5.0 mg of in 25.0 mL of methanol. Its working solution (100ng/mL) was prepared by appropriate dilution of the stock solution in methanol. Standard stock and working solutions used were stored at 5 °C, while CSs and QC samples in plasma were kept at -70 °C until use.

Sample extraction protocol

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 100 µL of spiked blood sample, 25 µL of internal standard was added and vortex-mixed for about 10 sec. Further, the mixture was pretreated with 500 µL of 0.1M zinc sulphate solution in water to lyse the cells, followed by vortex-mixing for 1.0 min. Following centrifugation at 1811 × g for 2 min, the supernatant was separated and applied to solid phase extraction (SPE) on a Lichrosep Sequence SPE cartridge which was previously conditioned with 1.0 mL of methanol followed by 1.0 mL of water. The cartridge was first washed with 1.0 mL 10% acetic acid in water and then with 1.0 mL, 10 % methanol in water. Elution was carried with 500 µL methanol in pre-labeled RIA vials. The solvent was then evaporated to dryness in a thermostatically controlled water bath maintained at 40 °C under a gentle stream of nitrogen. The dried sample was reconstituted in 100 µL of the mobile phase and 10 µL was used for injection into the chromatographic system.

Method validation procedures

The validation protocol and the acceptance criterion were essentially based on the USFDA guidelines [44].

System suitability was checked by injecting 6 successive injections of aqueous samples of sirolimus (25 ng/mL) and IS (100 ng/mL) at the beginning of each batch. The precision (%CV) in

the measurement of retention time it was in the range of 0.08-0.17 %, and 1.15 to 2.12 for area response of sirolimus and IS. The system performance was also verified with one processed blank sample, one upper limit of quantitation and one LLOQ along with the IS at the beginning and end of each batch. The S/N ratio was ≥ 70 for the analyte and IS. The auto sampler carry over for the analyte was checked by injecting the following sequence of injections: processed blank plasma, upper limit of quantitation (ULOQ) sample, processed blank plasma, LLOQ sample, and processed blank plasma.

The selectivity of the method was checked in eight different batches/lots of blank blood. The method linearity was evaluated from five linearity curves using least square weighted ($1/x^2$) linear regression. Intra-batch accuracy and precision was assessed by analyzing six replicates of LQC, MQC-1/2, and HQC samples from a single batch on the same day, while for inter-batch, five batches were analyzed on three consecutive days in a similar manner.

Ion suppression/enhancement effects on the method sensitivity and selectivity was studied by the post column analyte infusion experiment. A standard solution containing sirolimus (25 ng/mL) and IS (100 ng/mL) was infused post column via a 'T' connector into the mobile phase at 10μL/min employing an in-built infusion pump. Further, 10 μL aliquots of extracted control blood were then injected into the column and chromatogram was acquired for sirolimus and IS.

The extraction recovery and matrix effect were determined at four QC levels in six replicates as reported previously [45]. Relative recovery or extraction recovery was calculated by comparing the mean area response of extracted samples (spiked before extraction) to that of unextracted samples (spiked after extraction) at each QC level for the analyte and IS. Absolute matrix effect was computed by comparing the mean area response of unextracted samples (spiked after extraction) with mean area of neat standard solutions. Further, the effect of blood matrix on the analyte quantification, expressed as relative matrix effect was also checked in eight different batches/lots of blood. The assessment of relative matrix effect was based on the calculation of precision (% CV) values for slopes of calibration lines prepared from these batches. For a method to be practically free from relative matrix effect the % CV should not exceed 3-4 %.

The stability of sirolimus and IS was examined in stock solutions and for the analyte in matrix by comparing the area response ratio (sirolimus/IS) of the stability samples with freshly prepared comparison samples. Bench top stability at room temperature, wet extract (autosampler) stability at 5 °C, freeze-thaw and long-term stability (at -20°C and -70°C) in spiked blood samples were determined at LQC and HQC levels in six replicates.

Dilution reliability was established from six replicates of standards prepared at 1/2 (45.0 ng/mL) and 1/10th (9.00 ng/mL) dilution, by spiking standard stock solution of sirolimus having 90.0 ng/mL concentration in screened blank blood. The precision and accuracy were evaluated by comparing the results against freshly prepared calibration curve standards. Method ruggedness was ascertained by analyzing two precision and accuracy batches. The first batch was analyzed by different analysts while the second batch was studied on two different columns.

Application of the method

The method was applied for a clinical study in 16 healthy Indian subjects under fasting conditions. The design of the study was an open label, balanced, randomized, single dose, two treatment, two sequence, two period, crossover bioequivalence of a test (2.0 mg sirolimus tablets from an Indian Pharmaceuticals Company, India) and reference (2.0 mg sirolimus tablets, Rapamune from Wyeth Pharmaceuticals Inc., subsidiary of Pfizer Inc., Philadelphia, USA) formulation. The study was performed as per the International Conference on Harmonization and USFDA guidelines [46]. An Independent Ethics Committee approved the study protocol and a written consent was provided by all the subjects before enrolment in the study. Blood samples for the determination of sirolimus were collected in K3EDTA-vacuettes at pre-dose (0.00), 0.50, 1.00, 1.50, 2.00, 2.33, 2.67, 3.00, 3.33, 3.67, 4.00, 4.33, 4.67, 5.00, 5.33, 5.67, 6.00, 6.33, 6.67, 7.00, 8.00, 10.0, 12.0, 24.0, 36.0, 48.0, and 72.0 h after oral administration of the drug. The samples were divided into two aliquots and stored in two different pre-labeled RIA vials at –70 °C until analysis. The pharmacokinetic parameters of sirolimus were estimated by non-compartmental model using WinNonlin software version 5.2.1 (Pharsight Corporation, Sunnyvale, CA, USA). The C_{max} values and the time to reach maximum blood concentration (T_{max}) were estimated directly from the observed blood concentration vs. time data. The area under the blood concentration–time curve from time 0 to 72 h (AUC_{0-72}) was calculated using the linear trapezoidal rule. To determine whether the test and reference formulations were pharmacokinetically equivalent, C_{max}, and AUC_{0-72} and their ratios (test/reference) using log transformed data were assessed; their means and 90% CIs were analyzed by using SAS® software version 9.1.3 (SAS Institute Inc., Cary, NC, USA). The drugs were considered pharmacokinetically equivalent if the difference between the compared parameters was statistically non-significant ($P \geq 0.05$) and the 90% confidence intervals (CI) for these parameters fell within 80 to 125%.

The assay reproducibility was confirmed by reanalysis of 87 subject sample (10 % of total subject samples analyzed). The selection criteria included samples which were near the C_{max} and the elimination phase in the pharmacokinetic profile of sirolimus. The results obtained were compared with the initial values. The percent change in the value should not be more than ±20% [47].

RESULTS AND DISCUSSION

Method development

To achieve the desired sensitivity and selectivity, the extraction procedure, chromatography and mass detection parameters were suitably optimized. In the present work, electrospray ionization (ESI) source was used to maximize sensitivity and obtain good linearity in the regression curves. As observed in several reports [30, 35, 41 and 48] sirolimus has low affinity for protons and therefore the protonated precursor ions in the positive ionization mode have very low abundance and thus remain undetected. The Q1 scan for sirolimus and IS showed strong ability to bind with ammonium ions (from ammonium acetate used in the mobile phase) to give peaks at *m/z* 931.6 and 821.5 corresponding to ammonium ion adducts with much higher abundance. These adducts can be readily fragmented to give stable and consistent product ions. The most abundant product ions obtained from the ammonium ion adducts in the Q3 scan corresponded to *m/z* 864.4 (neutral loss of H_2O, NH_3 and CH_3OH) and 768.3 (neutral loss of $2H_2O$ and NH_3) for sirolimus and IS respectively (**Figure 1**). In addition to the quantification transition, a qualifying transition was also monitored for the identification of the analyte (*m/z* 931.6 → 896.4) and IS (*m/z*

821.5 → 786.7). A dwell time of 100 ms for sirolimus and IS was adequate to have sufficient no. of data points for quantification.

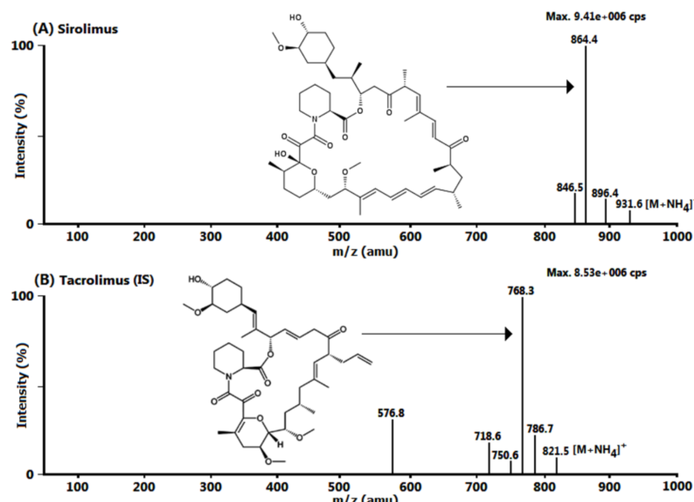

Figure 1 Product ion mass spectra of (A) sirolimus (m/z 931.6 → 864.4, scan range 50-1000 amu) and (B) tacrolimus (IS, m/z 821.5 → 768.3, scan range 50-1000 amu) in the positive ionization mode.

Several chromatographic methods have been reported for the analysis of sirolimus from whole blood using isocratic [17] as well as gradient conditions [27, 28, 35]. Initially, chromatography under isocratic conditions was tried using various combinations of acetonitrile/methanol-aqueous component with acidic and alkaline buffers (formic acid/acetic acid-ammonium formate/ ammonium acetate, ammonia-ammonium formate) to achieve good resolution and symmetric peak shapes as well as to have shorter run time on Acquity UPLC BEH C18 (50 mm × 2.1mm, 1.7µm) column. It was possible to have adequate retention, acceptable capacity factor and relatively good peak shape for sirolimus. Nevertheless, it was difficult to achieve the desired sensitivity of 0.05 ng/mL. Thus gradient conditions were tested and the best performance in terms of sensitivity, peak shape and short analysis time was possible using a binary step gradient with solvent A consisting of 10mM ammonium acetate, pH 5.00 adjusted with acetic acid and solvent B having premixed methanol and acetonitrile (60:40, *v/v*), by maintaining a flow rate of 0.400 mL/min. Initially up to 0.5 min, the ratio of A and B was kept at 98:2 (*v/v*) and from 0.5 min to 1.0 min the ratio was switched to 2:98 (*v/v*), before returning to the starting conditions [A:B, 98:2 (*v/v*)] up to 1.5 min. This ensured a retention time of 1.01 and 0.95 for sirolimus and IS respectively. Tacrolimus which is also an immunosuppressant drug was efficiently used as an internal standard in the present study. It had similar chromatographic elution pattern and did not affect the overall accuracy and precision of the method. The MRM ion chromatograms in **Figure 2** of extracted blank blood (double blank), blank blood fortified with IS, sirolimus at (0.05 ng/mL) and IS and a subject sample at C_{max} demonstrates the selectivity of the method.

Sample preparation is a decisive step for precise and accurate quantitation by LC-MS/MS methods. Majority of the methods available in literature have demonstrated a combination of protein precipitation followed by SPE for precise and quantitative recovery of sirolimus from whole blood [22, 42]. As sirolimus is sequestered within the erythrocytes, it is essential to break the binding with RBCs. Thus, a protein precipitant is required to lyse the cells and free the analyte. $ZnSO_4$ was used as protein precipitant as reported by several authors [17, 22, 27, 42], followed by SPE on LiChrosep Sequence extraction cartridges employing 100 µL blood samples. Washing of cartridges with 1.0 mL 10% acetic acid in water followed by 1.0 mL, 10 % methanol in water was adequate to remove endogenous compounds. The recovery of sirolimus was quantitative using 500 µL methanol for elution at all QC levels.

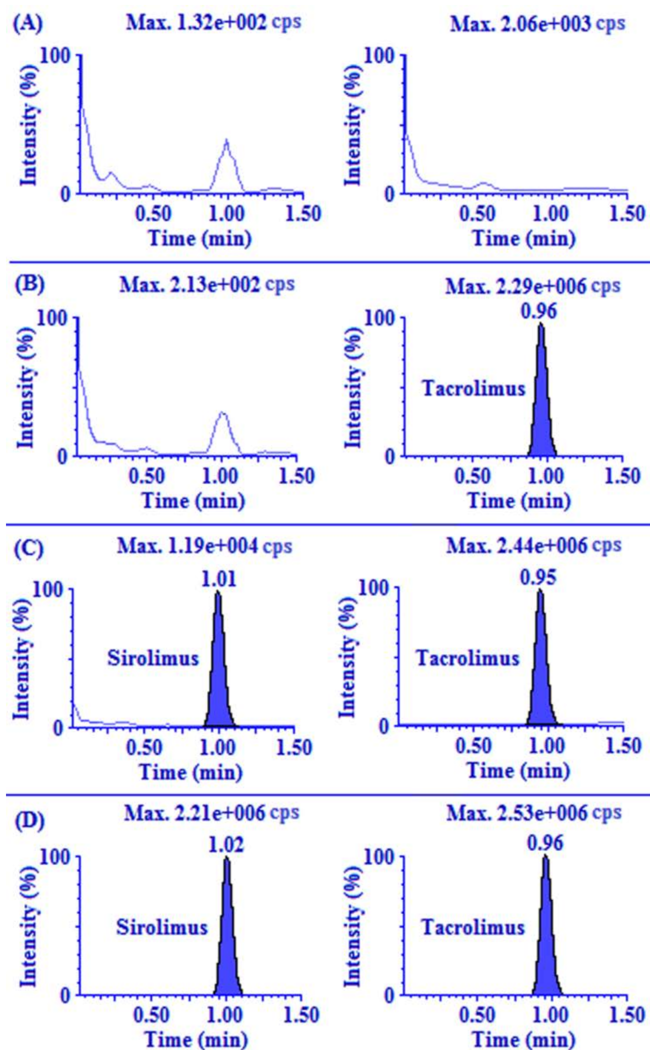

Figure 2 MRM ion-chromatograms of (A) double blank blood (without IS) (B) blank blood with tacrolimus (IS, m/z 821.5 → 768.3), (C) sirolimus (m/z 931.6 → 864.4) at LLOQ and IS (D) subject sample at Cmax after administration of 2.0 mg dose of sirolimus.

Results of post-column infusion experiment in Figure 3 indicate no ion suppression or enhancement at the retention time of sirolimus and IS. Though major ion suppression was observed around 0.5 min, however, it did not affect in the quantitation of sirolimus.

I. Assay validation results

Carryover evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. Practically, there was no carry-over observed during autosampler carryover experiment. No enhancement in the response was observed in double blank after subsequent injection of highest calibration standard (aqueous and

extracted) at the retention time of sirolimus and IS respectively as shown in **Figure 4**.

The calibration curves were linear over the concentration range of 0.050–50.0ng/mL. A straight-line fit was made through the data points by least square regression analysis and a constant proportionality was observed. The mean and standard deviation value for slope, intercept and correlation coefficient (r^2) observed were 0.0331280 and 0.001445; 0.0013210 and 0.000489; 0.9991237 and 0.000486 respectively. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 92.0 to 98.6 % and 1.18 to 6.17 % respectively. The LLOQ sample of 0.050ng/mL sirolimus concentration was measured with an acceptable accuracy and precision at a signal-to-noise ratio (S/N) of ≥ 70.

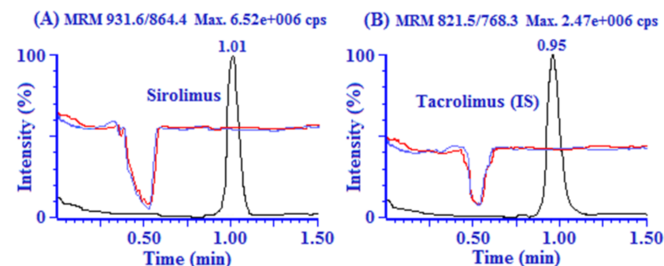

Figure 3 UPLC-MS/MS chromatograms of blank blood during post-column infusion of (A) sirolimus and (B) tacrolimus (IS)

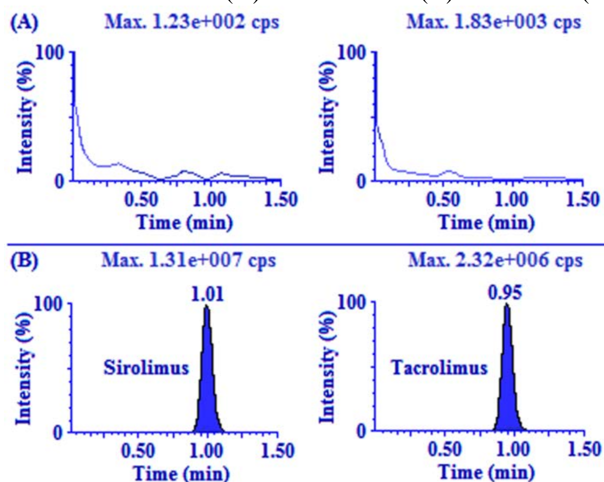

Figure 4 MRM ion-chromatograms for carry over test of sirolimus (*m/z* 821.5 → 768.3) and tacrolimus (IS, *m/z* 931.6 → 864.4) in (A) double blank blood (without analyte and IS), (B) sirolimus at ULOQ and IS (C) double blank blood (without analyte and IS) and (D) sirolimus at LLOQ and IS.

The intra-batch precision (%CV) ranged from 1.27 to 4.05 % and the accuracy was within 95.8 to 102.8 %. For the inter-batch experiments, the precision varied from 3.38 to 5.44 % and the accuracy was within 98 to 102 % (**Table 1**).

Table 1 Intra-batch and inter-batch precision and accuracy for sirolimus in human whole blood

QC level	Intra-batch (n = 6; single batch)			Inter-batch (n = 30; 6 from each batch)		
Nominal concentration (ng/mL)	Mean conc. found (ng/mL)	CV (%)	Accuracy (%)	Mean conc. found for 5 batches (ng/mL)	CV (%)	Accuracy (%)
HQC (45.0)	43.1	1.27	95.8	44.1	4.48	97.9
MQC-1 (25.0)	25.1	1.36	99.3	24.5	5.26	97.3
MQC-2 (2.50)	2.57	3.77	102.8	2.53	5.44	101.4
LQC (0.150)	0.148	4.05	98.7	0.148	3.38	98.7

Table 2 Absolute matrix effect and recovery of sirolimus from human whole blood (n = 6)

QC level	Mean area response			Absolute matrix effect*	Relative recovery**
	A (CV, %)	B (CV, %)	C (CV, %)		
HQC	1880970 (3.7)	1852620 (3.0)	1772300 (2.4)	98.5 (95.6)	95.7 (93.6)
MQC-1	1066071 (4.3)	1071812 (4.3)	990157 (3.6)	100.5 (97.2)	92.4 (96.2)
MQC-2	102908 (3.4)	99747 (4.3)	93062 (3.3)	96.9 (94.3)	93.3 (91.7)
LQC	6052 (1.5)	6116 (3.2)	5696 (3.0)	101.1 (97.5)	93.1 (94.4)

A: Reconstitution solution; B: Spiked in extracted sample; C: Spiked before extraction;
CV: coefficient of variation; * [B/A × 100], values in parenthèses are for tacrolimus;
** [C/B × 100], values in parenthèses are for tacrolimus

Further, another essential criteria to evaluate matrix effect, expressed as relative matrix in different blood lots /batches showed % CV values in the measurement of slope of standard curves was 2.82 (**Table 3**).

Table 3 Relative matrix effect in eight different lots of human blood

Blood lot	Slope of calibration curve
Lot-1	0.03225
Lot-2	0.03458
Lot-3	0.03322
Lot-4	0.03378
Lot-5	0.03214
Lot-6	0.03401
Lot-7	0.03298
Lot-8	0.03211
Mean	0.03313
Standard deviation	0.000935
% Coefficient of variation	2.82

Table 4 Stability of sirolimus under different conditions (n = 6) in human blood

Storage condition	Nominal conc. (ng/mL)	Mean, stability samples ± SD	Change (%) *
Bench top stability at room temperature; 9 h			
HQC	45.0	44.4 ± 3.22	-1.33
LQC	0.150	0.147 ± 0.007	-2.01
Wet extract stability at 5 °C; 50 h			
HQC	45.0	42.7 ± 0.97	-5.11
LQC	0.150	0.155 ± 0.016	3.33
Freeze & thaw stability; 5 cycles, -20°C			
HQC	45.0	43.4 ± 1.33	-3.56
LQC	0.150	0.145 ± 0.002	-3.33
Freeze & thaw stability; 5 Cycles, -70°C			
HQC	45.0	46.4 ± 1.83	3.11
LQC	0.150	0.146 ± 0.003	2.67
Long term matrix stability in matrix; 252 days, -20°C			
HQC	45.0	43.6 ± 0.65	-3.11
LQC	0.150	0.145 ± 0.005	-3.33
Long term matrix stability in matrix; 252 days, -70°C			
HQC	45.0	42.2 ± 1.40	-6.22

LQC	0.150	0.142 ± 0.002	5.33

$$*\text{Change } (\%) = \frac{\text{Mean stability samples} - \text{Mean comparison samples}}{\text{Mean comparison samples}} \times 100$$

The stability results for sirolimus in human blood under different storage conditions are summarized in Table 4. Stock solution samples kept for short-term stability remained unchanged up to 8 h, while the long term stability under refrigerated temperature below 8°C, the solutions of sirolimus and IS were stable for a minimum of 40 days. Sirolimus samples in control human blood for bench top stability were stable for atleast 9h at 25°C and for minimum of five freeze and thaw cycles at –20 °C and –70 °C. Spiked blood samples stored at these two temperatures for long term stability were found stable for a minimum period of 252 days. Autosampler stability (wet extract) of the spiked QC samples maintained at 5 °C was determined up to 50 h without significant drug loss.

The dilution test was performed to validate method reliability for analyte concentration above the ULOQ concentration which may be encountered during subject sample analysis. The precision for dilution reliability of 1/2 and 1/10th dilution were 1.87 and 1.36 %, while the accuracy results were 98.01 and 95.90 % respectively. This is within the acceptance limit of 15% for precision (% CV) and 85 to 115% for accuracy. Method ruggedness was evaluated using re-injection of analyzed samples on two different columns of the same make and also with different analysts. The precision (% CV) and accuracy values for two different columns ranged from 3.66 to 7.03% and 95.3 to 106.1 % respectively at all four quality control levels. For the experiment with different analysts, the results for precision and accuracy were within 2.78-5.10% and 96.0 to 105.7 % respectively.

Application to a pharmacokinetic/bioequivalence study

To the best of our knowledge there are no reports on the pharmacokinetics of sirolimus in Indian subjects. Thus, the validated method was applied to quantify sirolimus concentration in human samples after oral administration of 2 mg oral dose of sirolimus. **Figure 5** shows the blood concentration vs. time profile of sirolimus in 16 healthy subjects human. Around 1300 samples, including the calibration, QC and subject samples were analyzed during a period of 8 days and the precision and accuracy were well within the acceptable limits.

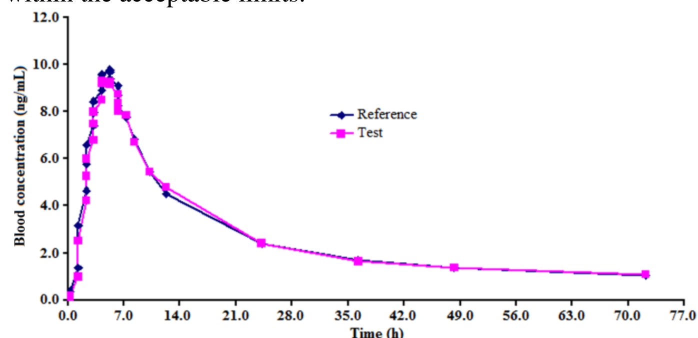

Figure 5 Mean blood concentration-time profile of sirolimus after oral administration of 2 mg (test and reference) tablet formulation to 16 healthy subjects.

The mean pharmacokinetic parameters for sirolimus (**Table 5**) and log transformed geometric least squares mean values for C_{max} and AUC_{0-72} under fasting conditions are summarized in **Table 6**. The 90 % confidence interval of individual ratio geometric mean for test/reference was within 89-108 %, which supports bioequivalence of the test formulation with the reference product in terms of rate and extent of absorption. Furthermore, there was no adverse event during the course of the study.

Table 5 Mean pharmacokinetic parameters following oral administration of 2 mg sirolimus in 16 healthy subjects

Parameter	Mean ± standard deviation	
	Test	Reference
Maximum blood concentration [C_{max} (ng/mL)]	10.594 ± 2.126	11.034 ± 2.583
Time point of maximum plasma concentration [T_{max} (h)]	4.437 ± 1.344	4.708 ± 1.088
Area under the blood concentration-time curve [AUC_{0-72h} (h.ng/ml)]	192.383 ± 39.392	193.715 ± 52.713

Table 6 Comparison of treatment ratios and 90% CIs of natural log(Ln)-transformed parameters sirolimus

Parameter	Ratio (test/reference),%	90% CI (Lower – Upper)	Power
Ln C_{max} (ng/mL)	97.06	89.73 – 104.98	0.9971
Ln AUC_{0-72h} (h.ng/mL)	100.99	94.52 – 107.89	0.9995

Incurred sample reanalysis (ISR) results are represented in Figure 6. Out of 87 incurred samples studied, 55 samples showed % change for assay reproducibility within ± 5 %, 22 samples were within 5 to 10 %, 8 samples showed a change of >10-15 %, while

the remaining 2 samples were between >15 to 20 %. This authenticates the reproducibility of the proposed method.

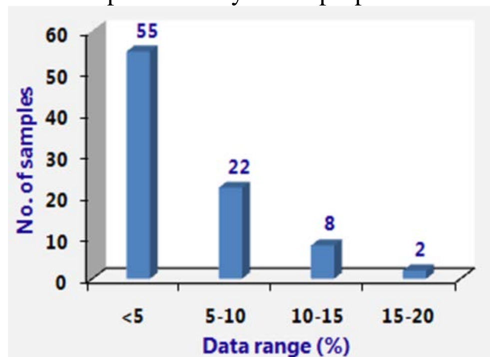

Figure 6 Incurred sample reanalysis results for sirolimus

CONCLUSIONS

The developed UPLC-MS/MS method for the quantitation of sirolimus in human blood was fully validated as per USFDA guidelines and can be efficiently used for therapeutic drug analysis. Despite the fact that there are several methods for the quantitation of sirolimus in whole blood, the present method offers significant advantages over those previously reported, in terms of sensitivity, reproducibility and overall analysis time. The chromatographic run time of 1.5 min per sample make it an attractive procedure in high-throughput bioanalysis of sirolimus. With dilution integrity up to 10-folds, it is possible to extend the upper limit of quantification to 90ng/mL. In addition, the method is practically free from matrix interference as evident from the results obtained for absolute and relative matrix effect and post column infusion study. Furthermore, the reproducibility is realistically proved through incurred sample reanalysis.

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