



Therapeutic Drug Monitoring of Sirolimus, Correlation With Laboratory Parameters In Transplant Patients

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Abstract

Sirolimus is a potent immunosuppressive agent administered as prophylactic agent to prevent rejection after organ transplantation. Sirolimus must be used within a narrow therapeutic window. Due to inter- and intra-variability, sirolimus blood concentrations may be affected, therefore, there is no possibility of predicting the sirolimus blood concentrations based on the dose patients received. Therapeutic drug monitoring (TDM) of whole blood is an important part of immunosuppressive therapy and is mandatory for sirolimus dosage individualization. The objective of this study was to present a validated method for the analysis of sirolimus in human blood by LC/MS spectrometry and also evaluation of correlation between blood sirolimus concentration and laboratory parameters. We examined a group of 32 patients receiving sirolimus at different stages after organ (kidney, liver or pancreas) transplantation. The mean sirolimus concentration was 10.2 ng/ml (range: 1.3-30.1 ng/ml). The assay was validated for a linear dynamic range of 1-50 ng/ml. The correlation coefficient (r) was 0.995. The within-run imprecision CV(%) for concentrations (1 and 10 ng/ml) were 14.7 and 2.2%, respectively. The between-run imprecision CV(%) for the same concentrations were 14.8 and 3.4%, respectively. Limit of quantification (LOQ) and limit of detection (LOD) were defined as 1 and 0.3 ng/ml, respectively. Analytic recovery was $98 \pm 2\%$ over a range of 1-50 ng/ml. Statistical results showed no correlation between sirolimus blood concentration and the dosage in patients receiving sirolimus. Also, no relationship between drug concentration in blood and laboratory parameters was seen.

Keywords: Immunosuppressive agent; Sirolimus; Therapeutic drug monitoring.

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1. Introduction

Allograft rejection by the recipient immune system still remains one of the most important obstacles in allogeneic organ transplantation.

Various immunosuppressive drugs are currently used to suppress the immune system and prevent tissue damage [1]. Sirolimus is a potent and the latest immunosuppressive agent [2, 3], approved in 1999 by the US FDA [4].

Sirolimus (Rapamune, rapamycin), is a macrocyclic lactone [5] derived from the natural fermentation of *Streptomyces hygroscopicus*, a member of the actinomyces bacterial group [6]. It was isolated by Sehgal from the soil of the Vai Atari region of Rapa Nui (Easter Island). It was later applied in animal transplant models by Calne *et al.* and Morris *et al.* [7]. Nowadays, It is used as immunosuppressant in patients after most organ transplantations (including heart, lung, liver, kidneys, bone marrow, and intestinal tract) [1, 8]. It shows synergistic immunosuppressive activity in combination with calcineurin inhibitors [8]. Kahan and co-workers have reported that the addition of

sirolimus to the cyclosporine immunosuppression regimen decreased renal allograft rejection episodes from 32% to 7.5% [9].

Sirolimus displays a unique immunosuppressive mechanism of action [7], distinct from that of cyclosporine and tacrolimus [10]. It acts during both co-stimulatory activation and cytokine-driven pathways, inhibiting the mammalian target of rapamycin (mTOR), an enzyme required for T- and B-cell proliferation and differentiation. The inhibition of this multifunctional serine-threonine kinase results in G1 cell cycle arrest of T- and B-lymphocytes [10]. In addition, sirolimus reduces intima proliferation of blood vessels, a major factor limiting long-term function and survival of transplant organs [8].

Sirolimus is metabolized in humans by hepatic and intestinal cytochrome P450-3A4, primarily leading to demethylated and hydroxylated metabolites [3]. It has a low oral bioavailability (around 14%) [11].

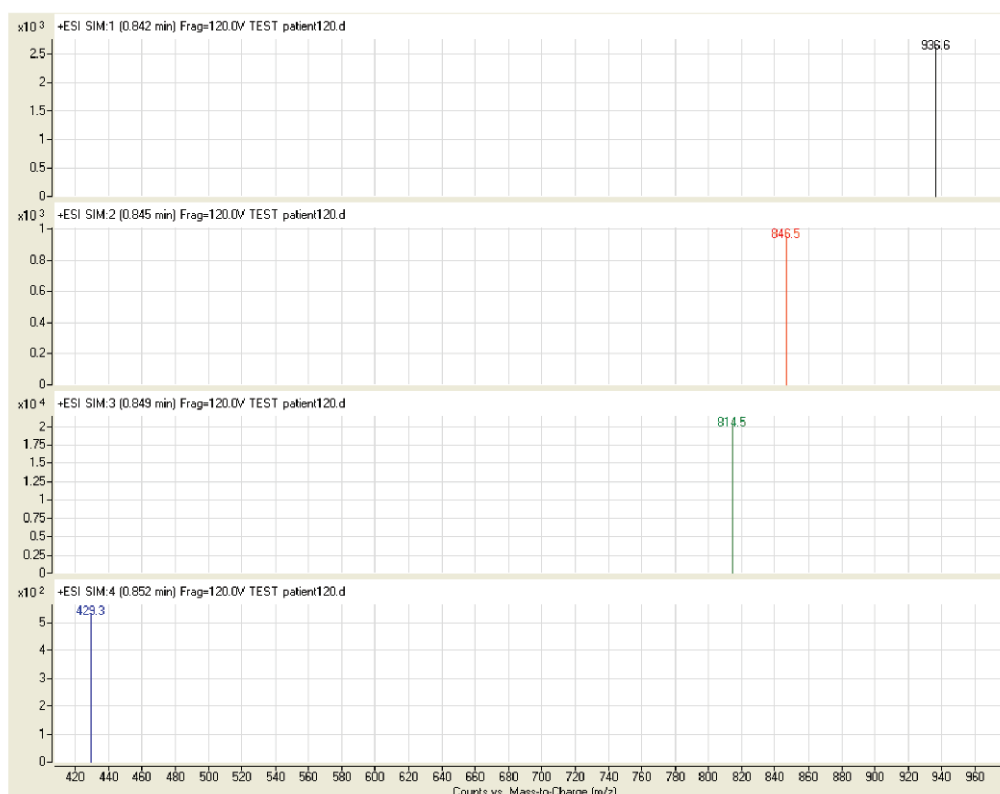


Figure 1. m/z quantification of sirolimus and internal standard.

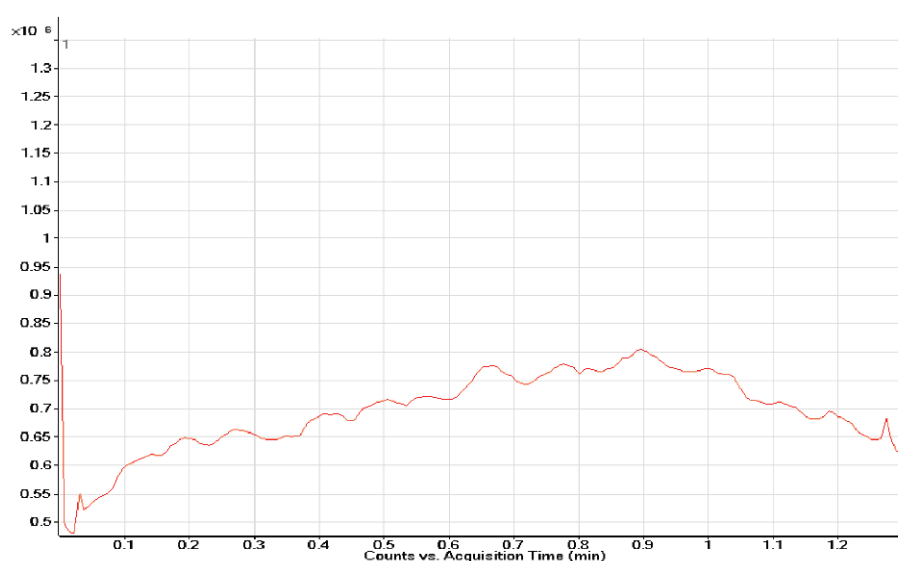
Table 1. The results of laboratory parameters.

Laboratory parameters	Control Mean±SD	Range	Patient Mean±SD	Range
Total Protein	8.26±0.6	7.5-9.6	6.9±0.6***	5.9-8.5
Albumin	4.5±0.2	4-4.8	4.1±0.3***	3.2-4.8
Golbulin	3.7±0.5	2.8-4.8	2.7±0.5***	1.9-4.2
AST	19±4.5	14-34	43±43**	11-216
ALT	17±57	9-35	35±39*	6-175
ALP	217±92	118-540	463±345***	134-1362
T.BILI	0.7±0.3	0.4-1.3	0.9±1.4	0.3-8.4
D.BILI	0.22±0.07	0.1-0.4	0.4±0.8	0.1-0.4
Calcium	9.2±0.5	8-9.9	9.37±0.73	7.6-11.5
Phosphorus	4.0±0.4	3-4.7	3.89±0.57	2.7-5.3
Triglyceride	149±45	88-259	184±77	83-419
Cholesterol	197±35	147-267	198.0±38.8	131-282
HDL	48.0±7.7	39-70	48±15	29-84
LDL	121±32	67-183	112±35	50-206
BUN	14.7±4.1	5-24	20.0±10.5*	5-51
Creatinine	0.9±0.2	0.5-1.4	1.2±0.5**	0.3-2.8
Na	140.0±2.7	135-145	141±3	135-147
K	4.0±0.2	3.6-4	4.1±0.5	3.4-5.2
WBC	6575±1562	3900-9000	5903±3773	2600-21000
Lymphocyte	1984±797	817-4100	1503±1030	100-4900
Hb	13.8±1.4	11.3-16.1	12.36±1.20*	7.3-17.6
Platelet	275105±64742	167000-400000	270300±224540	58000-1130000

*, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Clinical outcome and the side-effect profile are affected by under-dosing (when trough concentrations is approximately less than 5 µg/l) versus over-dosing (when trough concentrations exceed approximately 15 µg/l) [12] of the drug: namely, acute rejection episodes versus thrombocytopenia (platelet count $< 50000/\text{mm}^3$), leucopenia (count $< 3000/\text{mm}^3$),

hypertriglyceridemia (serum triglyceride value > 1000 mg/dl), hypercholesterolemia (serum cholesterol value > 750 mg/dl) [4, 5], cardiotoxicity, neurological effects and elevated risk of infections [7]. Therefore, TDM of whole blood is an important part of immunosuppressive therapy and is mandatory for sirolimus dosage individualization [1].

**Figure 2.** A chromatogram of drug-free whole blood.

Due to its long half-life (approximately around 59 ± 19 h) [6, 13], which may increase to >100 h in patients with liver dysfunction [11], minimal sirolimus concentrations should be monitored no sooner than 5 to 7 days after a change in dose [4] in order to reach the steady state [10]. Monitoring should be 1 to 2 days immediately post-transplant. For the first 3-6 months, 2-3 times a week until the patient is stable. Beyond 6 months, once every few months or whenever clinically indicated. In addition, other tests to check on the adverse effects of immunosuppressants include creatinine, liver function test and glucose [14]. Sometimes a transplant biopsy that showed criteria for the histopathologic diagnosis of rejection is needed [5].

An optimal target range of 5 to 15 ng/ml of sirolimus has been recommended [15]. In patients receiving cyclosporine-sparing regimens, higher concentrations may be necessary to achieve similar efficacy. The target through range of 12 to 20 mg/ml is recommended in this specific case [5, 10]. The sirolimus dose was only reduced in the presence of drug-induced toxicity [5].

Sirolimus is sequestered in red blood cells, leading to whole blood/plasma ratios of ≈ 38 . Because of low sirolimus concentrations in plasma and limited stability in that matrix, whole blood is the matrix of choice for determining sirolimus concentrations [14].

For an analytical method to be suitable for the TDM of sirolimus, it should be simple, sensitive (lower limit of quantification, <5 mg/l), and rapid (turn-around time, <24 h) [3].

Currently, several analytical methods have been developed for the determination of immunosuppressive drugs, among which the following immunoassays (IAs): fluorescence polarization immunoassay (FPIA), microparticle enzyme immunoassay (MEIA), enzyme multiplied immunoassay, radioimmunoassay, enzyme-linked immunosorbent assay and high-performance liquid chromatography HPLC/UV and HPLC-MS/MS (tandem mass spectrometry) methods [7]. The gold standard for evaluating sirolimus is the HPLC [10]. Among HPLC methods, LC/MS quantification has advantages over techniques such as immunoassay or HPLC/ultraviolet (UV) spectrophotometry include enhanced selectivity, lower detection limit, higher throughput, and lower cost per sample [16]. The major drawback of these assays is the requirement for tedious and time-consuming manual sample extraction procedures [8].

2. Materials and methods

2.1. Chemicals and reagents

Sirolimus powder was purchased from TCS (China industry LTD). Ascomycin as internal standard was purchased from Sigma-Aldrich (USA). Zinc sulfate for extraction solution, formic acid as mobile phase and ammonium oxalate as buffer were purchased from Merck (Germany). Acetonitrile for extraction and methanol as mobile phase were purchased from KALEDON (Canada). Human whole blood with EDTA and plasma were provided of healthy volunteers.

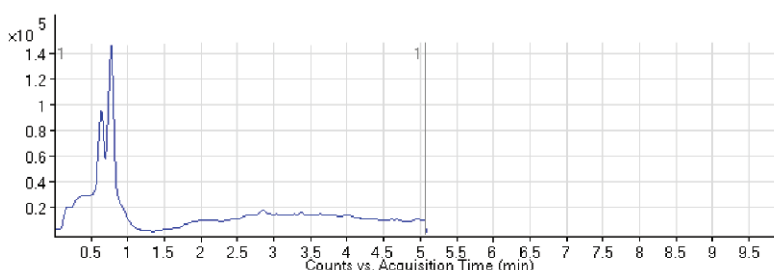


Figure 3. A chromatogram of sirolimus extracted from blood.

2.2. Sample collection

Thirty two transplant recipients (10 women and 22 men) receiving sirolimus were subjected. The consent ethic forms were taken from patients. The forms made us authorized to use whole-blood specimens that had been received for sirolimus analysis for TDM purposes. All specimens were taken during routine ambulatory visits without additional burden to patients. Specimens were collected just before the next dose of sirolimus for the corresponding trough level evaluation. Blood specimens were drawn into EDTA-anticoagulant collection screw caps tubes and stored at -20 °C. Also 5 ml of blood specimens were collected into clot tubes for testing biochemical parameters.

2.3. Instrumentation

The LC chromatograph was an Agilent 6460 series Triple Quadrupole LC/MS (USA). The biochemical autoanalyser was a Dirui CS-400 (China). The cell counter was a DREW- EXCELL 2280 (USA). The Deionizer was an ELGA (UK). The vortexer was an IKA (USA). The centrifuge was Hettich (Germany). The digital scale was a Precisa (Swiss, Precision; 0.001 g), and the filters were Sartorius (Germany, I.D.=0.45 µm).

2.4. Preparation of stock solution

2.4.1. Sirolimus stock solution

A stock solution of sirolimus was prepared as 10 µg/ml (stock solution 1) and 100 ng/ml (stock solution 2) in methanol. Analytical standard samples were prepared by spiking known quantities of the standard solutions into blank human whole blood with EDTA. The concentration range in whole blood was 1-50 ng/ml level for preparation of the standard curve.

Std-1 (1 ng/ml): 200 µl of stock solution 2 added to 20 ml drug-free whole blood.

Std-2 (5 ng/ml): 10 µl of stock solution 1 added to 20 ml drug-free whole blood.

Std-3 (10 ng/ml): 20 µl of stock solution 1 added to 20 ml drug-free whole blood.

Std-4 (20 ng/ml): 40 µl of stock solution 1 added to 20 ml drug-free whole blood.

Std-5 (50 ng/ml): 100 µl of stock solution 1 added to 20 ml drug-free whole blood.

2.4.2. Internal standard stock solution

A stock internal standard solution is prepared by dissolving 5 mg of ascomycin in a 10 ml graded volumetric flask with methanol and make up to 10 ml to give a stock solution of 500 µg/ml.

2.4.3. Preparation of precipitation reagent

To prepare 200 ml of precipitation reagent, 5 µl of internal standard stock (500 µg/ml) was dissolved in 200 ml of acetonitrile. The final concentration of internal standard is 12.5 ng/ml.

2.4.4. Preparation of mobile phase

A. Solution A (pH 2.8): To prepare 1 L of mobile phase A in a 1 L measuring cylinder add 0.154 g of ammonium acetate to 1.0 ml formic acid (Reagent grade) and make up to mark with water. This reagent is stable for 12 months at room temperature.

B. Solution B: To prepare 1 L of mobile phase B in a 1 L measuring cylinder add 0.154 g of ammonium acetate to 1.0 ml formic acid (Reagent grade) and make up to the mark with methanol. This reagent is stable for 12 months at RT.

C. Mobile phase: Mobile phase was solvent mixture of 10/90 (v/v) solution A and B.

2.5. Extraction Procedure

EDTA-treated whole blood samples (100 µl) were treated with 200 µl 0.1 M ZnSO₄ in polypropylene centrifuge capped vial and mixed well to lyse the cells. Then 500 µl of precipitating reagent was added. Samples were immediately vortexed for 2 min and centrifuged for 8 min at 15000 rpm and finally

the supernatant (20 µl) was manually injected into a HPLC column.

2.6. Chromatographic conditions

It is described as an isocratic LC/MS chromatography. The LC/MS system equipped with a quaternary pump. The HPLC elution mobile phase was a solvent mixture of 10/90 (v/v) solution A and B. A flow rate of 0.5 ml/min was used for sample analysis. A 50 mm×21 I.D. 3 µm C18 column ODSH (Germany) was used as the analytical column. The analytical column was maintained at a temperature of 50 °C and the injection volume was 20 µl. Dwell was 200 millisecond.

2.6.1. MS/MS detection

LC/MS detection was obtained using a model Agilent 6460 Triple Quadrupole instrument with a turbo ionspray ionization source. The QQQ mass selective detector was operated in electrospray-positive ionization mode and performed multiple reaction monitoring (MRM). The capillary voltage and charging voltage were 5500V and 2000 V, respectively. Gas temperature and vaporizer temperature were 350 and 250 °C, respectively. Nebulizer pressure was 60 psi, gas flow was 13 L/min. Fragmentation was produced by collision-activated dissociation using nitrogen as the collision gas. Under optimized fragmentation conditions several product ions were observed for each compound and the most intense was selected as the product ion to be monitored.

The peak areas obtained from multiple reaction monitoring of the following mass transitions: m/z 936.6-429.3 and m/z 846.5-814.5 was employed for the quantification of sirolimus and internal standard respectively as shown Figure 1.

2.7. Validation

2.7.1. Specificity study

The specificity of the method was determined by using 10 samples of drug-free

human blood with EDTA.

2.7.2. Precision study

Intra-assay precision was determined by performing 3 replicates at each QC level in a single analytical run. Inter-assay precision was assessed by analyzing the QC samples three times on five successive days. QC levels were human whole blood spiked with 1, 5, 10, 20 and 50 ng/ml sirolimus, respectively.

2.7.3. Accuracy study

The accuracy of the QC samples was determined by comparing the calculated value to the theoretical value with the result expressed as a percentage of the theoretical value.

2.7.4. Limit of detection (LOD)

The minimum detectable sirolimus concentration was defined as the concentration of sirolimus that could be distinguished from zero with 95% confidence. The LLOD (lower limit of detection) was assessed by the 2 SD method following repeated runs of $n = 10$ replicates of the drug-free zero calibrator.

2.7.5. Lower limit of quantification (LLOQ)

The lower limit of quantification was defined as the concentration of sirolimus at which a 20% between-run coefficient of variance (CV) was observed with the assay. LLOQ was assessed by performing 11 replicates of the lowest standard (1 ng/ml) in a single run and comparing them with the nominal concentration.

2.7.6. Linearity study

The evaluation of the linearity of the calibration curve was obtained from a set of calibration standards.

2.7.7. Recovery study

To determine the extraction recovery of sirolimus, three levels of known concentration spiking solutions prepared in the reconstitution

solvent were added to the extracted matrix of internal standard and normal human blood with EDTA. The calculated concentration for each of these samples was obtained using the standard calibration curve. The recovery was calculated by dividing the theoretical concentration by the calculated concentration.

3. Results and discussion

In this project, 32 transplant recipients that were transplanted from December 2004 to May 2011 have been studied, among which 10 women were between 26 to 52 years old and 22 men were between 5 to 61. The average duration of sirolimus treatment in the patients above was 16 ± 26 months.

A Typical chromatograms of drug-free whole blood, and patient samples are illustrated in Figure (2) and (3), respectively. Evaluation of the results showed interferences and ionization suppression were minimal. The actual retention times are 0.6 min. for sirolimus and 0.9 min. for the internal standard.

The average sirolimus concentration in transplanted patients' blood ($n = 32$) using LC/MS method was 10.2 ng/ml (range: 1.3 to 39.1 ng/ml). The assay was validated for a linear dynamic range of 1-50 ng/ml. The correlation coefficient (r) was 0.995. Limit of detection (LOD) was defined as 0.3 ng/ml. Limit of quantification (LOQ) was found to be approximately 1.0 ng/ml. The within-run imprecision CV (%) for concentrations (1, 10 ng/ml) were <15 and $<3\%$, respectively. The between-run imprecision CV (%) for the same concentrations were $<15\%$ and $<4\%$, respectively. Analytic recovery was $98 \pm 2\%$ over a range of 1–50 ng/ml. Within-run and Between-run relative standard deviations is 2.2- 14.7% and 3.4-14.8%, respectively.

Laboratory parameters including biochemical tests (LFT, Ca and P, TG and Cholesterol, BUN and Cr, Na and K) and CBC in the group of cases receiving sirolimus ($n=32$) and the control group were performed.

Table 1 summarizes the analysis results of these parameters.

The doses of sirolimus in the patients were as follows: one patient received 4 mg/day, 7 patients received 3 mg/day, among which 6 received all 3 mg once daily before or after their meals but one of them received 2 mg in the morning after breakfast and 1 mg in the evening after dinner; 22 patients received 2 mg/day, 2 patients received 1 mg a day. The average number of concomitant medications in these patients was 5.8 ± 2.9 . There has been no drug interaction between such drugs and Cytochrome P450. In most of the patients the drugs were taken with meals. None of the patients in these series received herbal drugs or fruits with known effects on Cytochrome P450, such as grapefruits. The above-mentioned patients received sirolimus 1 mg tablet (Wyeth Pharmaceutical, UK) which was available during the research period. Test results showed that the average sirolimus trough concentration in men was 11 ± 8 ng/ml while in women the number was 8.4 ± 5.1 ng/ml. Despite the fact that there was a difference in the average concentration between men and women, statistically, this difference was not significant. In both men and women, sirolimus trough concentration in blood was measured. The results showed that in 15 men, it was within the therapeutic range (5-15 ng/ml), in 4 patients, it was more than the therapeutic range and in 3, it was less. On the other hand, in women, sirolimus trough concentration in blood was within the therapeutic range in 6 patients, more than the therapeutic range in 2 patients and less than the therapeutic range in again 2 patients.

There is no correlation between sirolimus blood concentration and the dosage. As no significant difference was observed between the average blood concentration of drug and the dosage in patients receiving 1, 2, 2.5, 3 and 4 mg doses of medication ($r=0.27$ and $p=0.133$).

The comparison of blood parameters

(CBC) showed that, except hemoglobin, there was no significant difference between healthy people and transplant recipients. There was also no correlation between blood concentrations of drug and blood parameters. The comparison of biochemical parameters in healthy people and the transplant recipients (liver, kidney, pancreas) showed that there was no dramatic difference in the parameters except for total protein ($p<0.001$), albumin ($p<0.0001$), globulin, aminotransferases (AST, ALT) (respectively $p<0.05$ & $p<0.01$), alkaline phosphatase (ALP) ($p<0.001$), urea ($p<0.05$) and creatinine ($p<0.01$) that were significantly different. Although the total protein and albumin were significantly different between controls and patients, but there was no relationship between drug concentration in blood and these two parameters.

4. Conclusion

The results of blood drug concentrations in patients discussed in this study showed that there was no possibility of predicting the sirolimus blood concentrations based on the dose patients received, hence it must be mandatory for specialists to perform TDM on transplant recipients receiving sirolimus.

So, it is recommended to increase the number of samples and TDM and as a result create standard treatment guidelines (STG) in order to prevent different treatment strategies. Considering the fact that blood concentrations of sirolimus can be affected by many factors such as food, concomitant medications, drug formulation, etc. which can cause large fluctuations in drug concentration, it is suggested that TDM should be performed 1-2 days immediately post-transplant, and for the first 3-6 months, 2-3 times a week until the patient is stable. Beyond 6 months, TDM is needed once every few months or whenever clinically indicated to reduce the fluctuations and risks in such patients. It must be noted that in addition to dose adjustment of sirolimus

based on TDM, measurement of laboratory parameters, clinical status and biopsy along with TDM should be considered. And finally, the sirolimus blood concentration measurement in forensic toxicology may also help to determine the cause of death in those who had been received sirolimus.

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