Drug Monitoring of Sirolimus and Everolimus

Drug-Monitoring von Sirolimus und Everolimus

Victor W. Armstrong, Frank Streit

Summary: Sirolimus (Rapamune®) and everolimus (Certican™) are macrocyclic lactones with a novel mechanism of immunosuppressive action that act in synergy with the calcineurin inhibitors cyclosporine and tacrolimus. Both sirolimus and everolimus are rapidly absorbed (t_{max} 1–2 h), but they have a relatively low systemic bioavailability. Sirolimus has a prolonged half-life (ca. 60 h) and is therefore administered once daily, whereas everolimus has a shorter half-life (ca. 17.5 h) and is normally given twice daily. Both drugs show substantial inter- and intraindividual variation in their pharmacokinetics. They are metabolized by the cytochrome P-450 3A (CYP3A) system and they are also substrates for P-glycoprotein. Drug-drug interactions are therefore observed with inhibitors or inducers of CYP3A/P-glycoprotein. The major side effects associated with sirolimus and everolimus appear to be hypercholesterolaemia, hypertriglyceridaemia, thrombocytopenia and leucopenia. However, in contrast to the calcineurin inhibitors, they are not nephrotoxic. Several analytical procedures based on HPLC-UV, liquid chromatography-mass spectrometry (LC-MS), or liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been described for the quantification of sirolimus. In the case of everolimus, LC-MS and LC-MS/MS methods have been reported. As yet, immunoassays are not available. A provisional therapeutic range for trough whole-blood sirolimus concentrations, measured using a chromatographic procedure, in renal transplant recipients receiving cyclosporine and corticosteroids as concomitant immunosuppression is 4–12 mg/L. If cyclosporine therapy is discontinued, a target trough range of 12–20 µg/L is recommended. A therapeutic range for everolimus has not yet been established. A significantly increased risk of acute rejection has been documented at everolimus trough levels below 3 µg/L when used in combination with cyclosporine and steroids.

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Keywords: therapeutic drug monitoring; sirolimus; everolimus; tandem mass spectrometry.

Zusammenfassung: Sirolimus (Rapamune®) und Everolimus (Certican™) sind cyclische Makrolide mit einer α,β-Diketoamid-Bindung. Sie weisen einen anderen Mechanismus der Immunsuppression als die bisher verwendeten Calcineurin-Inhibitoren (Cyclosporine und Tacrolimus) auf, welche jedoch mit einer synergistischen Wirkung in Kombinationstherapie eingesetzt werden können. Sirolimus und Everolimus werden beide schnell adsorbiert (t_{max} 1–2 Std), haben jedoch eine geringe Bioverfügbarkeit. Sirolimus wird aufgrund seiner relativ langen Halbwertszeit (ca. 60 Std) nur einmal täglich gegeben, während Everolimus mit einer kürzeren Halbwertszeit (ca. 17,5 Std) zweimal täglich gegeben wird. Beide Substanzen zeigen sehr große inter- und intraindividuelle Schwankungen in ihrer Pharmakokinetik. Sie werden beide durch das Cytochrome P450 3A (CYP3A)-System metabolisiert und sind Substrate des P-Glycoproteins. Arzneimittelinteraktionen mit Inhibitoren oder Induktoren des CYP3A/P-Glycoproteins sind bekannt. Die Hauptnebenwirkungen von Sirolimus und Everolimus sind Hypercholesterolämie, Hypertriglyceridämie, Thrombocytopenie und Leukopenie. Im Vergleich zu den Calcineurininhibitoren sind sie jedoch nicht nephrotoxisch. Zur Quantifizierung von Sirolimus sind verschiedene Methoden basierend auf HPLC-UV, Liquid Chromatography-Mass Spectrometry (LC-MS) oder Liquid Chromatography-Tandem Mass Spectrometry beschrieben. Für Everolimus sind nur massenspektrometrische Methoden beschrieben. Zur Zeit ist kein Immunoassay verfügbar. Der vorläufige therapeutische Bereich für den Vollblut-Talspiegel von Sirolimus für nierentransplantierte Patienten, die eine Triple-Therapie aus Ciclosporin und Corticoiden erhalten, liegt bei 4–12 mg/L. Bei einer Immunsuppression ohne Cyclosporin wird ein therapeutischer Bereich von 12–20 µg/L empfohlen. Ein therapeutischer Bereich für Everolimus ist derzeit noch nicht erstellt. Ein signifikanter Anstieg des Risikos einer akuten Abstoßung bei Everolimus-Talspiegeln kleiner als 3 µg/L bei einer Kombinationstherapie mit Cyclosporin und Corticoiden wurde berichtet.

Schlüsselwörter: Drug-Monitoring; Sirolimus; Everolimus; Tandem-Massspektrometrie.

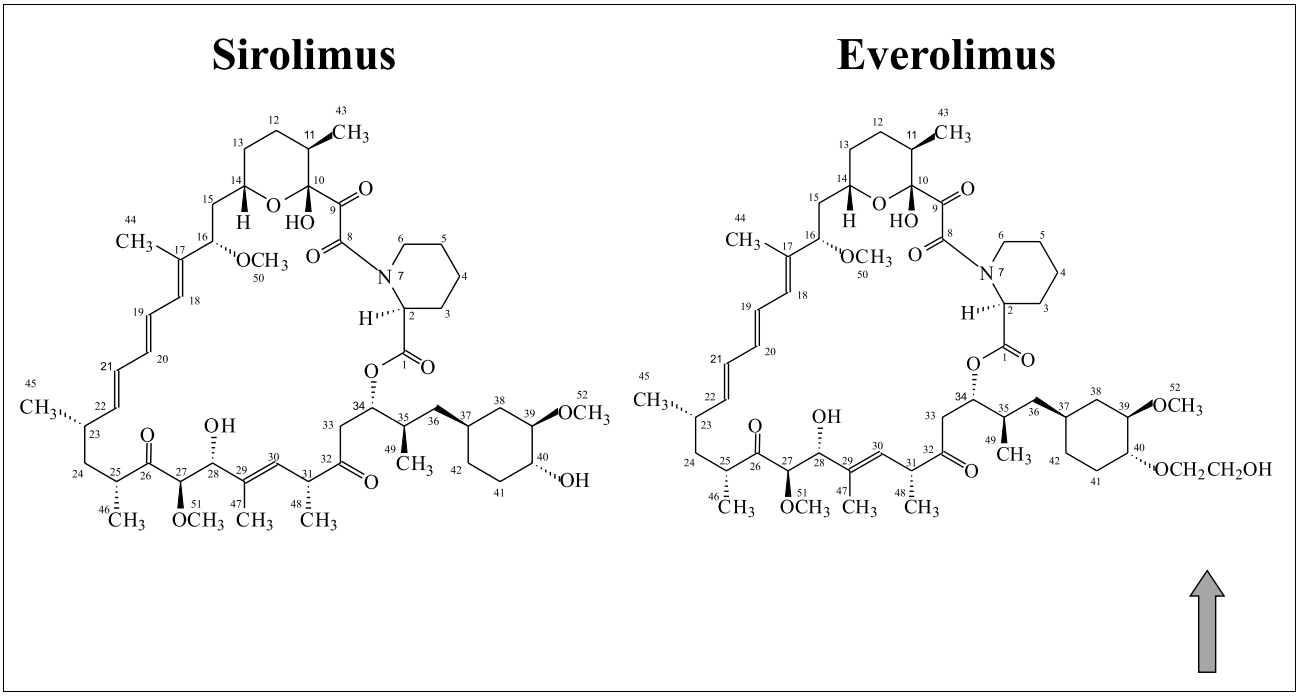

Figure 1 Chemical structures of sirolimus and everolimus. The 2-hydroxyethyl group of the latter is indicated by an arrow.

Introduction

Sirolimus (Rapamune®) and everolimus (Certican™) are macrocyclic lactones that display a novel mechanism of immunosuppressive action. Sirolimus is a macrocyclic triene antibiotic that is produced by the actinomycete *Streptomyces hygroscopicus*, which was first isolated from soil samples collected from the Vai Atari region of Rapa Nui (Easter Island). Everolimus is the 40-O-(2-hydroxyethyl) derivative of sirolimus (Fig. 1). Sirolimus was approved in September 1999 by the United States Food and Drug Administration and in December 2000 by the Committee for Proprietary Medicinal Products (CPMP), the scientific advisory body of the European Medicines Evaluation Agency (EMEA) for use in renal transplant recipients. Everolimus is currently still an investigational drug that is awaiting regulatory approval.

Mechanism of action

These new immunosuppressive agents inhibit T-lymphocyte activation and proliferation that occur in response to antigenic and cytokine stimulation. Their mechanism of action is, however, distinct from that of the other immunosuppressive drugs. Interaction with at least two intracellular proteins is required to elicit their antiproliferative activity. Sirolimus and everolimus first bind to the cytosolic immunophilin FK-binding protein 12 (FKBP12). In contrast to the tacrolimus-FKBP12 complex, the complex of sirolimus or everolimus with FKBP12 does not inhibit calcineurin activity. Instead, this complex binds to and inhibits the activation of the mammalian target of rapamycin (mTOR), a key regulatory kinase. This inhibition suppresses cytokine mediated T-cell proliferation inhibiting the progression from the G_1 to the S phase of the cell cycle. Thus, sirolimus and everolimus act at a later stage in the cell cycle than do the calcineurin inhibitors cyclosporine and tacrolimus. In the case of the latter two drugs, their complexes with the respective immunophilins inhibit the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin, thereby suppressing production of interleukin-2 and the transition of T lymphocytes from the G_0 to the G_1 phase of the cell cycle. The mTOR inhibitors can, therefore, be used in combination with the calcineurin inhibitors to produce a synergistic effect. Initially it was thought that sirolimus and tacrolimus could not be used together since they compete for the same immunophilin. However, it is now known that only 5–10 % of intracellular FKBP12 is bound at immunosuppressive concentrations of tacrolimus or sirolimus/everolimus [1]

Metabolism and pharmacokinetics

Sirolimus and everolimus are metabolized by enzymes of the cytochrome P3A4/5 (CYP3A4/5) family. They are also substrates for the MDR1 gene product P-glycoprotein, a membrane protein that is involved in the en-

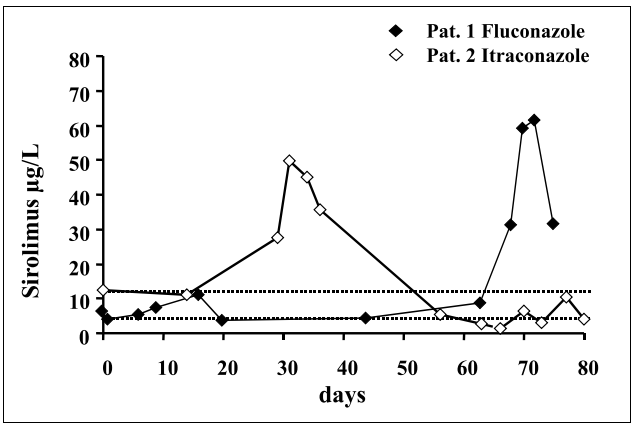

Figure 2 Time course of whole-blood sirolimus concentrations in two patients illustrating an interaction between sirolimus and the antifungal agents fluconazole and itraconazole. The dotted lines indicate the upper and lower limits of the therapeutic range when sirolimus is used in triple therapy together with cyclosporine and corticosteroids.

ergy-assisted transport of compounds out of cells. Consequently, both drugs can interact with inhibitors and inducers of either CYP3A4/5 or P-glycoprotein. Studies in healthy volunteers have revealed significant pharmacokinetic interactions with several drugs. Diltiazem, ketoconazole and cyclosporine increased the sirolimus area under the concentration-time curves (AUC) by 60 %, 990 % and 80 %, respectively, whereas rifampin reduced the sirolimus AUC by 82 % [2]. Concomitant administration of itraconazole and fluconazole without appropriate adjustment of the sirolimus dose was associated with a marked increase in sirolimus trough levels (Fig. 2). Itraconazole was also found to reduce the clearance of everolimus by 74 % in one patient [3].

Sirolimus is extensively metabolized by O-demethylation and/or hydroxylation [4, 5]. In kidney transplant recipients, trough whole-blood samples contained 56 ± 9 % of sirolimus metabolites, in particular the 12-hydroxy-, 16-O-demethyl-, 39-O-demethyl, 27, 39-O-di-demethyl- and dihydroxy sirolimus. The metabolites of sirolimus, however, display < 10 % of the immunosuppressive activity of the parent compound [6]. Sirolimus is rapidly absorbed and reaches a peak concentration approximately 2 h (t_{max}) after oral administration in renal transplant recipients. The oral bioavailability of sirolimus is approximately 15 % in humans receiving concurrently cyclosporine [7]. When the dosing of the two immunosuppressants was spaced 4 h apart, the exposure of sirolimus was reduced by approximately one-third [8]. The bioavailability of cyclosporine was, however, unaffected by the coadministration of sirolimus. A high-fat meal was found to decrease the mean peak (C_{max}) sirolimus concentration by 34 %, whereas total exposure (AUC) was increased by 35 % compared with fasting values [9]. The time to peak concentration was increased 3.5-fold after the high-fat breakfast. It is recommended that patients take the drug consistently with or without food to minimize unnecessary fluctuations in trough whole-blood sirolimus concentrations.

The t_{max} of everolimus at steady state in renal transplant recipients receiving cyclosporine and corticosteroids as concomitant immunosuppression is 1.5–2 h [10]. A high-fat meal was found to delay the time to maximum concentration by a median of 1.75 h, in renal transplant recipients receiving multiple doses of everolimus [11]. The peak blood concentration was reduced by 60 % and the AUC by 21 %. As with sirolimus, there is also a clinically significant interaction between cyclosporine and everolimus. Concomitant administration of either Neoral (microemulsion cyclosporine formulation) or Sandimmune (conventional cyclosporine formulation) with everolimus led to increases in the AUC for everolimus of 168 % and 74 %, respectively [12].

Sirolimus in whole blood is partitioned independently of temperature principally into red blood cells (94.5 %) with much lower portions in plasma (3.1 %), lymphocytes (1 %) and granulocytes (1 %) [13]. The elimination half-life of sirolimus is approximately 60 h with a wide interindividual variation [14, 15]. The long terminal half-life of the drug necessitates a loading dose if steady-state concentrations are to be rapidly achieved. However, on account of this long half-life, sirolimus need only be administered as a single dose once daily. Everolimus has a much shorter half-life than that of sirolimus. The average terminal elimination half-life in renal transplant recipients was reported to be around 25 h after a single dose, while a multiple-dose study provided an estimate of approximately 17.5 h [10]. Everolimus is therefore usually administered as twice-daily doses.

Hepatic impairment leads to a reduction in the apparent clearance of both sirolimus and everolimus with an increase in both drug exposure and half-life. In the case of sirolimus, patients with Child-Pugh classification A or B displayed an increase in the AUC of 61 % compared with individuals with normal hepatic function [2]. The apparent clearance of everolimus was significantly reduced by 53 % in subjects with moderate hepatic impairment compared with healthy subjects [16]. This was manifested by a 2-fold increase in the area under the concentration-time curve and an 84 % increase in the half-life.

Clinical studies

Two multicentre, randomized double-blind phase III clinical trials demonstrated the safety and efficacy of sirolimus for the prevention of acute rejection after renal transplantation. Both studies compared two doses of sirolimus (2 and 5 mg) with either placebo [17, 18] or azathioprine [18] when administered in combination with cyclosporine and steroids. The primary end-point in both studies was the rate of efficacy failure, defined

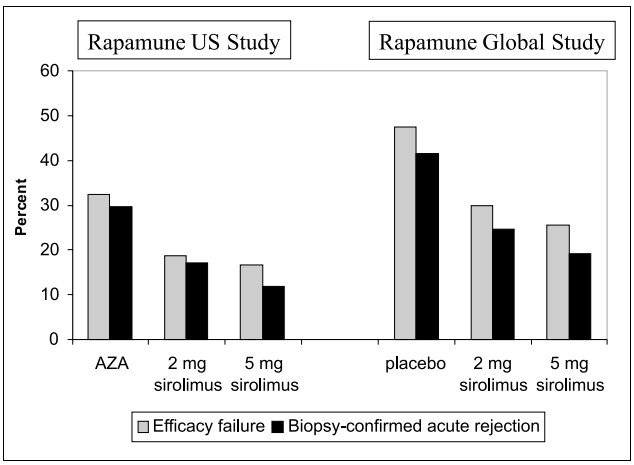

Figure 3 Rates of efficacy failure and biopsy-proven acute rejection in two pivotal multicentre safety and efficacy studies using a combination of sirolimus with cyclosporine and corticosteroids for the prevention of acute rejection after renal transplantation. In the US Rapamune study [18], two doses of sirolimus (2 or 5 mg/day) were compared with azathioprine (AZA). In the global Rapamune study [17], the comparison group received a placebo (in addition to cyclosporine and corticosteroids). The primary end point of efficacy failure was a composite of acute rejection, graft loss, or death during the first 6 months after transplantation.

as acute graft rejection, graft loss or death, during the first 6 months after transplantation. The major findings are summarized in Fig. 3. A significant reduction in the rate of efficacy failure was observed for both sirolimus doses compared with either placebo or azathioprine. Everolimus is being investigated in double-blind, randomized, parallel group studies designed to evaluate efficacy equivalence with mycophenolate mofetil (MMF). Initial results show that everolimus and MMF produce similar low rates of acute rejection in renal transplant recipients receiving triple immunosuppressive therapy with conventionally dosed cyclosporine and corticosteroids [19].

Nephrotoxicity, one of the major problems of calcineurin-based therapy, is not a side effect of either of the mTOR inhibitors. The major side effects associated with the latter immunosuppressive drugs appear to be hypercholesterolaemia, hypertriglyceridaemia, thrombocytopenia and leucopenia [1, 19].

Drug monitoring

Sirolimus

From the pharmacokinetic and clinical data it is apparent that sirolimus is a critical-dose drug, which requires therapeutic drug monitoring to minimize drug-related toxicity and maximize efficacy. In its approval of the use of sirolimus, the Committee for Proprietary Medicinal Products of the European Union stated that optimal therapy requires therapeutic drug monitoring (TDM) in all patients. In the United States approval, TDM was not mandatory. However, drug monitoring was recommended in patients with hepatic impairment, during concurrent administration of strong inducers/inhibitors of CYP3A4 (e.g. rifampin, rifabtin) and/or if cyclosporine is markedly reduced or discontinued.

There have been several reports of a strong correlation between sirolimus trough concentrations and the sirolimus AUC, suggesting that trough concentration monitoring is an adequate pharmacokinetic measure of drug exposure [20]. Clinical studies have demonstrated a significant negative correlation between sirolimus trough levels and the incidence of acute rejection after renal transplantation. A significant positive correlation was seen between trough levels and the occurrence of hypertriglyceridaemia, thrombocytopenia and leucopenia [21]. A provisional therapeutic range for trough whole-blood sirolimus concentrations, measured using a chromatographic procedure, in renal transplant recipients receiving cyclosporine and corticosteroids as concomitant immunosuppression is 4–12 mg/L. If cyclosporine therapy is discontinued, a target trough range of 12–20 µg/L is recommended.

Methods of analysis

A consensus conference recommended that the analytical assay for sirolimus should have the following performance criteria [22]:

- the interday CV should be ≤ 10 % at 5 µg/L
- the analytical measuring range should be between 1 and 75 µg/L
- when compared with a validated specific assay, the slope of the regression analysis should be 0.9 to 1.1 and the standard error of the residuals ($S_{y|x}$) should be no more than 5 µg/L

Because 95 % of the drug is sequestered in red blood cells, the preferred matrix for sirolimus measurement is whole blood with EDTA as anticoagulant. Since sirolimus is sensitive to light, blood samples should be shielded to prevent degradation.

Immunoassays

An automated microparticle enzyme immunoassay using the IMx analyser (Abbott) was used for the quantification of sirolimus in some of the major pivotal clinical studies. The method required manual pretreatment of the blood sample with a precipitation reagent and transfer of the supernatant after centrifugation to the sample well. The major drawback with this method was a substantial overestimation of the sirolimus concentrations in patient samples when compared to a more specific HPLC procedure [23, 24]. This was presumably due to cross-reactivity of the antibody with sirolimus metabolites. This immunoassay was, however, withdrawn and is not commercially available. Nevertheless, other commercial immunoassays are currently under development.

Table 1 Mass transitions used for the quantification [26] of the four immunosuppressants sirolimus, everolimus, tacrolimus and cyclosporine. Ascomycin and cyclosporin D were used as internal standards.

	Precursor ion *m/z*	Product ion *m/z*
Sirolimus	931.5 [M + NH_4^+]	864.5
Everolimus	975.5 [M + NH_4^+]	908.5
Tacrolimus	821.5 [M + NH_4^+]	768.5
Ascomycin	809.5 [M + NH_4^+]	756.5
Cyclosporine	1202.8 [M + H^+]	425.4
Cyclosporin D	1216.8 [M + H^+]	425.4

HPLC with UV detection

Several methods have been reported for the quantification of sirolimus using reversed-phase HPLC and UV detection at 278 or 276 nm [20]. Sample preparation is based on liquid-liquid extraction using either ether or 1-chlorobutane. Both C8 and C18 reversed-phase columns have been used for chromatographic separation using combinations of methanol, water, or acetonitrile as the mobile phase. Run times are generally longer than 30 min, although one procedure in which liquid-liquid extraction was combined with solid-phase extraction reported a run time of 10 min. The lower limit of quantification of these methods is around 1–2 µg/L. HPLC/UV methods appear to require more sample integrity than do LC-MS methods (Kim Napoli, personal communication), and blood should not be left at room temperature for more than 4 h. For overnight shipping, it is recommended that blood is shipped on dry ice or with cold packs. Blood samples are stable at 4 °C for up to 7 days and can be stored indefinitely when frozen.

HPLC with mass spectrometric (MS) detection

Methods have been developed more recently for the quantification of sirolimus using HPLC with single-stage (LC-MS) or tandem mass spectrometric (LC-MS/MS) detection. The first application of HPLC electrospray MS for the quantification of sirolimus and four of its metabolites appeared in 1996 [4]. This method involved solid-phase extraction of the blood samples (1 mL) followed by isocratic elution on a C18 analytic column. The HPLC system was connected to a triple-stage quadrupole mass spectrometer with an electrospray interface and positive ions [M + Na^+] were detected. The linear range for sirolimus was 0.25–250 µg/L and analysis time was 6 min. An LC-MS/MS procedure for quantification of sirolimus was first reported in 1998 [25]. Sample pretreatment involved protein precipitation of whole blood (0.5 mL) using an organic solvent followed by C18 solid-phase extraction. Sirolimus was detected in the positive ion mode [M + NH_4^+] by multiple reaction monitoring of the mass transition *m/z* 931.8→864.6. The method was linear over the range 0.25–100 µg/L. Total chromatographic run time was 10 min. Methods have also been developed to simultaneously measure two or more immunosuppressants. This is of particular interest since patients are often treated with a combination of a calcineurin inhibitor and an mTOR inhibitor. A rapid, sensitive liquid chromatography tandem mass spectrometry procedure has been developed that allows the simultaneous quantification of sirolimus, everolimus, tacrolimus and cyclosporine in whole blood [26]. The procedure only requires 100 µL of whole blood and is linear over a working range of 0.5–100 µg/L for sirolimus, everolimus and tacrolimus, and 5–2500 µg/L for cyclosporine. Samples are pretreated with a methanol/0.3 M $ZnSO_4$ solution to precipitate proteins. After centrifugation, the supernatants are applied to an Aqua Perfect C18 reversed-phase column. The eluate is then introduced into a Sciex API 2000 triple quadrupole mass spectrometer with a turbo-ion spray interface. The drugs are detected in the positive ion mode by multiple reaction monitoring of the mass transitions shown in Table 1. Although LC-MS and in particular LC-MS/MS have the greatest inherent selectivity and sensitivity compared with other quantitative analytical methodologies for the measurement of immunosuppressive drugs, it is important to realize that results may be adversely affected due to ion suppression caused by the sample matrix [27, 28]. This problem may be particularly acute if sample preparation is simplified or even eliminated and/or if very short chromatographic times are used. It is therefore essential when establishing LC-MS and LC-MS/MS assays for sirolimus and everolimus, that potential ion suppression is investigated and excluded [26, 29]. This has not always been the case.

When using LC-MS/MS, it has been shown that blood samples retain their analytical integrity for up to 3 days at 30 °C when kept in the dark [30]. At 35 °C, there was a 9 % loss of the drug after 3 days of storage and a 13 % loss after 6 days [29].

Everolimus

Although it is not yet fully established whether routine drug monitoring will be necessary in the general kidney population, current evidence suggests that it will likely be a useful adjunct to better individualize treatment. A significant relationship has been demonstrated between everolimus trough concentrations and freedom from acute rejection in renal transplantation [3]. There was a significantly increased risk of acute rejection at everolimus trough levels below 3 µg/L. In this study, the highest trough concentrations were around 15 µg/L.

Methods of analysis

Currently, the only methods that have been described for the quantification of everolimus are high performance liquid chromatographic-electrospray mass spectrometric and tandem mass spectrometric assays [26, 31–33]. These methods typically involve protein precipitation followed by C18 reversed-phase chromatography and detection in the positive ion mode.

Conclusion

Current evidence indicates that monitoring the blood concentrations of sirolimus or everolimus is an effective tool for optimizing therapy when either of these two drugs is used in immunosuppressive drug protocols.

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