9

Immunosuppressive Drugs

Pharmacokinetics, Preanalytic Variables, and Analytical Considerations

Anthony W. Butch, PhD

Contents

Summary

To optimize therapeutic effectiveness and minimize unwanted adverse effects, reliable and precise methods are required for monitoring blood concentrations of immunosuppressive drugs. Therapeutic monitoring of cyclosporine, tacrolimus, and sirolimus is currently considered an integral part of organ transplant programs, and compelling arguments have been made for monitoring mycophenolic acid. Although high-performance liquid chromatography (HPLC) is considered the reference method for monitoring immunosuppressive drugs, most laboratories currently measure these drugs by immunoassay. Immunoassays have gained widespread use because they can be automated, have low start-up costs, and do not require specialized testing personnel. Unfortunately, immunoassays exhibit significant metabolite cross-reactivity that differs among immunoassays and is dependent on the transplanted organ as well as time post-transplant. The advantage of HPLC methods is that they are highly specific and can separate drug metabolites from parent compound. However, HPLC methods can require extensive sample cleanup, have long analytical run times, and require specialized training. Some of these drawbacks can be partially overcome by using HPLC with mass spectrometry (MS) detection systems, although the instrumentation is currently expensive. In view of the high cost of immunoassay reagents, HPLC-MS systems are becoming more cost effective, especially when considering that they can simultaneously measure multiple immunosuppressive drugs in a single whole blood specimen.

Key Words: Therapeutic drug monitoring; immunosuppressive drugs; cyclosporine; tacrolimus; sirolimus; mycophenolic acid.

From: *Handbook of Drug Monitoring Methods*
Edited by: A. Dasgupta © Humana Press Inc., Totowa, NJ

1. INTRODUCTION

It has now been more than 50 years since the first successful kidney transplant was performed between monozygotic twins *(1)*. At that time, the field of immunology was in its infancy, and transplants between non-identical twins ended in organ failure because of acute graft rejection. It was not until the introduction of azathioprine (a nucleotide analogue less toxic than 6-mercaptopurine) in the early 1960s that chemical immunosuppression and prolonged kidney allograft survival became possible *(2)*. Azathioprine by itself was not potent enough to prevent acute graft rejection. However, the combination of azathioprine and corticosteroids was shown to provide effective chemical immunosuppression, with 1-year kidney allograft survival rates ranging from 40 to 50% *(3)*. This combination of chemical immunosuppression continued to be the cornerstone of transplant programs for the next 20 or so years until cyclosporine (CsA) entered the transplantation arena in the late 1970s *(4)*.

In the late 1980s, other immune cell modulators such as tacrolimus and sirolimus were discovered and added to the arsenal of chemical immunosuppressive agents *(5,6)*. Mycophenolic acid (MPA) (as the prodrug mycophenolate mofetil) became available in the mid 1990s based on reports from multicenter clinical trials demonstrating that it could further reduce the incidence of renal graft rejection when used in combination with CsA and steroids *(7–9)*.

The number of solid organ transplants performed in the USA continues to increase each year (Table 1) *(10)*. There has been a 17% increase in kidney, a 29% increase in liver, a 2% reduction in heart, and an overall increase of 17% over the last 5 years, when comparing organ transplants performed in 2005 with 2001 *(10)*. Sadly, the limiting factor in the number of transplanted organs is the availability of donor organs. There were more than 94,000 patients on the U.S. organ transplant waiting list at the end of 2005 *(11)*.

The discovery that CsA had immunosuppressive activity that specifically targeted T lymphocytes was a major breakthrough in organ transplantation because it dramatically reduced acute graft rejection and improved long-term graft and patient survival *(12,13)*. The identification of other immunosuppressive drugs that modulate immune responses by additional molecular pathways enabled treatment options to evolve and has permitted combination therapies to be individualized based on patient requirements. Classes of immunosuppressive drugs along with generic and brand names currently approved

Table 1
Solid Organ Transplants in the USA

	Year				
Organ Transplanted	*2001*	*2002*	*2003*	*2004*	*2005*
Kidney	14,100	14,527	14,856	15,671	16,477
Liver	4984	5061	5364	5780	6441
Heart	2171	2112	2026	1961	2126
All Organs[a]	23,942	24,552	25,083	26,539	28,098

[a] Includes pancreas, kidney-pancreas, intestine, lung, and heart-lung transplants.

Table 2
Immunosuppressive Drugs Used in Solid Organ Transplantation

Drug Class	*Generic Name*	*Brand Names*
Corticosteroids	Prednisone	Orasone, Deltasone
	Methylprednisolone	Solu-Medrol, A-methaPred, Medrol
	Dexamethasone	Decadron
Anti-metabolites	Azathioprine	Imuran
	Cyclophosphamide	Cytoxan, Neosar
	Mycophenolate mofetil	CellCept
	Mycophenolate sodium	Myfortic
Calcineurin inhibitors	Cyclosporine A	Sandimmune, Neoral, many generic forms of Cyclosporines
	Tacrolimus (FK-506)	Prograf
mTOR inhibitors	Sirolimus (Rapamycin)	Rapamune
	Everolimus[a] (RAD0001)	Certican

mTOR, mammalian target of rapamycin.
[a] Everolimus is currently in phase III clinical trials in the USA and has not been approved by the Food and Drug Administration (FDA) for use as an immunosuppressive agent.

by the United States Food and Drug Administration (FDA) for use in solid organ transplantation are listed in Table 2.

2. RATIONALE FOR IMMUNOSUPPRESSIVE DRUG MONITORING

A prerequisite for optimizing and individualizing immunosuppressive therapy is a reliable and precise method for monitoring drug concentrations. However, not all immunosuppressive drugs require routine monitoring of blood concentrations. For instance, corticosteroids are dosed based on empirical guidelines and are not routinely monitored. Although methods have been developed to measure blood concentrations of azathioprine *(14–16)*, this antiproliferative agent is seldom monitored by transplant centers. Blood concentrations of CsA, tacrolimus, sirolimus, and MPA are routinely monitored at transplant centers for the following reasons: (a) there is a clear relationship between drug concentration and clinical response; (b) these drugs have a narrow therapeutic index; (c) these drugs exhibit a high degree of inter- and intrapatient variability; (d) the pharmacological response can be difficult to distinguish from unwanted side effects; (e) there is a risk of poor or non-compliance because the drugs are administered for the lifetime of the graft or patient; and (f) there are significant drug–drug interactions.

The potential for drug interactions is not limited to non-immunosuppressive agents but can also occur among the various classes of immunosuppressive drugs. For instance, CsA inhibits transport of an MPA metabolite from the liver to bile resulting in lower MPA concentrations when the two drugs are used together for immunosuppressive therapy *(17,18)*. The combination of CsA and sirolimus or tacrolimus and sirolimus results in increased blood concentrations of sirolimus *(17,19)*. In 2004, the majority of kidney, liver, and heart transplant patients were receiving tacrolimus and MPA followed

by CsA and MPA for immunosuppression, before hospital discharge *(20)*. Tacrolimus and sirolimus or CsA and sirolimus were less commonly used, and sirolimus and mycophenolate mofetil (MMF) were the least common immunosuppressive regimens. All these drug regimens typically included corticosteroids *(20)*. This illustrates the widespread use of combination immunosuppression and the importance of therapeutic drug monitoring, given the potential for various drug interactions.

This chapter will focus primarily on FDA-approved immunosuppressive drugs that are routinely monitored by clinical laboratories supporting solid organ transplant programs. These include CsA, tacrolimus, sirolimus, and MPA. Everolimus will be briefly discussed because it is currently in phase III clinical trials. Other drugs that are not commonly monitored, such as corticosteroids, azathioprine, and cyclophosphamide, will not be discussed further. Clinical pharmacokinetics, unwanted adverse effects, and various drug interactions will be provided for each of the chemical immunosuppressive agents. A comprehensive review of analytical methods will also be provided, along with detailed information regarding limitations and potential sources of error associated with each of the testing methodologies.

3. CALCINEURIN INHIBITORS

The chemical structures of CsA and tacrolimus, calcineurin inhibitors commonly used in organ transplantation, are shown in Fig. 1. The calcineurin inhibitors block the activation and proliferation of $CD4^+$ and $CD8^+$ T lymphocytes by inhibiting IL-2 production *(21,22)*. Under normal circumstances, binding of major histo compatibility complex–peptide complexes to T-cell receptors results in the formation of an activated form of the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin. This leads to de-phosphorylation of the nuclear factor of activated T cells (NF-AT) (among others) and nuclear translocation of NF-AT. Once in the nucleus, NF-AT binds genes encoding pro-inflammatory cytokines such as IL-2, resulting in up-regulated gene transcription *(23)*. CsA and tacrolimus freely cross lymphocyte membranes and form complexes with specific cytoplasmic binding proteins called

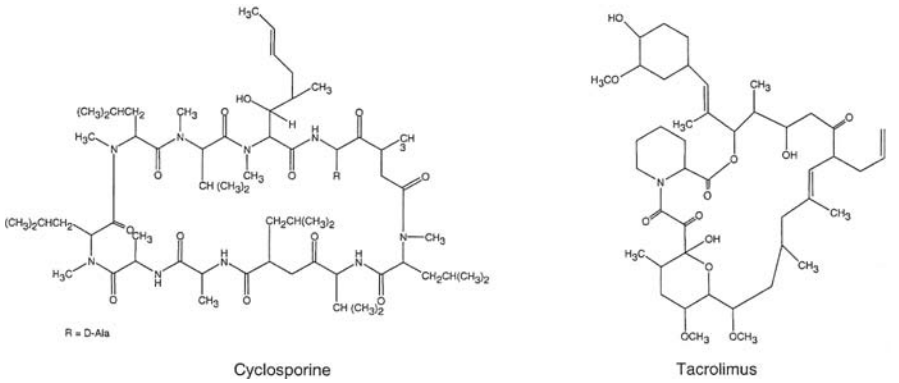

Fig. 1. Chemical structures of the calcineurin inhibitors, cyclosporine (CsA) and tacrolimus. This figure was published in Pharmacology & Therapeutics, Volume 112, Masuda S, Inui KI, an up-date review on individualized dosage adjustment of calcineurin inhibitors in organ transplant patients, page 186, Copyright Elsevier 2006.

immunophilins. CsA binds to the immunophilin cyclophilin and tacrolimus binds to the immunophilin FK506-binding protein-12 *(24,25)*. The drug–immunophilin complexes inhibit calcineurin activity, which prevents nuclear translocation of NF-AT. The end result is down-regulated cytokine gene transcription *(26–28)*.

3.1. Cyclosporine

CsA is a small cyclic polypeptide (molecular weight of 1204) that was originally isolated from fungal cultures of *Tolypocladium inflatum Gams* in 1970 *(29)*. It is currently approved in the USA as an immunosuppressive drug to prolong organ and patient survival in kidney, liver, heart and bone marrow transplants. CsA is available for both oral and intravenous administration (Sandimmune). A microemulsion formulation of CsA, called Neoral, exhibiting more reproducible absorption characteristics is also available for oral administration *(30)*. In addition, several generic microemulsion formulations are now available and are often referred to as CsA modified *(31,32)*.

3.1.1. Pharmacokinetics

Oral absorption of Sandimmune is low (5–30%) and highly variable, ranging from 4 to 89% in renal and liver transplant patients *(33,34)*. Absorption of the microemulsion formulation is more consistent, averaging approximately 40% *(35)*. Peak blood concentrations typically occur between 1–3 and 2–6 h following oral administration of Neoral and Sandimmune, respectively *(33,36,37)*. Absorption can be delayed for several hours in a subgroup of patients. Because CsA is lipophilic, it crosses most biologic membranes and has a wide tissue distribution *(38)*. CsA is highly bound to plasma proteins (>90% to lipoproteins), with the majority of CsA localizing in erythrocytes. The distribution of CsA between plasma and erythrocytes is temperature-dependent and varies with changes in hematocrit *(39)*. Because of the potential for artifactural redistribution of CsA during specimen processing because of ambient temperature fluctuations, ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood should be used to measure CsA concentrations *(40–42)*.

CsA is extensively metabolized by cytochrome P450 enzymes (CYP3A isoenzymes) located in the small intestine and liver *(43)*. There is also a cellular transporter of immunosuppressive drugs, called P-glycoprotein, that influences metabolism by regulating CsA bioavailability. P-glycoprotein pumps some of the CsA out of enterocytes back into the lumen of the gut *(44,45)*. This efflux pump probably contributes to the poor absorption rates observed after oral administration of CsA. CYP3A isoenzymes and P-glycoprotein genetic polymorphisms can also influence the oral bioavailability of CsA and are probably involved in the delayed absorption that has been noted in a subset of patients *(44)*. CsA is oxidized or N-demethylated to more than 30 metabolites *(46,47)*. Most of the metabolites do not possess immunosuppressive activity and are not clinically significant *(48)*. However, there is growing evidence to indicate that a few of the inactive metabolites may contribute to CsA toxicity *(48)*. Two of the hydroxylated metabolites, AM1 and AM9, exhibit 10–20% of the immunosuppressive activity of the parent compound *(49,50)* and can account for as much as 33% of the whole blood CsA concentration *(51)*. The major route of CsA elimination is biliary excretion into the feces. As expected, dosage adjustments are necessary in patients with

hepatic dysfunction. Only a small fraction (6%) of CsA and metabolites appear in the urine *(36)*, making dosage adjustments unnecessary in patients with renal insufficiency.

3.1.2. Adverse Effects

Serious side effects related to CsA treatment are concentration-dependent and include nephrotoxicity, neurotoxicity, hepatotoxicity, hirsutism, hypertrichosis, gingival hypertrophy, glucose intolerance, hypertension, hyperlipidemia, hypomagnesemia, hyperuricemia, and hypokalemia. In general, over-suppression leads to an increased risk for viral infections and lymphoproliferative disease, especially in children *(52)*.

3.1.3. Drug Interactions

Numerous drugs influence the absorption and metabolism of CsA. Any drug that inhibits the cytochrome P-450 system or the P-glycoprotein efflux pump increases blood CsA concentrations because of increased absorption and decreased metabolism. Drugs having the opposite effect (P-450 and/or P-glycoprotein inducers) produce decreased CsA concentrations. Drugs causing increased CsA blood concentrations include calcium channel blockers, several antifungal agents, and the antibiotic erythromycin. Several anticonvulsants and antibiotics, including antituberculosis agents, reduce blood CsA concentrations. In addition, there are many other drugs that synergize with CsA and potentiate nephrotoxicity. There are several excellent reviews that discuss specific drug interactions with CsA *(53,54)*. Not all of the interactions are caused by pharmaceuticals as various foods and herbal remedies can influence CsA concentrations. For instance, grapefruit juice increases CsA blood concentrations by increasing absorption whereas St John's wort decreases CsA concentrations by increasing metabolism *(55)*.

3.1.4. Preanalytic Variables

Whole blood anticoagulated with EDTA is the recommended sample type based on numerous consensus documents *(40–42)*. CsA in EDTA whole blood is stable at least 11 days at room temperature or higher temperatures (37°C) *(56)*. For long-term storage, whole blood samples should be placed at −20°C and are stable for at least 3 years *(57)*. As previously mentioned, CsA should only be measured in whole blood samples. Plasma is considered generally not acceptable because partitioning of CsA between plasma and erythrocytes is a temperature- and time-dependent process that can be altered during in vitro specimen processing *(41)*. In addition, plasma CsA concentrations are twofold lower than whole blood concentrations and results in poor analytical precision at low plasma CsA concentrations.

The timing of specimen collection has always been right before administration of the next dose (i.e., trough levels) *(40,41)*. For standardization purposes, the timing should be within 1 h before the next dose *(42)*. However, the introduction of Neoral in 1995, a microemulsion CsA formulation with more predictable absorption kinetics, has resulted in higher peak concentrations and increased drug exposure, based on area under the concentration time curves *(58)*. The highest and most variable CsA concentrations typically occur within the first 4 h after Neoral dosing *(59)*. However, similar trough concentrations are observed for both the conventional and the microemulsion CsA formulations, demonstrating that trough concentrations are not predictive of total

drug exposure *(60)–(62)*. Increased exposure to CsA using Neoral results in decreased rejection rates with slightly higher serum creatinine concentrations compared with conventional CsA therapy *(58,63,64)*. Thus, a better predictor of immunosuppressive efficacy was needed when administering Neoral. Pharmacokinetic and pharmacodynamic studies demonstrated that maximal inhibition of calcineurin and IL-2 production was correlated with the highest CsA concentrations 1–2 h after dosing *(59,65)*, indicating that drug levels shortly after dosing may be a better predictor of total drug exposure and clinical outcome *(66)*. Because multiple time points after dosing are not practical in a clinical setting, different time points were examined and CsA concentrations 2 h after dosing (called C2 monitoring) was shown to correlate best with total drug exposure and result in better clinical outcomes *(67–70)*. These findings have resulted in C2 monitoring of CsA becoming standard practice at many transplant centers. Unfortunately, this creates various nursing/ phlebotomy challenges because blood samples have to be drawn very close to the 2-h time point after dosing, ideally 10 min on either side of the 2-h mark *(71)*. At the author's institution, C2 testing is performed on 16% of all whole blood samples (annual volume ~14,000) received in the laboratory for CsA testing. To avoid confusion and prevent testing delays because of the need for sample dilution of C2 specimens, our laboratory has created a separate test for C2 monitoring and reports all CsA C2 results in μg/mL to avoid mis-interpreting C2 results as tough levels. We still report CsA trough results in ng/mL.

3.1.5. Methods of Analysis

Monitoring of CsA is critical for optimizing immunosuppression and organ survival while minimizing unwanted toxic side effects. Improvements in immunosuppressive regimens, along with demands for narrower and tighter control of CsA blood levels, have placed greater demand on clinical laboratories to provide timely and reliable drug concentrations. There are many methods currently available to measure CsA. Factors that need to be considered when selecting a CsA assay include metabolite cross-reactivity, cost of instrumentation and reagents, ease of operation, level of technical expertise required to perform testing, test volume, expected turnaround times, the current method being used when switching methods, and the history/preferences of the transplant physicians. For example, turnaround times can be a critical issue in an outpatient setting when it is desirable to have CsA test results available when patients are being seen by their physicians. Depending on the institution, this may require 2–4 h turnaround times for anywhere from 10 to 50 specimens that have been drawn a few hours before the scheduled clinic visit.

CsA can be measured by radioimmunoassay (RIA), semi-automated and automated non-isotopic immunoassays, and high-performance liquid chromatography (HPLC) with UV (HPLC-UV) or mass spectrometry detection systems (HPLC-MS). There are four companies manufacturing six different CsA assays currently being used in the USA. Assays for CsA and the percentage of laboratories using each method based on the College of American Pathologists Immunosuppressive Drugs Monitoring 1st Survey of 2006 are summarized in Table 3. The Cyclo-Trac SP RIA by Diasorin (Still water, MN, USA) is the least popular and is used by only 1% of all laboratories, most likely because of the manual format and need to handle radioisotopes. Interestingly, the Abbott monoclonal fluorescence polarization immunoassay (FPIA) (Abbott Park, IL, USA) is used by >70% of all laboratories. This is somewhat surprising because the

Table 3
Currently Used Methods to Measure Cyclosporine (CsA)

Method	*Assay*	*Manufacturer*	*Laboratories Using Assay (%)*[a]
Radioimmunoassay	Cyclo-Trac SP	DiaSorin	1
Immunoassay			
Semi-automated	Polyclonal FPIA	Abbott	2
	Monoclonal FPIA	Abbott	71
	CEDIA PLUS	Microgenics	8
	Syva EMIT 2000	Dade-Behring	5
Automated	Dimension ACMIA	Dade-Behring	5
HPLC-UV			2
HPLC-MS			6

FPIA, fluorescence polarization immunoassay; CEDIA, cloned enzyme donor immunoassay; EMIT, enzyme-multiplied immunoassay technique; ACMIA, antibody-conjugated magnetic immunoassay; HPLC-UV, high-performance liquid chromatography with ultraviolet detection; HPLC-MS, high-performance liquid chromatography with mass spectrometry detection.

[a] Percentages are based on the College of American Pathologists Immunosuppressive Drug Monitoring 1st survey of 2006.

Abbott monoclonal FPIA has considerable cross-reactivity with CsA metabolites, and recommendations by numerous consensus panels specify that the analytical method should be specific for parent compound *(40–42)*. HPLC methods to measure CsA are specific for parent compound and, because of this, are considered the "gold standard" for CsA quantitation. Yet, HPLC methods are used by only 8% of all laboratories and are primarily restricted to larger transplant centers. The lack of widespread acceptance of HPLC methods to measure CsA may reflect high initial equipment costs for MS detection systems and the need for specialized training for test performance. HPLC systems with UV detection are considerably less expensive and easier to operate but can suffer from a wide variety of chemical interferences depending on the specific protocol utilized. There are several excellent protocols to measure CsA using HPLC-MS and HPLC-MS/MS systems *(72,73)*. Because sample requirements are the same for analysis of many of the immunosuppressants (CsA, tacrolimus, sirolimus, everolimus), simultaneous measurement of two or more immunosuppressive drugs in a single specimen can be performed using HPLC-MS *(74)*. As therapeutic drug monitoring applications continue to emerge, the use of HPLC-MS will continue to increase and may become commonplace equipment in clinical laboratories in the not too distant future.

All the immunoassays, with the exception of the Dimension antibody conjugated magnetic immunoassay (ACMIA) (Dade Behring, Dearfield, IL, USA), are semi-automated because they require a whole blood pretreatment step. This typically involves preparing a whole blood hemolysate by adding an extraction reagent such as methanol to an aliquot of whole blood. The hemolysate is then centrifuged and the separated supernatant is analyzed by the FPIA or Syva enzyme-multiplied immunoassay (EMIT) (Dade Behring). The cloned enzyme donor immunoassay (CEDIA) PLUS (Microgenics Comp., Fremont, CA, USA) pretreatment step is simpler because a centrifugation step is not required after addition of the extraction reagent. Bayer (Bayer Health care, Tarrytown, NY, USA) has also developed a CsA assay with a simplified pretreatment

Table 4
Instrument Applications for Cyclosporine (CsA) Immunoassays

Immunoassay	*Instrument Application*	*Manufacturer*
Monoclonal FPIA	TDx, AxSYM	Abbott Laboratories
CEDIA PLUS	MGC240	Microgenics Corp.
	SYNCHRON LX, UniCel Dx	Beckman Coulter
	Hitachi 902, 911, 912, 917, Modular P	Roche Diagnostics
	AU 400, 640, 2700, 5400	Olympus America
	Aeroset	Abbott Laboratories
Syva EMIT 2000	COBAS Mira[a], INTEGRA 400, 800	Roche Diagnostics
	Dimension RxL Max, Xpand, Xpand Plus, V-Twin, Viva, Viva-E	Dade-Behring
Dimension ACMIA	Dimension RxL Max, Xpand, Xpand Plus, V-twin,Viva, Viva-E	Dade-Behring

FPIA, fluorescence polarization immunoassay; CEDIA, cloned enzyme donor immunoassay; EMIT, enzyme-multiplied immunoassay technique; ACMIA, antibody-conjugated magnetic immunoassay.
[a] This instrument is no longer manufactured or supported by the company.

step that is pending FDA approval for use on the ADVIA Centaur *(75)*. The Dimension ACMIA does not require a pretreatment step allowing whole blood samples to be placed directly on the instrument. Instruments that currently have applications for the various CsA immunoassays are provided in Table 4.

3.1.6. Metabolite Cross-Reactivity

The Abbott polyclonal antibody-based FPIA is non-specific and has extensive cross-reactivity with CsA metabolites. The use of this assay has been declining over the years, and only about 2% of all laboratories currently use this assay (Table 3). CsA results using the Abbott polyclonal FPIA are approximately four times higher than those obtained using HPLC methods *(76)*. Because of the magnitude of metabolite

Table 5
Cyclosporine (CsA) Metabolite Cross-Reactivity of Immunoassays

	Percentage CsA Metabolite Cross-Reactivity[a]			
Immunoassay	*AM1*	*AM4n*	*AM9*	*AM19*
Monoclonal FPIA	6–12	≤ 6	14–27	≤ 4
CEDIA PLUS	8	30	18	2
Syva EMIT 2000	≤ 5	8–13	≤ 4	0
Dimension ACMIA	0	4	0	0

FPIA, fluorescence polarization immunoassay; CEDIA, cloned enzyme donor immunoassay; EMIT, enzyme-multiplied immunoassay technique; ACMIA, antibody-conjugated magnetic immunoassay.
[a] Each metabolite was evaluated at 1000 μg/L except AMI, which was tested at 500 μg/L in the CEDIA PLUS assay. Data are derived from references *77–81*.

cross-reactivity and the poor correlation with clinical outcomes and toxicity, the use of this polyclonal assay should be discouraged. Cross-reactivity of the monoclonal immunoassays with CsA metabolites is summarized in Table 5. The Dimension ACMIA has the least overall metabolite cross-reactivity whereas the monoclonal CEDIA PLUS is reported to have the highest overall metabolite cross-reactivity. CsA metabolites, AM1 and AM9, are typically present in the highest concentrations after transplantation *(51)* and cross-reacts the least in the Dimension ACMIA and Syva EMIT, and the most in the monoclonal FPIA (Table 5). The magnitude of metabolite cross-reactivity contributes to the degree of CsA overestimation when comparing immunoassays with HPLC. Mean CsA concentrations have been found to be approximately 12, 13, 17, 22, and 40% higher than HPLC when measured by the Dimension ACMIA, Syva EMIT, CEDIA PLUS, FPIA on the TDx, and FPIA on the AxSYM, respectively *(77–81)*. Thus, it is important to consider metabolite cross-reactivity and the degree of CsA overestimation when selecting the "right" CsA immunoassay to support a solid organ transplant program.

3.1.7. Analytical Considerations

Consensus conference recommendations for CsA immunoassays are that the slope of the line should be 1.0 ± 0.1, with a *y*-intercept and $S_{y/x} \leq 15\,\mu g/L$, when compared with HPLC *(41)*. None of the current immunoassays satisfy all these requirements *(76–81)*. For instance, the Dimension ACMIA satisfies the slope and intercept requirements but exceeds the $S_{y/x}$ limit, whereas the CEDIA PLUS and Syva EMIT satisfies only one requirement. The FPIA fails to satisfy any of the requirements. Between-day precision recommendations require a coefficient of variation (CV) of $\leq$10% at a CsA concentration of 50 μg/L and a CV of $\leq$5% at 300 μg/L *(41,42)*. Most of the immunoassays satisfy the precision recommendation at 300 μg/L, but it is important that each laboratory determine between-day precision studies at CsA concentrations around 50 μg/L. This is particularly important because recent immunosuppressive drug regimens are designed to reduce CsA trough concentrations to minimize toxicity. Another potential problem is bias because of incorrect assay calibration. Results from the 2003 International Proficiency Testing Scheme have shown that the FPIA using the TDx and CEDIA PLUS overestimates CsA concentrations by 5–10%, whereas the Syva EMIT and Dimension ACMIA slightly underestimate target CsA concentrations by $\leq$5% *(82)*. Lastly, for assays involving a manual extraction step, poor technique can significantly contribute to the overall imprecision of the assay. Careful attention to detail and good technique can minimize variations at this important preanalytical step. This holds true for all whole blood immunosuppressive drug assays requiring a manual extraction step (tacrolimus, sirolimus, and everolimus).

3.1.8. C2 Monitoring and Specimen Dilution

Therapeutic ranges for CsA are often organ-specific and can vary widely between transplant centers. They also differ based on various immunosuppressive drug combinations, the time after transplant, and during periods of toxicity and organ rejection. Trough whole blood CsA levels following kidney transplants are typically between 150–250 μg/L shortly after transplant and are tapered down to $<$150 μg/L during maintenance therapy. Recommended levels after liver and heart transplants are

250–350 μg/L shortly after transplant and <150 μg/L during maintenance therapy. These target ranges were determined using HPLC and will vary considerably when measured using immunoassay, depending on the amount of metabolite cross-reactivity.

For C2 monitoring, target concentrations vary between 600 and 1700 μg/L depending on the type of graft and the time after transplantation *(66)*. C2 concentrations often exceed the analytical range of most immunoassays because typical calibration curves are designed to measure trough CsA levels. The FPIA and Syva EMIT have analytical ranges up to 1500 and 500 μg/L, respectively. The CEDIA PLUS and Dimension ACMIA have separate calibration curves for C2 monitoring, with an analytical range from 450 to 2000 and 350 to 2000 μg/L, respectively. However, 28% of laboratories using the CEDIA PLUS reported using only the low-range calibration curve and would have to dilute samples above 450 μg/L *(83)*. Sample dilution can lead to major inaccuracies in test results, and dilution protocols need to be carefully validated before implementation *(83,84)*. This is because CsA metabolites may not dilute in a linear fashion, and there may be differences in the amount of time needed for diluted samples to re-equilibrate, depending on the immunoassay and dilution protocol. Proficiency testing programs have demonstrated that laboratories produce widely varying results when challenged with samples with CsA concentrations outside the analytical range of immunoassays. For instance, at a CsA parent concentration of 2000 μg/L, 125 laboratories participating in the survey reported CsA values ranging from 1082 to 3862 μg/L *(84)*. These findings indicate that laboratories need to develop carefully controlled validated dilution protocols. A validated dilution protocol for the monoclonal FPIA on the TDx has recently been described *(85)*.

Another concern with C2 monitoring is metabolite concentrations and the need for therapeutic ranges that are assay-specific. This clearly is necessary when measuring trough CsA concentrations. A recent study monitoring C2 concentrations in kidney and liver transplant patients found equivalent CsA results when measured using the FPIA, CEDIA PLUS, and Syva EMIT *(86)*. As expected, paired trough samples produced CsA concentrations that differed among the immunoassays. These data indicate that for C2 monitoring, assay-specific therapeutic ranges may not be necessary.

3.2. Tacrolimus

Tacrolimus (also known as FK-506) is a macrolide antibiotic with a molecular weight of 822 (Fig. 1) that was originally isolated from the fungus *Streptomyces tsukubaensis (5)*. In the USA, tacrolimus (brand name Prograf) was approved for use in liver transplantation in 1994 and in kidney transplantation in 1997. It is approximately 100 times more potent than CsA and is associated with a decrease in acute and chronic rejection, and better long-term graft survival *(87)*. In 2004, more than two-thirds of all kidney and liver transplant recipients, and approximately one-half of all heart transplant recipients, were receiving tacrolimus before hospital discharge *(20)*. At the author's institution, approximately 3.5 times more tacrolimus tests are performed compared with CsA.

3.2.1. Pharmacokinetics

Tacrolimus is available for both oral and intravenous administration. Similar to CsA, oral absorption of tacrolimus from the gut is poor and highly variable, averaging

25% *(88)*. Peak blood concentrations occur within 1.5–4 h. Tacrolimus is primarily bound to albumin, α_1-acid glycoprotein, and lipoproteins in the plasma. However, the majority of tacrolimus is found within erythrocytes *(89)*.

Tacrolimus is metabolized using cytochrome P450 isoenzymes (CYP3A) located in the small intestine and liver. Similar to CsA, the bioavailability of tacrolimus is influenced by CYP3A and the multidrug efflux pump (P-glycoprotein) located in intestinal enterocytes. Biotransformation of tacrolimus occurs by demethylation, hydroxylation, and oxidative reactions *(90)*. At least nine metabolites have been identified based on in vitro studies *(91)*, and all, with the exception of 31-*o*-demethyl tacrolimus (M-II), have very little immunosuppressive activity. M-II has been shown in vitro to have the same immunosuppressive activity as parent compound *(92)*. Metabolites represent 10–20% of whole blood tacrolimus concentrations *(93)*. Tacrolimus is eliminated primarily by biliary excretion into the feces. Patients with hepatic dysfunction require dosage adjustments. Very little tacrolimus is found in urine, and blood concentrations are not altered in renal dysfunction.

3.2.2. Adverse Effects

Tacrolimus shares many dose-dependent side effects with CsA *(94)*. These include nephrotoxicity, neurotoxicity, hepatotoxicity, hypertension, and glucose intolerance. Nephrotoxicity with tacrolimus may be less of a problem than with CsA, especially in renal transplantation *(95)*. Diabetogenesis is approximately three times more common with tacrolimus than with CsA *(96)*. Hyperkalemia, hyperuricemia, hyperlipidemia, hirsutism, and gingival hypertrophy are also observed following tacrolimus use, but less commonly than with CsA *(97)*. Alopecia is also associated with tacrolimus use *(94)*.

3.2.3. Drug Interactions

Because tacrolimus is metabolized mainly by the cytochrome P450 system, the majority of drug interactions described for CsA also apply to tacrolimus *(88)*. St John's wort also decreases blood tacrolimus concentrations.

3.2.4. Preanalytic Variables

For quantitation of tacrolimus, EDTA-anticoagulated whole blood is the specimen of choice for the same reasons provided for CsA. Whole blood samples are stable for 1 week when shipped by mail without coolant *(98,99)*, 1–2 weeks at room temperature *(99,100)*, 2 weeks at refrigerator temperatures *(100)*, and almost 1 year at −70°C *(100)*.

Trough blood tacrolimus concentrations are almost exclusively used for routine monitoring and are believed to be a good indicator of total drug exposure *(101)*. However, recent experience with CsA has challenged this notion, and alternative draw times 1–6 h after dosing have been proposed *(102)*. Whereas some investigators have found a poor correlation between trough tacrolimus concentrations and total drug exposure, others have found good correlation *(103,104)*. Overall, the findings suggest that trough tacrolimus concentrations are predictive of total drug exposure and that measuring tacrolimus at specified times after dosing may not result in dramatic improvements. Until this issue is fully resolved, trough levels will continue to be used for reasons of convenience and reproducibility.

3.2.5. Methods of Analysis

Monitoring of tacrolimus is an integral part of any organ transplant program because of variable dose-to-blood concentrations and the narrow therapeutic index. Tacrolimus can be measured using enzyme-linked immunosorbent assay (ELISA), semi-automated and automated immunoassay, and HPLC-MS (Table 6). The ELISA and semi-automated immunoassays require a manual whole blood pre-treatment step. The Dimension ACMIA does not require a pretreatment step allowing whole blood samples to be directly placed on the instrument. Sample extraction can be semi-automated using modern HPLC-MS systems *(105)*.

The ELISA takes about 4 h to complete, requires numerous manual steps, and is used by few clinical laboratories. The Abbott microparticle enzyme immunoassay (MEIA) II on the IMx instrument is currently used by 88% of the laboratories in the USA that participate in the College of American Pathologists immunosuppressive proficiency testing program (Table 6). The MEIA II has a reported detection limit of 2 μg/L and replaced an earlier version (MEIA I) with a detection limit of 5 μg/L. The tacrolimus Syva EMIT has applications for Dade Behring instrumentation, the COBAS Integra 400 *(106)*, the Beckman Synchron LX20 PRO *(107)*, and the Bayer ADVIA 1650 *(108)*. However, the Syva EMIT is currently available only outside the USA. Microgenics has just released a CEDIA for tacrolimus in the USA that has applications for several Hitachi, Olympus, and Beckman instruments. Dade-Behring has just launched (July 2006) an ACMIA to measure tacrolimus using the Dimension family of analyzers and the V-Twin and Viva-E drug-testing analyzers. It uses the same monoclonal antibody used in the Syva EMIT to measure tacrolimus. Lastly, Abbott is developing a chemiluminescent immunoassay for use on their ARCHITECH system *(109)*.

Table 6
Analytical Methods to Measure Tacrolimus

Method	*Assay*	*Manufacturer*	*Laboratories Using Method (%)*[a]
ELISA	Pro-Trac II	DiaSorin	≤ 3
Immunoassay			
Semi-Automated	MEIA II	Abbott	88
	Syva EMIT	Dade-Behring	–[b]
	CEDIA	Microgenics	≤ 3
Automated	Dimension ACMIA	Dade-Behring	–[c]
HPLC-MS			9

ELISA, enzyme-linked immunosorbent assay; MEIA, microparticle enzyme immunoassay; EMIT, enzyme-multiplied immunoassay technique; CEDIA, cloned enzyme donor immunoassay; ACMIA, antibody-conjugated magnetic immunoassay; HPLC-MS, high-performance liquid chromatography with mass spectrometry detection.

[a] Percentages are based on the College of American Pathologists Immunosuppressive Drugs Monitoring Survey of 2006.

[b] Currently available only outside the USA.

[c] This assay received Food and Drug Administration (FDA) clearance and was launched in July 2006.

HPLC-MS methods are used by most of the laboratories not using the MEIA II. Tacrolimus cannot be measured by HPLC-UV because the molecule does not possess a chromophore. It is noteworthy that HPLC-MS is the only method that is specific for parent drug and meets the recommendations set forth in Consensus documents *(42)*. There are numerous recently reported assays to quantitate tacrolimus by using HPLC-MS or HPLC-MS/MS with detection limits <0.5 ng/mL *(105,110)*. A major advantage of HPLC-MS over immunoassays is the ability to simultaneously measure other immunosuppressant drugs in the same whole blood sample, such as CsA, sirolimus, and everolimus *(111)*.

3.2.6. Metabolite Cross-Reactivity

All the immunoassays have significant cross-reactivities with tacrolimus metabolites. The ELISA, MEIA II, and EMIT cross-react with M-II (31-*o*-demethyl), M-III (15-*o*-demethyl) and M-V (15,13-di-*o*-demethyl) metabolites of tacrolimus *(112)*. The CEDIA has significant cross-reactivity with M-I (13-*o*-demethyl) but does not cross-react with M-II or M-III. Cross-reactivity of the CEDIA with M-V has not been examined *(113)*. The ACMIA is expected to have metabolite cross-reactivity similar to the EMIT because both assays use the same monoclonal antibody. The extent of positive bias because of metabolite cross-reactivity is dependent on the transplant group studied. Metabolite cross-reactivity in patients with good liver function is typically not a problem because metabolite concentrations are relatively low compared with parent drug *(114)*. However, metabolites tend to accumulate during reduced liver function and immediately after liver transplant, resulting in significant assay interference and falsely high blood tacrolimus concentrations *(115)*. Overall, the MEIA II produces tacrolimus results that are 15–20% higher, the EMIT produces results 17% higher, and the CEDIA produces results 19% higher than those obtained by HPLC-MS, in kidney and liver transplant patients *(107,112,113,116,117)*. Calibration error may also contribute to some of the overall positive bias.

3.2.7. Analytical Considerations

The recommended therapeutic range for whole blood tacrolimus concentrations after kidney and liver allograft transplants is 5–20 μg/L when measured using HPLC-MS *(118)*. When tacrolimus is used with other immunosuppressive agents such as sirolimus, the desired target concentration for tacrolimus can be considerably <5 μg/L. In view of this, it is important for each laboratory to determine performance characteristics of their tacrolimus assay at concentrations <5 μg/L and make transplant services aware of the lower limit of detection and the imprecision (%CV) at this concentration. The functional sensitivity (between-day CV <20%) of the MEIA II and CEDIA is reported to be around 2 μg/L *(112,116,119,120)*, whereas the detection limit of the EMIT is around 3 μg/L *(107)*. At our institution, we examined functional sensitivity of the MEIA II tacrolimus assay by measuring whole blood pools at various concentrations in duplicate during a 10-day period. As shown in Fig. 2, a 20% CV was observed at a tacrolimus concentration of approximately 2 μg/L. In addition, we found that the MEIA II produced tacrolimus concentrations ranging from 0.8 to 1.7 μg/L when testing samples from patients not receiving tacrolimus ($n = 8$). Homma et al. *(121)* also found false-positive results when measuring tacrolimus in whole blood samples

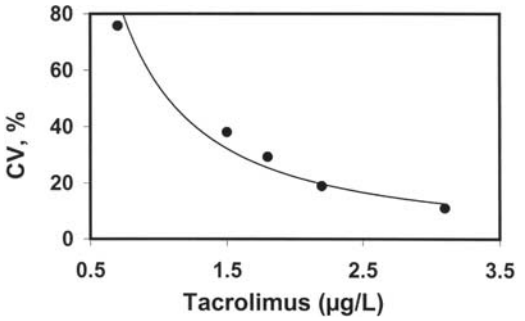

Fig. 2. Functional sensitivity of the Abbott tacrolimus microparticle enzyme immunoassay (MEIA) II on the IMx instrument. Whole blood patient pools at varying tacrolimus concentrations were analyzed in duplicate on 10 separate days. The coefficient of variation (CV) is the standard deviation of the mean tacrolimus concentration divided by the mean. The value is multiplied by 100 and is expressed as a percentage (%).

from patients not receiving tacrolimus using the MEIA. Based on our data, we use a cutoff of 2 µg/L for tacrolimus and report values lower than this cutoff as <2 µg/L.

The MEIA II has been shown to produce falsely elevated tacrolimus concentrations when the hematocrit is <25% *(122,123)*. The EMIT for tacrolimus is not affected by changes in hematocrit values *(123)*. Hematocrit bias in the MEIA II could result in therapeutic tacrolimus blood concentrations in under-immunosuppressed patients because of low hematocrit values. This would potentially be most problematic shortly after transplant when hematocrit values are typically at their lowest concentrations. This tacrolimus bias could also make it difficult to appropriately dose patients with widely fluctuating hematocrit values.

The reliability of the MEIA II at low whole blood tacrolimus concentrations has recently been questioned. At tacrolimus concentrations <9 µg/L, the MEIA II exhibited greater between-day imprecision and a weaker correlation with results obtained by HPLC-MS/MS *(124)*. Recovery experiments also demonstrated that the degree of over-estimation of tacrolimus using the MEIA II was more pronounced at lower drug concentrations *(124)*. Poor precision at low tacrolimus concentrations was also noted in the College of American Pathologists longitudinal immunosuppressive drug study. The study found that the major source of imprecision was within-laboratory variation over time, and it was postulated that the variation might be due to changes in assay standardization or reagent lot-to-lot changes *(125)*. Taken together, these performance variables are important to consider when selecting an assay to monitor whole blood tacrolimus concentrations.

4. MAMMALIAN TARGET OF RAPAMYCIN INHIBITORS

The chemical structures of the mammalian target of rapamycin (mTOR) inhibitors, sirolimus and everolimus, are shown in Fig. 3. Both are macrocyclic lactones. Sirolimus (also known as rapamycin) is a lipophilic molecule (molecular weight of 914) derived from *Streptomyces hygroscopicus*. This actinomycete fermentation product was identified in the early 1970s and was approved by the FDA in 1999 for use with CsA to reduce the incidence of acute rejection in renal transplantation *(126)*. Everolimus is a

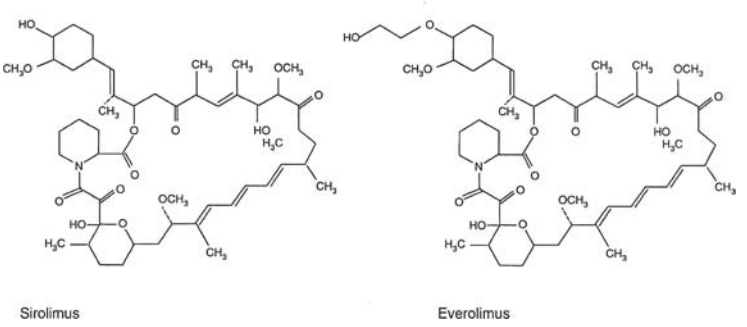

Fig. 3. Chemical structures of the mammalian target of rapamycin (mTOR) inhibitors, sirolimus and everolimus. This figure was published in Critical Reviews in Oncology/Hematology, Volume 56, Taylor AL, Watson CJE, Bradley JA, Immunosuppressive agents in solid organ transplantation: mechanisms of action and therapeutic efficacy, page 34, Copyright Elsevier 2005.

chemically modified version that is more hydrophilic than sirolimus and has improved pharmacokinetic characteristics and improved bioavailability *(127)*. Everolimus is still in phase III trials and is only available for investigational use in the USA.

Sirolimus and everolimus readily cross the lymphocyte plasma membrane and bind to the intracellular immunophilin, FK506-binding protein-12 *(128)*. In contrast to tacrolimus, sirolimus–immunophilin and everolimus–immunophilin complexes do not inhibit calcineurin activity. Instead, the complexes are highly specific inhibitors of the mTOR, a cell cycle serine/threonine kinase involved in the protein kinase B-signaling pathway. This results in suppressed cytokine-induced T-lymphocyte proliferation, with a block in progression from the G1 to S phase of the cell cycle *(129)*. The mTOR inhibitors work synergistically with the calcineurin inhibitors to produce a profound immunosuppressive effect on T lymphocytes.

4.1. Sirolimus

4.1.1. Pharmacokinetics

Sirolimus is available for both oral and intravenous administration. Its long half-life of approximately 60 h allows once-a-day dosing *(130)*. Sirolimus is rapidly absorbed from the gastrointestinal tract, and peak blood concentrations occur 2 h after an oral dose *(131)*. Oral bioavailability is low, ranging from 5 to 15% *(132)* and is considerably reduced (approximately fivefold) when administered within 4 h or concomitantly with CsA *(133)*. There is considerable interpatient variability in total drug exposure that can vary by as much as 50% *(133)*. Sirolimus is primarily found within erythrocytes (95%), with approximately 3 and 1% partitioning into the plasma and lymphocytes/granulocytes, respectively *(134)*. Almost all of the plasma sirolimus is bound to proteins, with lipoproteins being the major binding protein.

Similar to the calcineurin inhibitors, sirolimus is metabolized in the intestine and liver by cytochrome P450 enzymes (CYP3A) *(135)*. The multidrug efflux pump P-glycoprotein in the gastrointestinal tract also controls metabolism by regulating bioavailability. Sirolimus is hydroxylated and demethylated to more than seven metabolites with the hydroxyl forms being the most abundant *(136)*. Metabolites represent

approximately 55% of whole blood sirolimus levels *(136)*. The pharmacological activity of metabolites has not been fully investigated because of difficulties associated with their isolation. However, preliminary studies indicate that the immunosuppressive activity of metabolites is <30% of that observed for the parent compound *(137)*. Sirolimus is eliminated primarily by biliary and fecal pathways, with small quantities appearing in urine *(135)*. As with the calcineurin inhibitors, dosage adjustments are needed in patients with hepatic dysfunction.

4.1.2. Adverse Effects

The incidence of adverse effects is dose-related and includes metabolic, hematological, and dermatological effects *(138)*. Metabolic side effects include hypercholesterolemia, hyper- and hypokalemia, hypophosphatemia, hyperlipidema, and increased liver function tests. Anemia can be problematic, with decreases in leukocyte, erythrocytes, and platelet counts being the most common. Skin rashes, acne, and mouth ulcers are also observed in patients being switched to mTOR inhibitors. As with other immunosuppressive drugs, there is an increased risk of infection and an association with lymphoma development. Interstitial pneumonitis is also associated with sirolimus therapy *(139)*.

4.1.3. Drug Interactions

CYP3A inhibitors such as antifungal agents (itraconazole, ketoconazole), clarithromycin, erythromycin, and verapamil increase blood levels of sirolimus. CYP3A inducers such as carbamazepine, phenobarbital, phenytoin, and rapamycin may decrease sirolimus blood levels. Grapefruit juice can increase sirolimus by decreasing drug clearance. St John's wort can decrease sirolimus levels. As previously noted, the concomitant use of CsA can result in increased sirolimus concentrations *(140)*. Although tacrolimus and sirolimus compete for sites on the same binding protein, the two drugs do not appear to have significant drug–drug interactions in clinical practice *(104)*.

4.1.4. Preanalytic Variables

EDTA-anticoagulated whole blood is the recommended specimen matrix *(132)*. This is because almost all of the sirolimus (~95%) is concentrated in erythrocytes, and plasma levels are too low for most analytical methods *(134)*. Whole blood samples are stable for 10 days at ambient temperature *(141)*, at least 1 week at 30–34°C *(141, 142)*, 30 days at 4°C *(143)*, and at least 2 months at −40°C *(143)*. Whole blood samples can withstand three freeze-thaw cycles without altering measured sirolimus concentrations *(141,142)*.

In contrast to the calcineurin inhibitors, there is good correlation between predose sirolimus concentrations and total drug exposure based on area under the curve measurements *(104,144)*. This also holds true when sirolimus is used in combination with CsA or tacrolimus *(104,144)*. Thus, whole blood 24-h trough specimens are recommended when monitoring sirolimus *(132)*.

4.1.5. Methods of Analysis

Therapeutic monitoring of sirolimus is critical because the administered dose is a poor predictor of total drug exposure because of individual patient variables. Because of the long drug half-life, daily monitoring of sirolimus is typically not necessary. Weekly

monitoring of levels may be needed shortly after transplantation followed by monthly monitoring. Target concentrations for sirolimus range between 4 and 12 μg/L when used in combination with a calcineurin inhibitor *(145)*. Similar to tacrolimus, these relatively low whole blood concentrations can be a challenge analytically for some of the currently available methods of analysis. As combination immunosuppressant therapies continue to evolve, target concentrations for sirolimus may become lower, further challenging the analytical performance of some of the currently utilized assays.

Sirolimus can be measured by immunoassay and HPLC with UV or MS detection. According to the College of American Pathologist proficiency testing program (1st survey of 2006), more than 130 laboratories in the USA currently perform sirolimus testing. Approximately 60% of the laboratories measure whole blood sirolimus by the Abbott IMx MEIA that became commercially available in 2004. The original Abbott MEIA kit was only used experimentally to support early clinical studies (investigational use only) and was never available commercially for routine monitoring of sirolimus. The "investigational use only" Abbott immunoassay was discontinued in 2001. A CEDIA for sirolimus (Microgenics) has recently become commercially available for use on several Roche automated analyzers (Hitachi 911, 912, 917, and modular P). The Microgenics sirolimus immunoassay is currently not used by many laboratories in the USA. The majority of laboratories not using the Abbott MEIA (approximately 34%) measure sirolimus by HPLC-MS. The major advantage of HPLC-MS is increased sensitivity and specificity, despite the need for highly skilled personnel. A few laboratories measure sirolimus by HPLC-UV, although this method requires elaborate sample cleanup procedures and long chromatographic run times *(146–148)*. This results in higher labor costs, making HPLC-UV methods unsuitable for laboratories supporting large transplant programs.

4.1.6. Metabolite Cross-Reactivity

Both of the currently available immunoassays have significant cross-reactivity with sirolimus metabolites. The MEIA method has 58 and 63% cross-reactivity with 41-*o*-demethyl-sirolimus and 7-*o*-demethyl-sirolimus, respectively *(149)*. The CEDIA has 44% cross-reactivity with 11-hydyroxy-sirolimus and 73% cross-reactivity with 41- and 32-*o*-demethyl-sirolimus *(150)*. This degree of metabolite cross-reactivity results in significant bias between assays. The MEIA produces whole-blood sirolimus concentrations that are 9–49% higher than those obtained by HPLC-UV and HPLC-MS, depending on the study and transplant group studied *(149,151–155)*. One study found that the CEDIA method produces whole blood sirolimus levels with a mean positive bias of 20.4% compared with HPLC-MS *(156)*. However, immunoassay metabolite cross-reactivity may be less of an issue from a clinical standpoint because the distribution of metabolites in whole blood are similar among patients and are relatively stable over long periods of time *(157)*.

4.1.7. Analytical Considerations

The therapeutic window for sirolimus appears to be between 5 and 15 μg/L when used in combination with CsA and between 12 and 20 μg/L when used alone *(130)*. Sirolimus levels slightly below the currently used therapeutic range can be a challenge for some of the HPLC-UV methods, with functional sensitivities (based on between-day

CVs of <20%) of 2–3 μg/L *(147,148)*. This is also true for the two currently available immunoassays. The MEIA method has a functional sensitivity that varies among laboratories, with values ranging from 1.3 to 3.0 μg/L *(149,151–155)*. Technical variations at the manual extraction step most likely contribute to the differences in functional sensitivity that were observed among laboratories evaluating the MEIA. One study found that the CEDIA has a functional sensitivity of 3.0 μg/L *(156)*. HPLC-MS methods have excellent sensitivity, with functional sensitivities <1 μg/L *(158,159)*. As previously mentioned, a further advantage of HPLC-MS methods is the ability to measure multiple immunosuppressants in the same whole blood sample. It is important that laboratories experimentally determine their own lower limit of detection based on long-term between day imprecision data (using whole blood samples) and not rely on package insert information or published data.

The sirolimus MEIA is prone to error that is dependent on hematocrit levels. There is an inverse relationship between hematocrit and measured sirolimus levels. At a sirolimus concentration of 5 μg/L, results can be 20% higher for hematocrits of <35% and as much as 20% lower for hematocrits >45% *(149,160)*. When the hematocrit is between 35 and 45%, MEIA bias is <10% at sirolimus concentrations ranging from 5 to 22 μg/L. Incomplete extraction of sirolimus from erythrocyte-binding proteins is the most probable mechanism leading to the hematocrit interference. The CEDIA does not appear to be affected by variations in hematocrit between 20 and 60% *(150)*; however, there are no independently published studies supporting the manufacturer's claim.

4.2. Everolimus

Everolimus (also known as SZD RAD) is a structural analogue of sirolimus with an additional hydroxyethyl group (Fig. 3). Everolimus is currently in phase III clinical trials in the USA and has not received FDA approval for use as an immunosuppressive agent. Because everolimus is still in the experimental stage it will only be briefly discussed.

4.2.1. Pharmacokinetics

Everolimus has improved bioavailability *(161,162)* and a shorter elimination half-life (~24 h) than sirolimus *(163)*. Everolimus also has lower intrapatient drug variability than sirolimus *(144,164)*. Concomitant use of CsA results in increased everolimus blood concentrations due to inhibition of everolimus metabolism *(165)*. Similar to sirolimus, everolimus is metabolized in the intestine and liver by cytochrome P450 enzymes. At least 20 metabolites have been identified *(166)*, with mono-hydroxyl, di-hydroxyl, demethylated, and an open ring form being the major metabolites *(167)*. Metabolites are in relatively low concentrations when monitoring trough blood concentrations *(167)*.

4.2.2. Methods of Analysis

Immunoassays to measure everolimus are not currently available in the USA and most likely will lag behind FDA approval of the drug. Seradyn has developed an FPIA (Innofluor Certican Assay System) to measure whole blood everolimus outside the USA on Abbott TDx instrumentation *(168)*. The FPIA method has a functional sensitivity of 2 μg/L *(168)*, which is just below the therapeutic trough blood concentration lower limit of 3 μg/L *(169)*. When compared with HPLC-MS, the FPIA has a positive

mean bias of 24.4% in renal transplant recipients *(170)*. The positive bias is due to differences in calibrator-assigned values and antibody cross-reactivity with everolimus metabolites *(170)*. Cross-reactivity with metabolites ranges from 5 to 72% *(168)*. HPLC-UV and HPLC-MS methods are also available to measure everolimus *(171,172)*.

5. MYCOPHENOLIC ACID

MPA is a fermentation product of *Penicillium* species that was originally shown to have antibacterial, antifungal, and immunosuppressive potential in animal studies *(173)*. To improve the bioavailability of MPA, mycophenolate mofetil (brand name CellCept), the 2-morpholinoethyl ester of MPA was developed for oral and intravenous administration *(174)*. Mycophenolate mofetil received FDA approval for use as an immunosuppressant with corticosteroids and CsA to prevent organ rejection in 1995. The sodium salt of MPA, mycophenolate sodium (brand name Myfortic), has recently become available for oral administration as delayed-release tablets. MPA has primarily replaced azathioprine in organ transplantation. The chemical structure of the active compound MPA and the two parent compounds are shown in Fig. 4.

MPA is a potent non-competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH) enzymatic activity *(175)*. IMPDH is the rate-limiting enzyme in the production of guanosine nucleotides that are required for DNA synthesis and

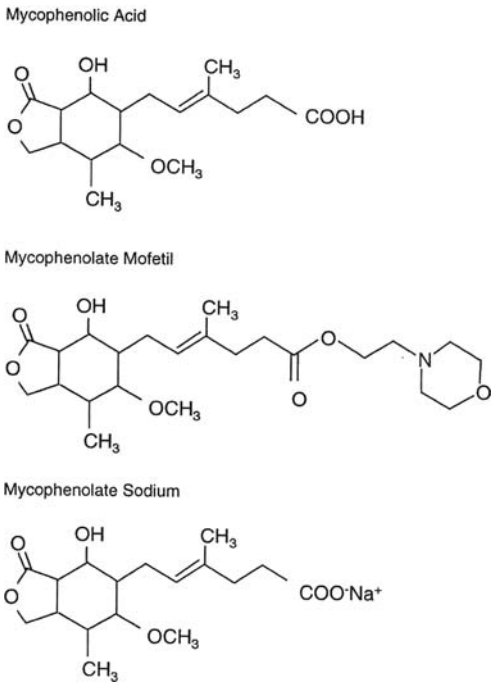

Fig. 4. Chemical structures of the active compound mycophenolic acid (MPA), and the two prodrugs, mycophenolate mofetil and mycophenolate sodium. This figure was published in Critical Reviews in Oncology/Hematology, Volume, Taylor AL, Watson CJE, Bradley JA, Immunosuppressive agents in solid organ transplantation: mechanisms of action and therapeutic efficacy, page 29, Copyright Elsevier 2005.

cellular proliferation. Guanosine nucleotides are synthesized in most cell types using the IMPDH pathway and a separate salvage pathway. However, the salvage pathway is not found in lymphocytes, and MPA blockage of the IMPDH pathway selectively inhibits lymphocyte proliferation *(176,177)*. There are two isoforms of IMPDH and MPA selectively inhibit the type II isoform, which is predominantly expressed by activated and not resting lymphocytes *(178)*.

5.1. Pharmacokinetics

Mycophenolate mofetil and mycophenolate sodium are rapidly and completely absorbed, and quickly de-esterified in the blood and tissues to MPA, the active form of the drug. The half-life of mycophenolate mofetil during intravenous administration is <2 min *(179)*. Following an oral dose of mycophenolate mofetil, MPA reaches a maximum concentration within 1 h *(180)*. Almost all the drug (>99%) can be found in the plasma compartment *(181)*. For this reason, serum or plasma MPA concentrations are used for routine therapeutic drug monitoring of MPA.

MPA has an elimination half-life of 18 h and is glucuronidated in the liver to the primary inactive metabolite, 7-*o*-glucuronide mycophenolic acid (MPAG) *(182)*. Small quantities of the inactive metabolite 7-*o*-glucoside are also produced in the liver *(180,183)*. Another metabolite produced in small quantities is acyl glucuronide, an active metabolite that may contribute to the adverse gastrointestinal effects of MPA *(184)*. MPAG exhibits significant enterohepatic recirculation with a second MPA plasma peak occurring 4–12 h after drug administration. The kidneys primarily clear MPAG with concentrations rapidly accumulating in patients with severe renal impairment (glomerular filtration rates <25 mL/min) *(185)*. MPA is extensively bound in the circulation to albumin with typical concentrations of free or unbound MPA ranging from 1.25 to 2.5% of the total concentration *(181)*. Free MPA concentrations are increased in hypoalbuminemia, hyperbilirubinemia, and uremia *(186)*. It has been shown that the immunosuppressive effects of MPA are related to free MPA and not the total drug concentration *(181)*. In chronic renal failure, the free concentration of MPA can increase dramatically indicating over immunosuppression when the total MPA concentration is within the therapeutic range *(186,187)*.

5.2. Adverse Effects

Adverse effects from mycophenolate mofetil and mycophenolate sodium are similar. The most common dose-limiting unwanted side effects are diarrhea, nausea, vomiting, and abdominal pain *(188)*. Marrow suppression and anemia can also occur *(94)*. An increased risk of cytomegalovirus, candida, and herpes simplex infections has also been reported *(94,189)*.

5.3. Drug Interactions

Coadministration of CsA results in significantly lower trough concentrations of MPA *(190)*, most likely because of diminished enterohepatic recirculation of MPAG and MPA *(191)*. The antibiotics mycostatin, tobramycin, and cefuroxime also decrease MPA bioavailability by a similar mechanism *(192)*. Tacrolimus may increase the bioavailability of MPA by inhibiting MPAG formation *(193)*; however, additional

studies are needed to confirm this potential drug interaction. Steroids such as dexamethasone lower MPA concentrations by augmenting the activity of the enzyme responsible for MPA metabolism. Several non-steroidal inflammatory drugs such as niflumic acid, diflunisal, flufenamic acid, mefenamic acid, and salicylic acid increase MPA concentrations by inhibiting MPA glucuronidation *(194)*. Antacids (aluminum and magnesium hydroxide) lower total MPA exposure by reducing drug absorption in the gastrointestinal tract. Other drugs such as calcium polycarbophil and iron ion preparations also result in decreased MPA concentrations by the same mechanism *(195)*. Lastly, salicylic acid and furosemide increase the free fraction of MPA by altering albumin binding.

5.4. Preanalytic Variables

Plasma or serum can be used to measure MPA and free MPA blood concentrations *(187)*. However, plasma from EDTA-anticoagulated whole blood is the recommended specimen of choice because the same sample can be used to measure whole blood CsA, tacrolimus, and sirolimus *(196)*. MPA and MPAG are stable in whole blood and plasma samples at room temperature for at least 4 h *(197)*. Plasma samples are stable at 4°C for 4 days and at least 11 months when stored at −20°C *(196)*. Free MPA is stable for at least 6 months when stored at −20°C *(198)*. Thawing and refreezing of plasma samples can be performed up to four times without significant loss of MPA *(199)*. When monitoring MPA during intravenous infusion of mycophenolate mofetil, whole blood samples should be immediately placed in ice and the plasma separated within 30 min *(200,201)*. This is because mycophenolate mofetil is very unstable and rapidly undergoes temperature-dependent degradation to MPA in whole blood samples placed at room temperature *(200)*.

Trough concentrations of MPA are routinely used for drug monitoring and are generally believed to be a relatively good indicator of total drug exposure *(202)*. This is somewhat surprising as numerous studies have shown that area under the curve (0–12 h) measurements are more predictive of total drug exposure and acute graft rejection than trough concentrations *(203–205)*. In addition, MPA trough concentrations can vary considerably depending upon time after transplantation *(205)*. Nevertheless, the superiority of area under the curve measurements is probably overshadowed by practical considerations such as additional testing costs and difficulties associated with the collection of multiply timed samples.

5.5. Methods of Analysis

When MPA was originally approved for use (as mycophenolate mofetil), therapeutic drug monitoring was considered unnecessary. However, recent studies have found wide variations in total drug exposure (as high as 10-fold) following a fixed dose, suggesting that individualized dosing may be of considerable benefit *(206,207)*. A roundtable meeting recently recommended therapeutic drug monitoring based on the interpatient variability and the significant drug interactions associated with combination immunosuppressive therapy *(208)*.

At the present time, fewer than 30 laboratories in the USA measure MPA (1st CAP proficiency survey of 2006). Roughly half the laboratories measure MPA using HPLC,

with the majority of remaining laboratories using HPLC-MS methods. Numerous HPLC methods with UV, fluorimetric, and MS detection systems have been described to measure MPA in plasma samples *(198,199,209–211)*. The HPLC methods primarily differ in sample extraction, analytical column, run-time, and lower limit of detection. Free MPA can be measured using HPLC methods after separation of protein-bound MPA by ultrafiltration *(185,212)*. However, free MPA is typically more difficult to measure and does not appear to be superior to total MPA in predicting clinical outcomes in most transplant patients *(213)*.

Automated assays to measure MPA are currently not available in the USA. Several companies are developing product applications for various automated instruments for either serum and/or plasma samples. For instance, Dade-Behring is developing an enhanced turbidimetric inhibition immunoassay to measure MPA for use on Dimension clinical chemistry analyzers *(214)*. Microgenics is developing a CEDIA to measure MPA on Hitachi, Olympus, and Microgenics (MGC 240) clinical chemistry analyzers *(215)*. Lastly, Roche (Roche Diagnostics, Indianapolis, IN, USA) is developing an enzyme receptor assay to measure total MPA and free MPA using the COBAS INTEGRA system (Roche Diagnostics, Indianapolis, IN, USA) *(216)*. At the time of this writing, none of these assays have been submitted to the FDA for review.

Dade-Behring has an EMIT 2000 MPA immunoassay that is widely used outside the USA. The assay can be performed on Dade-Behring Dimension instruments, the Roche COBAS, and Hitachi automated chemistry analyzers. The antibody used in the EMIT assay has cross-reactivity with acyl glucoronide *(217)* and produces MPA values that are approximately 10–30% higher than those obtained using HPLC *(218–221)*. The bias can be considerably higher in patients with impaired renal function because of accumulation of acyl glucoronide *(218,222)*. The positive bias because of acyl glucoronide cross-reactivity may turn out to be advantageous because metabolite has in vitro anti-IMPDH activity *(206,223)*.

5.6. Analytical Considerations

The generally accepted therapeutic range for trough MPA plasma concentrations is 1.0–3.5 mg/L *(196,224,225)*. This range of values can be easily measured by currently available analytical methods with good precision. Concentrations of free MPA are typically 2% of the total MPA level and can be analytically challenging for some of the HPLC-UV methods *(226)*. In these situations, the functional sensitivity of the free MPA assay needs to be carefully validated.

HPLC is the reference method for measuring MPA that other methods are validated against. This is because HPLC is highly specific for parent compound and is free from coadministered drug interferences *(200,209–211)*. As immunoassays to measure MPA become available in the USA, metabolite cross-reactivity and assay bias will have to be taken into account when interpreting MPA concentrations.

6. CONCLUSION

Advances in immunosuppressive therapy are largely responsible for the success and improved outcomes that are now obtained following allogeneic organ transplantation. Today, very few allografts are lost to immune-mediated acute rejection, and there

is remarkable improvement in patient and graft survival. A major goal of immunosuppressive drug therapy is to optimize therapeutic effectiveness while minimizing unwanted adverse effects. Therefore, therapeutic drug monitoring plays a central role because a "one size fits all" approach for immunosuppressive drugs has proved unsuccessful, with optimal drug therapy requiring individualized dosing. Therapeutic monitoring of CsA, tacrolimus, and sirolimus is now considered an integral part of organ transplant programs, and several arguments have been made for monitoring MPA.

Although HPLC is considered the reference method for monitoring immunosuppressive drugs, the majority of laboratories in the USA are currently using immunoassays. Immunoassays are attractive because they can be automated, have low start-up costs, and do not require highly skilled testing personnel. Their major drawback is metabolite cross-reactivity, which results in varying degrees of positive bias that is unique to each immunoassay. Furthermore, cross-reactivity is not always predictable and can vary depending on post-transplant time and type of organ transplanted. The advantage of HPLC is high specificity and the ability to separate metabolites from parent compound. Drawbacks of HPLC include the need for extensive sample cleanup, long analytical run times, and specialized training. This can be partially overcome by using HPLC with MS detection, which requires less sample preparation and has shorter run times than HPLC with UV detection. Unfortunately, HPLC-MS systems are currently very expensive and require highly trained operating personnel. New HPLC-MS systems with automated sample preparation are emerging that are considerably easier to operate. Given the cost of immunoassay reagents, these newer systems are becoming more cost effective, especially when one considers that HPLC-MS can simultaneously measure multiple immunosuppressive drugs in a single whole blood specimen.

Note: At the time of this book's printing, the Roche total and free MRA assays had just been cleared for use in the United States.

REFERENCES

1. Merrill JP, Murray JE, Harrison JH, Guild WR. Successful homotransplantation of the human kidney between identical twins. *JAMA* 1956;160:277–282.
2. Murray JE, Merrill JP, Harrison JH, Wilson RE, Dammin GJ. Prolonged survival of human-kidney homographs by immunosuppressive drug therapy. *N Engl J Med* 1963;268:1315–1323.
3. Starzl TE, Marchioro TL, Waddell WR. The reversal of rejection in human renal homografts with subsequent development of homograft tolerance. *Surg Gynecol Obstet* 1963;117:385–395.
4. Calne RY, White DJ, Thiru S, Evans DB, McMaster P, Dunn DC, Craddock GN, Pentlow BD, Rolles K. Cyclosporin A in patients receiving renal allografts from cadaver donors. *Lancet* 1978;2:1323–1327.
5. Goto T, Kino T, Hatanaka H, Nishiyama M, Okuhara M, Kohsaka M, Aoki H, Imanaka H. Discovery of FK-506, a novel immunosuppressant isolated from *Streptomyces tsukubaensis*. *Transplant Proc* 1987;19:4–8.
6. Singh K, Sun S, Vezina C. Rapamycin (AY-22,989), a new antifungal antibiotic. IV. Mechanism of action. *J Antibiot (Tokyo)* 1979;32:630–645.
7. Sollinger HW. Mycophenolate mofetil for the prevention of acute rejection in primary cadaveric renal allograft recipients. U.S. Renal Transplant Mycophenolate Mofetil Study Group. *Transplantation* 1995;60:225–232.

8. A blind, randomized clinical trial of mycophenolate mofetil for the prevention of acute rejection in cadaveric renal transplantation. The Tricontinental Mycophenolate Mofetil Renal Transplantation Study Group. *Transplantation* 1996;61:1029–1037.
9. Placebo-controlled study of mycophenolate mofetil combined with cyclosporin and corticosteroids for prevention of acute rejection. European Mycophenolate Mofetil Cooperative Study Group. *Lancet* 1995;345:1321–1325.
10. Organ Procurement and Transplant Network. Transplants by organ and donor type, 1995-2004, and National transplant statistics, national reports. In: *OPTN/SRTR 2005 Annual Report: Summary Table 7 and Organ Summary Tables* (Accessed August 10, 2006, at http://www.ustransplant.org).
11. Organ Procurement and Transplant Network. National transplant statistics, national reports. In: *OPTN/SRTR 2005 Annual Report: Organ Summary Tables* (Accessed August 10, 2006, at http://www.ustransplant.org).
12. Lindholm A, Albrechtsen D, Tufveson G, Karlberg I, Persson NH, Groth CG. A randomized trial of cyclosporine and prednisolone versus cyclosporine, azathioprine, and prednisolone in primary cadaveric renal transplantation. *Transplantation* 1992;54:624–631.
13. Cyclosporin in cadaveric renal transplantation: one-year follow-up of a multicentre trial. *Lancet* 1983;2:986–989.
14. Bruunshuus I, Schmiegelow K. Analysis of 6-mercaptopurine, 6-thioguanine nucleotides and 6-thiuric acid in biological fluids by high-performance liquid chromatography. *Scand J Clin Invest* 1989;49:779–784.
15. Kreuzenkamp-Jansen CW, De Abreu RA, Bokkerink JPM, Trijbels JMF. Determination of extracellular and intracellular thiopurines and methylthiopurines with HPLC. *J Chromatogr* 1995;672:53–61.
16. Rabel SR, Stobaugh JF, Trueworthy R. Determination of intracellular levels of 6-mercaptopurine metabolites in erythrocytes utilizing capillary electrophoresis with laser-induced fluorescence detection. *Anal Biochem* 1995;224:315–322.
17. Filler G, Lepage N, Delisle B, Mai I. Effect of cyclosporine on mycophenolic acid area under the concentration-curve in pediatric kidney transplant recipients. *Ther Drug Monit* 2001;23:514–519.
18. van Gelder T, Klupp J, Barten MJ, Christians U, Morris RE. Comparison of the effects of tacrolimus and cyclosporine on the pharmacokinetics of mycophenolic acid. *Ther Drug Monit* 2001;23:119–128.
19. Undre NA. Pharmacokinetics of tacrolimus-based combination therapies. *Nephrol Dial Transplant* 2003;18(Suppl 1):i12-i15.
20. Organ Procurement and Transplant Network. Immunosuppression: evolution in practice and trends, 1994-2004. In: *OPTN/SRTR 2005 Annual Report: Table 2* (Accessed June 19, 2006, at http://www.ustransplant.org).
21. Shibasaki F, Hallin U, Uchino H. Calcineurin as a multifunctional regulator. *J Biochem (Tokyo)* 2002;131:1–15.
22. Siekierka JJ, Hung SH, Poe M, Lin CS, Sigal NH. A cytosolic binding protein for the immunosuppressant FK506 has peptidylprolyl isomerase activity but is distinct from cyclophilin. *Nature* 1989;341:755–757.
23. Schreiber SL, Crabtree GR. The mechanism of action of cyclosporin A and FK-506. *Immunol Today* 1992;13:136–142.
24. Flanagan WM, Corthesy B, Bram RJ, Crabtree GR. Nuclear association of a T-cell transcription factor blocked by FK506 and cyclosporin A. *Nature* 1991;352:803–807.
25. Clipstone NA, Crabtree GR. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* 1992;357:695–697.
26. Schreiber SL. Chemistry and biology of immunophilins and their immunosuppressive ligands. *Science* 1991;251:283–287.
27. Gummert JF, Ikonen T, Morris RE. Newer immunosuppressive drugs: a review. *J Am Soc Nephrol* 1999;10:1366–1380.
28. Jorgensen KA, Koefoed-Nielsen PB, Karamperis N. Calcineurin phosphatase activity and immunosuppression. A review on the role of calcineurin phosphatase activity and the immunosuppressive effect of cyclosporin A and tacrolimus. *Scand J Immunol* 2003;57:93–98.
29. Borel JF, Feurer C, Gubler HU, Stahelin H. Biological effects on cyclosporin A: a new antilymphocytic agent. *Agents Actions* 1976;6:468–475.

30. Vonderscher J, Meinzer A. Rationale for the development of Sandimmune Neoral. *Transplant Proc* 1994;26:2925–2927.
31. Bartucci MR. Issues in cyclosporine drug substitution: implications for patient management. *J Transpl Coord* 1999;9:137–142.
32. Alloway RR. Generic immunosuppressant use in solid organ transplantation. *Transplant Proc* 1999;31(Suppl 3A):2S–5S.
33. Kovarik JM, Mueller EA, van Bree JB, Fluckinger SS, Lange H, Schmidt B, Boesken WH, Lison AE, Kutz K. Cyclosporine pharmacokinetics and variability from a microemulsion formulation-a multicenter investigation in kidney transplant patients. *Transplantation* 1994;58:658–663.
34. Ptachcinski RJ, Venkataramanan R, Burckart GJ. Clinical pharmacokinetics of cyclosporin. *Clin Pharmacokinet* 1986;11:107–132.
35. Hoppu K, Jalanko H, Laine J, Holmberg C. Comparison of conventional oral cyclosporine and cyclosporine microemulsion formulation in children with a liver transplant. *Transplantation* 1996;62:66–71.
36. Faulds D, Goa KL, Benfield P. Cyclosporin. A review of its pharmacodynamic and pharmacotherapeutic properties, and therapeutic use in immunoregulatory disorders. *Drugs* 1993;45:953–1040.
37. Noble S, Markham A. Cyclosporin. A review of the pharmacokinetic properties, clinical efficacy and tolerability of a microemulsion-based formulation (Neoral). *Drugs* 1995;50:924–941.
38. Fahr A. Cyclosporin clinical pharmacokinetics. *Clin Pharmacokinet* 1993;24:472–495.
39. Wenk M, Follath F, Abisch E. Temperature dependency of apparent cyclosporine A concentrations in plasma. *Clin Chem* 1983;29:1865.
40. Kahan BD, Shaw LM, Holt D, Grevel J, Johnston A. Consensus document: Hawk's meeting on therapeutic drug monitoring of cyclosporine. *Clin Chem* 1990;36:1510–1516.
41. Shaw LM, Yatscoff RW, Bowers LD, Freeman DJ, Jeffery JR, Keown PA, McGilveray IJ, Rosano TG, Wong PY. Canadian consensus meeting on cyclosporine monitoring: Report of the consensus panel. *Clin Chem* 1990;36:1841–1846.
42. Ollerich M, Armstrong VW, Kahan B, Shaw L, Holt DW, Yatscoff R, Lindholm A, Halloran P, Gallicano K, Wonigeit K, Schutz E, Schran H, Annesley T. Lake Louise consensus conference on cyclosporin monitoring in organ transplantation: report of the consensus panel. *Ther Drug Monit* 1995;17:642–654.
43. Zhang YC, Benet L. The gut as a barrier to drug absorption. *Clin Pharmacokinet* 2001;40:159–168.
44. Wu CY, Benet LZ, Hebert MF, Gupta SK, Rowland M, Gomez DY, Wacher VJ. Differentiation of absorption and first-pass gut and hepatic metabolism in humans: studies with cyclosporine. *Clin Pharmacol Ther* 1995;58:492–497.
45. Lown KS, Mayo RR, Leichtman AB, Hsiao HL, Turgeon DK, Schmiedlin-Ren P, Brown MB, Guo W, Rossi SJ, Benet LZ, Watkins PB. Role of intestinal P-glycoprotein (mdr1) in interpatient variation in the oral bioavailability of cyclosporine. *Clin Pharmacol Ther* 1997;62:248–260.
46. Wallemacq PE, Lhoest G, Dumont P. Isolation, purification and structure elucidation of cyclosporine A metabolites in rabbit and man. *Biomed Mass Spectrom* 1989;18:48–56.
47. Christians U, Sewing KF. Cyclosporin metabolism in transplant patients. *Pharmacol Ther* 1993;57:291–345.
48. Yatscoff RW, Rosano TG, Bowers LD. The clinical significance of cyclosporine metabolites. *Clin Biochem* 1991;24:23–35.
49. Radeke HH, Christians U, Sewing KF, Resch K. The synergistic immunosuppressive potential of cyclosporin metabolite combinations. *Int J Immunopharmacol* 1992;14:595–604.
50. Rosano TG, Brooks CA, Dybas MT, Cramer SM, Stevens C, Freed BM. Selection of an optimal assay method for monitoring cyclosporine therapy. *Transplant Proc* 1990;22:1125–1128.
51. Ryffel B, Foxwell BM, Mihatsch MJ, Donatsch P, Maurer G. Biologic significance of cyclosporine metabolites. *Transplant Proc* 1988;20(Suppl 2):575–584.
52. Smets F, Sokal EM. Lymphoproliferation in children after liver transplantation. *J Pediatr Gastroenterol Nutr* 2002;34:499–505.
53. Scott JP, Higenbottam TW. Adverse reactions and interactions of cyclosporin. *Med Toxicol* 1988;3:107–127.

54. Yee GC, McGuire TR. Pharmacokinetic drug interactions with cyclosporin. *Clin Pharmacol* 1990;19:319–332 and 400–415.
55. Durr D, Stieger B, Kullak-Ublick GA, Rentsch KM, Steinert HC, Meier PJ, Fattinger K. St John's Wort induces intestinal P-glycoprotein/MDR1 and intestinal and hepatic CYP3A4. *Clin Pharmacol Ther* 2000;68:598–604.
56. Smith MC, Sephel GC. Long-term in vitro stability of cyclosporine in whole-blood samples. *Clin Chem* 1990;36:1991–1992.
57. Schran HF, Rosano TG, Hasse AE, Pell MA. Determination of cyclosporine concentrations with monoclonal antibodies. *Clin Chem* 1987;33:2225–2229.
58. Keown P, Landsberg D, Hollaran P, Shoker A, Rush D, Jeffery J, Russell D, Stiller C, Muirhead N, Cole E, Paul L, Zaltzman J, Loertscher R, Daloze P, Dandavino R, Boucher A, Handa P, Lawen J, Belitsky P, Parfrey P. A randomized, prospective multicenter pharmacoepidemiologic study of cyclosporine microemulsion in stable renal graft recipients. Report of the Canadian Neoral Renal Transplantation Study Group. *Transplantation* 1996;27:1744–1752.
59. Halloran PF, Helms LM, Noujaim J. The temporal profile of calcineurin inhibition by cyclosporine in vivo. *Transplantation* 1999;15:1356–1361.
60. Lindholm A, Kahan BD. Influence of cyclosporine pharmacokinetics, trough concentrations, and AUC monitoring on outcome after kidney transplantation. *Clin Pharmacol Ther* 1993;54: 205–218.
61. Lindholm A. Review: factors influencing the pharmacokinetics of cyclosporine in man. *Ther Drug Monit* 1991;13:465–477.
62. Grevel J, Welsh MS, Kahan B. Cyclosporine monitoring in renal transplantation: area under the curve monitoring is superior to trough-level monitoring. *Ther Drug Monit* 1989;11:246–248.
63. Belitsky P, Levy GA, Johnston A. Neoral absorption profiling: an evolution in effectiveness. *Transplant Proc* 2000;32(Suppl 3A):S45–S52.
64. Keown P, Niese D. Cyclosporine microemulsion increases drug exposure and reduces acute rejection without incremental toxicity in de novo renal transplantation. International Sandimmune Neoral Study Group. *Kidney Int* 1998;54:938–944.
65. Stein CM, Murray JJ, Wood AJ. Inhibition of stimulated interleukin-2 production in whole blood: a practical measure of cyclosporine effect. *Clin Chem* 1999;45:1477–1484.
66. Oellerick M, Armstrong VW. Two-hour cyclosporine concentration determination: An appropriate tool to monitor neoral therapy? *Ther Drug Monit* 2002;24:40–46.
67. Mahalati K, Belitsky P, Sketris I, West K, Panek R. Neoral monitoring by simplified sparse sampling area under the concentration-time curve. *Transplantation* 1999;68:55–62.
68. Grant D, Kneteman N, Tchervenkow J, Roy A, Murphy G, Tan A, Hendricks L, Guilbault N, Levy G. Peak cyclosporine levels (Cmax) correlate with freedom from liver graft rejection: results of a prospective, randomized comparison of neoral and sandimmune for liver transplantation (NOF-8). *Transplantation* 1999;67:1133–1137.
69. Cantarovick M, Barkun JS, Tchervenkov JI, Besner JG, Aspeslet L, Metrakos P. Comparison of Neoral dose monitoring with cyclosporine through levels versus 2-hour postdose levels in stable liver transplant patients. *Transplantation* 1998;66:1621–1627.
70. Cantarovick M, Elstein E, de Varennes B, Barkun JS. Clinical benefit of Neoral dose monitoring with cyclosporine 2-hour post-dose levels compared with trough levels in stable heart transplant patients. *Transplantation* 1999;68:1839–1842.
71. Wallemacq PE. Therapeutic monitoring of immunosuppressant drugs. Where are we? *Clin Chem Lab Med* 2004;42:1204–1211.
72. Whitman DA, Abbott V, Fregien K, Bowers LD. Recent advances in high-performance liquid chromatography/mass spectrometry and high-performance liquid chromatography/tandem mass spectrometry: detection of cyclosporine and metabolites in kidney and liver tissue. *Ther Drug Monit* 1993;15:552–556.
73. Zhou L, Tan D, Theng J, Lim L, Liu YP, Lam KW. Optimized analytical method for cyclosporine A by high-performance liquid chromatography-electrospray ionization mass spectrometry. *J Chromatogr B Biomed Sci Appl* 2001;754:201–207.

74. Taylor PJ. Therapeutic drug monitoring of immunosuppressant drugs by high-performance liquid chromatography-mass spectrometry. *Ther Drug Monit* 2004;26:215–219.
75. Belensky A, Lazzaro P, Shuaib A, Natrajan A, Costello J, Barbarakis M, Connolly P, Hartl T. Bayer ADVIA Centaur® cyclosporine assay: an analytical evaluation. *Clin Chem* 2006;52(Suppl):A60 (Abstract).
76. Tredger JM, Roberts N, Sherwood R, Higgins G, Keating J. Comparison of five cyclosporin immunoassays with HPLC. *Clin Chem Lab Med* 2000;38:1205–1207.
77. Steimer W. Performance and specificity of monoclonal immunoassays for cyclosporine monitoring: How specific is specific? *Clin Chem* 1999;45:371–381.
78. Schutz E, Svinarov D, Shipkova M, Niedmann PD, Armstrong VW, Wieland E, Oellerich M. Cyclosporin whole blood immunoassays (AxSYM, CEDIA, and Emit): a critical overview of performance characteristics and comparison with HPLC. *Clin Chem* 1998;44:2158–2164.
79. Hamwi A, Veitl M, Manner G, Ruzicka K, Schweiger C, Szekeres T. Evaluation of four automated methods for determination of whole blood cyclosporine concentrations. *Am J Clin Pathol* 1999;112:358–365.
80. Terrell AR, Daly TM, Hock KG, Kilgore DC, Wei TQ, Hernandez S, Weibe D, Fields L, Shaw LM, Scott MG. Evaluation of a no-pretreatment cyclosporin A assay on the Dade Behring Dimension RxL clinical analyzer. *Clin Chem* 2002;48:1059–1065.
81. Butch AW, Fukuchi AM. Analytical performance of the CEDIA®cyclosporine PLUS whole blood immunoassay. *J Anal Toxicol* 2004;28:204–210.
82. Immunosuppressive Drugs International Proficiency Testing Scheme URL (Accessed August 16, 2006, at http://www.bioanalytics.co.uk).
83. Morris RG, Holt DW, Armstrong VW, Griesmacher A, Napoli KL, Shaw LM. Analytical aspects of cyclosporine monitoring, on behalf of the IFCC/IATDMCT joint working group. *Ther Drug Monit* 2004;26:227–330.
84. Holt DW, Johnston A, Kahan BD, Morris RG, Oellerich M, Shaw LM. New approaches to cyclosporine monitoring raise further concerns about analytical techniques. *Clin Chem* 2000;46:872–874.
85. Juenke JM, Brown PI, Urry FM, McMillin GA. Specimen dilution for C2 monitoring with the Abbott TDxFLx cyclosporine monoclonal whole blood assay. *Clin Chem* 2004;50:1430–1433.
86. Johnston A, Chusney G, Schutz E, Oellerich M, Lee TD, Holt DW. Monitoring cyclosporin in blood: between-assay differences at trough and 2 hours post-dose (C2). *Ther Drug Monit* 2003;25:167–173.
87. First MR. Tacrolimus based immunosuppression. *J Nephrol* 2004;17:25–31.
88. Venkataramanan R, Swaminathan A, Prasad T, Jain A, Zuckerman S, Warty V, McMichael J, Lever J, Burckart G, Starzl T. Clinical pharmacokinetics of tacrolimus. *Clin Pharmacokinet* 1995;29:404–430.
89. Zahir H, Nand RA, Brown KF, Tattam BN, McLachlan AJ. Validation of methods to study the distribution and protein binding of tacrolimus in human blood. *J Pharmacol Toxicol Methods* 2001;46:27–35.
90. Iwasaki K, Shiraga T, Nagase K, Tozuka Z, Noda K, Sakuma S, Fujitsu T, Shimatani K, Sato A, Fujioka M. Isolation, identification, and biological activities of oxidative metabolites of FK506, a potent immunosuppressive macrolide lactone. *Drug Metab Dispos* 1993;21:971–977.
91. Kelly P, Kahan BD. Review: metabolism of immunosuppressant drugs. *Curr Drug Metab* 2002;3:275–287.
92. Tamura K, Fujimura T, Iwasaki K, Sakuma S, Fujitsu T, Nakamura K, Shimomura K, Kuno T, Tanaka C, Kobayashi M. Interaction of tacrolimus (FK506) and its metabolites with FKBP and calcineurin. *Biochem Biophys Res Commun* 1994;202:437–443.
93. Beysens J, Wigner RMH, Beuman GH, van der Heyden J, Kootstra G, van As H. FK 506: Monitoring in plasma or in whole blood? *Transplant Proc* 1991;23:2745–2747.
94. Taylor AL, Watson CJE, Bradley JA. Immunosuppressive agents in solid organ transplantation: mechanisms of action and therapeutic efficacy. *Crit Rev Oncol Hematol* 2005;56:23–46.
95. Artz MA, Boots JM, Ligtenberg G, Roodnat JI, Christiaans MH, Vos PF, Moons P, Borm G, Hilbrands LB. Conversion from cyclosporine to tacrolimus improves quality-of-life indices, renal graft function and cardiovascular risk profile. *Am J Transplant* 2004;4:937–945.

96. Mentzer RM Jr, Jahania MS, Lasley RD. Tacrolimus as a rescue immunosuppressant after heart and lung transplantation. The U.S. Multicenter FK506 Study Group. *Transplantation* 1998;65:109–113.
97. Laskow DA, Neylan JF, Shapiro RS, Pirsch JD, Vergne-Marini PJ, Tomlanovich SJ. The role of tacrolimus in adult kidney transplantation: a review. *Clin Tranplant* 1998;12:489–503.
98. Annesley TM, Hunter BC, Fidler DR, Giacherio DA. Stability of tacrolimus (FK 506) and cyclosporine G in whole blood. *Ther Drug Monit* 1995;17:361–365.
99. Alak AM, Lizak P. Stability of FK506 in blood samples. *Ther Drug Monit* 1996;18:209–211.
100. Freeman DJ, Stawecki M, Howson B. Stability of FK 506 in whole blood samples. *Ther Drug Monit* 1995;17:266–267.
101. Holt DW. Therapeutic drug monitoring of immunosuppressive drugs in kidney transplantation. *Curr Opin Nephrol Hypertens* 2002;11:657–663.
102. Staatz CE, Tett SE. Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplant. *Clin Pharmacokinet* 2004;43:623–653.
103. Wong KM, Shek CC, Chau KF, Li CS. Abbreviated tacrolimus area-under-the-curve monitoring for renal transplant recipients. *Am J Kidney Dis* 2000;35:660–666.
104. McAlister VC, Mahalati K, Peltekian KM, Fraser A, MacDonald AS. A clinical pharmacokinetic study of tacrolimus and sirolimus combination immunosuppression comparing simultaneous to separated administration. *Ther Drug Monit* 2002;24:346–350.
105. Lensmeyer GL, Poquette MA. Therapeutic monitoring of tacrolimus concentrations in blood: semi-automated extraction and liquid chromatography-electrospray ionization mass spectrometry. *Ther Drug Monit* 2001;23:239–249.
106. Akbas SH, Yavuz A, Tuncer M, Yurdakonar E, Akcit F, Gurkan A, Demirbas A, Gultekin M, Ersoy F, Akaydin M. Evaluation of the new EMIT tacrolimus assay in kidney and liver transplant recipients. *Transplant Proc* 2004;36:86–88.
107. LeGatt DF, Shalapay CE, Cheng SB. The EMIT 2000 tacrolimus assay: an application protocol for the Beckman Synchron LX20 PRO analyzer. *Clin Biochem* 2004;37:1022–1030.
108. Stephen DWS, Rooke P, Clark S, Coutts M, Crowe L, Docherty D. Evaluation of the Dade Behring Syva® EMIT 2000 tacrolimus assay on the Bayer Advia 1650. *Ann Clin Biochem* 2003;40: 697–700.
109. Baugher BW, Drengler S, Rahn T, Ramp J, Liao M, Wong PY. Evaluation of an assay in development for tacrolimus on the Abbott ARCHITECH® analyzer. *Clin Chem* 2006;52(Suppl):A59 (Abstract).
110. Keevil BG, McCann SJ, Cooper DP, Morris MR. Evaluation of a rapid micro-scale assay for tacrolimus by liquid chromatography-tandem mass spectrometry. *Ann Clin Biochem* 2002;39:487–492.
111. Deters M, Kirchner G, Rewsch K, Kaever V. Simultaneous quantification of sirolimus, everolimus, tacrolimus and cyclosporine by liquid chromatography-mass spectrometry (LM-MS). *Clin Chem Lab Med* 2002;40:285–292.
112. Iwasaki K, Shiraga T, Matsuda H, Nagase K, Tokuma Y, Hata T, Fujii Y, Sakuma S, Fujitsu T, Fujikawa A, Shimatani K, Sato A, Fujioka M. Further metabolism of FK506 (tacrolimus). Identification and biological activities of the metabolites oxidized at multiple sites of FK506. *Drug Metab Dispos* 1995;23:28–34.
113. CEDIA® Tacrolimus Assay (package insert). Microgenics Corporation, Fremont, CA, 2005.
114. Staatz CE, Taylor PJ, Tett SE. Comparison of an ELISA and an LC/MS/MS method for measuring tacrolimus concentrations and making dosage decisions in transplant recipients. *Ther Drug Monit* 2002;24:607–615.
115. Gonschior AK, Christians U, Winkler M, Linck A, Baumann J, Sewing KF. Tacrolimus (FK506) metabolite patterns in blood from liver and kidney transplant patients. *Clin Chem* 1996;42:1426–1432.
116. Oellerich M, Armstrong VW, Schutz E, Shaw LM. Therapeutic drug monitoring of cyclosporine and tacrolimus. *Clin Biochem* 1998;31:309–316.
117. Cogill JL, Taylor PJ, Westley IS, Morris RG, Lynch SV, Johnson AG. Evaluation of the tacrolimus II microparticle enzyme immunoassay (MEIA II) in liver and renal transplant recipients. *Clin Chem* 1998;44:1942–1946.
118. Busuttil RW, Klintmalm GB, Lake JR, Miller CM, Porayko M. General guidelines for the use of tacrolimus in adult liver transplant patients. *Transplantation* 1996;61:845–847.

119. Schambeck CM, Bedel A, Keller F. Limit of quantification (functional sensitivity) of the new IMx tacrolimus II microparticle enzyme immunoassay. *Clin Chem* 1998;44:2317.
120. Tacrolimus II (package insert). Abbott Laboratories, Diagnostics Division, Abbott Park, IL, 2003.
121. Homma M, Tomita T, Yuzawa K, Takada Y, Kohda Y. False positive blood tacrolimus concentration in microparticle enzyme immunoassay. *Biol Pharm Bull* 2002;25:1119–1120.
122. Kuzuya T, Ogura Y, Motegi Y, Moriyama N, Nabeshima T. Interference of hematocrit in the tacrolimus II microparticle enzyme immunoassay. *Ther Drug Monit* 2002;24:507–511.
123. Akbas SH, Ozdem S, Caglar S, Tuncer M, Gurkan A, Yucetin L, Senol Y, Demirbas A, Gultekin M, Ersoy FF, Akaydin M. Effects of some hematological parameters on whole blood tacrolimus concentration measured by two immunoassay-based analytical methods. *Clin Biochem* 2005;38:552–557.
124. Ghoshal AK, Soldin SJ. IMx tacrolimus II assay: Is it reliable at low blood concentrations? A comparison with tandem MS/MS. *Clin Biochem* 2002;35:389–392.
125. Steele BW, Wang E, Soldin SJ, Klee G, Elin RJ, Witte DL. A longitudinal replicate study of immunosuppressive drugs. A College of American Pathologists Study. *Arch Pathol Lab Med* 2003;127:283–288.
126. Miller JL. Sirolimus approved with renal transplant indication. *Am J Health Syst Pharm* 1999;56:2177–2178.
127. Sedrani R, Cottens S, Kallen J, Schuler W. Chemical modification of rapamycin: the discovery of SZD RAD. *Transplant Proc* 1998;30:2192–2194
128. Abraham RT, Wiederrecht GJ. Immunopharmacology of rapamycin. *Annu Rev Immunol* 1996;14:483–510.
129. Kimball PM, Derman RK, Van Buren CT, Lewis RM, Katz S, Kahan BD. Cyclosporine and rapamycin affect protein kinase C induction of intracellular activation signal, activator of DNA replication. *Transplantation* 1993;55:1128–1132.
130. Mahalati K, Kahan BD. Clinical pharmacokinetics of sirolimus. *Clin Pharmacokinet* 2001;40:573–585.
131. Zimmerman JJ, Kahan BD. Pharmacokinetics of sirolimus in stable renal transplant patients after multiple oral dose administration. *J Clin Pharmacol* 1997;37:405–415.
132. Yatscoff RW, Boeckx R, Holt DW, Kahan BD, LeGatt DF, Sehgal S, Soldin SJ, Napoli K, Stiller C. Consensus guidelines for therapeutic drug monitoring of rapamycin: report of the consensus panel. *Ther Drug Monit* 1995;17:676–680.
133. Cattaneo D, Merlini S, Pellegrino M, Carrara F, Zenoni S, Murgia S, Baldelli S, Gaspari F, Remuzzi G, Perico N. Therapeutic drug monitoring of sirolimus: effect of concomitant immunosuppressive therapy and optimization of drug dosing. *Am J Transplant* 2004;4:1345–1351.
134. Yatscoff R, LeGatt D, Keenan R, Chackowsky P. Blood distribution of rapamycin. *Transplantation* 1993;56:1202–1206.
135. Sattler M, Guengerich FP, Yun CH, Christians U, Sewing KF. Cytochrome P-450 3A enzymes are responsible for biotransformation of FK506 and rapamycin in man and rat. *Drug Metab Dispos* 1992;20:753–761.
136. Gallant-Haidner HL, Trepanier DJ, Freitag DG, Yatscoff RW. Pharmacokinetics and metabolism of sirolimus. *Ther Drug Monit* 2000;22:31–35.
137. Leung LY, Zimmeman J, Lim HK. Metabolic disposition of [14C]-rapamycin (sirolimus) in healthy male subjects after a single oral dose. *Proc Int Soc Stud Xenobiotics* 1997;12:26 (Abstract).
138. Montalbano M, Neff GW, Yamashiki N, Meyer D, Bettiol M, Slapak-Green G, Ruiz P, Manten E, Safdar K, O'Brien C, Tzakis AG. A retrospective review of liver transplant patients treated with sirolimus from a single center: an analysis of sirolimus related complications. *Transplantation* 2004;78:264–268.
139. Haydar AA, Denton M, West A, Rees J, Goldsmith DJ. Sirolimus-induced pneumonitis: three cases and a review of the literature. *Am J Transplant* 2004;4:137–139.
140. Kaplan B, Meier-Kriesche HU, Napoli KL, Kahan BD. The effects of relative timing of sirolimus and cyclosporine microemulsion formulation coadministration on the pharmacokinetics of each agent. *Clin Pharmacol Ther* 1998;63:48–53.
141. Jones K, Saadat-Lajevardi S, Lee T, Horwatt R, Hicks D, Johnston A, Holt DW. An immunoassay for the measurement of sirolimus. *Clin Therapeutics* 2000;22(Suppl):B49–B61.

142. Salm P, Tresillian MJ, Taylor PJ, Pillans PI. Stability of sirolimus (rapamycin) in whole blood. *Ther Drug Monit* 2000;22:423–426.
143. Yatscoff RW, Faraci C, Bolingbroke P. Measurement of rapamycin in whole blood using reverse-phase high-performance liquid chromatography. *Ther Drug Monit* 1992;14:138–141.
144. Kahan BD, Napoli KL, Kelly PA, Podbielski J, Hussein I, Urbauer DL, Katz SH, Van Buren CT. Therapeutic drug monitoring of sirolimus: correlations with efficacy and toxicity. *Clin Transplant* 2000;14:97–109.
145. Holt DW, Denny K, Lee TD, Johnston A. Therapeutic monitoring of sirolimus: its contribution to optimal prescription. *Transplant Proc* 2003;35(Suppl 3):157S–161S.
146. Napoli KL, Kahan BD. Routine clinical monitoring of sirolimus (rapamycin) whole-blood concentrations by HPLC with ultraviolet detection. *Clin Chem* 1996;42:1943–1948.
147. Napoli KL. A practical guide to the analysis of sirolimus using high-performance liquid chromatography with ultraviolet detection. *Clin Ther* 2000;22(Suppl):B14–B24.
148. Maleki S, Graves S, Becker S, Horwatt R, Hicks D, Stroshane RM, Kincaid H. Therapeutic monitoring of sirolimus in human whole-blood samples by high-performance liquid chromatography. *Clin Ther* 2000;22(Suppl):B25–B37.
149. Wilson D, Johnston F, Holt D, Moreton M, Engelmayer J, Gaulier J-M, Luthe H, Marquet P, Moscato D, Oellerich M, Mosso R, Streit F, Brunet M, Brunet M, Fillee C, Schmid R, Wallemacq P, Barnes G. Multi-center evaluation of analytical performance of the microparticle enzyme immunoassay for sirolimus. *Clin Biochem* 2006;39:378–386.
150. CEDIA® Sirolimus Assay (package insert). Microgenics Corporation, Fremont, CA, 2004.
151. Vicente FB, Smith FA, Peng Y, Wang S. Evaluation of an immunoassay of whole blood sirolimus in pediatric transplant patients in comparison with high-performance liquid chromatography/tandem mass spectrometry. *Clin Chem Lab Med* 2006;44:497–499.
152. Holt DW, Laamanen MK, Johnston A. A microparticle enzyme immunoassay to measure sirolimus. *Transplant Proc* 2005;37:182–184.
153. Zochowska D, Bartlomiejczyk I, Kaminska A, Senatorski G, Paczek L. High-performance liquid chromatography versus immunoassay for the measurement of sirolimus: comparison of two methods. *Transplant Proc* 2006;38:78–80.
154. Fillee C, Mourad M, Squifflet JP, Malaise J, Lerut J, Reding R, Borghgraef P, Vanbinst R, Wallemacq PE. Evaluation of a new immunoassay to measure sirolimus blood concentrations compared to a tandem mass-spectrometric chromatographic analysis. *Transplant Proc* 2005;37:2890–2891.
155. Morris RG, Salm P, Taylor PJ, Wicks FA, Theodossi A. Comparison of the reintroduced MEIA® assay with HPLC-MS/MS for the determination of whole-blood sirolimus from transplant recipients. *Ther Drug Monit* 2006;28:164–168.
156. Westley IS, Morris RG, Taylor PJ, Salm P, James MJ. CEDIA® sirolimus assay compared with HPLC-MS/MS and HPLC-UV in transplant recipient specimens. *Ther Drug Monit* 2005;27:309–314.
157. Holt DW, McKeown DA, Lee TD, Hicks D, Cal P, Johnston A. The relative proportions of sirolimus metabolites in blood using HPLC with mass-spectrometric detection. *Transplant Proc* 2004;36:3223–3225.
158. Christians U, Jacobsen W, Serkova N, Benet LZ, Vidal C, Sewing KF, Manns MP, Kirchner GI. Automated, fast and sensitive quantification of drugs in blood by liquid chromatography-mass spectrometry with on-line extraction: immunosuppressants. *J Chromatogr B Biomed Sci Appl* 2000;748:41–53.
159. Holt DW, Lee T, Jones K, Johnston A. Validation of an assay for routine monitoring of sirolimus using HPLC with mass spectrometric detection. *Clin Chem* 2000;46:1179–1183.
160. Salm P, Taylor PJ, Pillans PI. The quantitation of sirolimus by high-performance liquid chromatography-tandem mass spectrometry and microparticle enzyme immunoassay in renal transplant recipients. *Clin Ther* 2000;22(Suppl):B71–B85.
161. Kirchner GI, Meier-Wiedenbach I, Manns MP. Clinical pharmacokinetics of everolimus. *Clin Pharmacokinet* 2004;43:83–95.
162. Hoyer PF, Ettenger R, Kovarik JM, Webb NJ, Lemire J, Mentser M, Mahan J, Loirat C, Niaudet P, Van Damme-Lombaerts R, Offner G, Wehr S, Moeller V, Mayer H; Everolimus

Pediatric Study Group. Everolimus in pediatric de nova renal transplant patients. *Transplantation* 2003;75:2082–2085.
163. Kahan BD, Kaplan B, Lorber MI, Winkler M, Cambon N, Boger RS. RAD in de novo renal transplantation: comparison of three doses on the incidence and severity of acute rejection. *Transplantation* 2001;71:1400–1406.
164. Kovarik JM, Kahan BD, Kaplan B, Lorber M, Winkler M, Rouilly M, Gerbeau C, Cambon N, Boger R, Rordorf C; Everolimus Phase 2 Study Group. Longitudinal assessment of everolimus in de novo renal transplant recipients over the first post-transplant year: pharmacokinetics, exposure-response relationships, and influence on cyclosporine. *Clin Pharmacol Ther* 2001;69:48–56.
165. Kahan BD, Podbielski J, Napoli KL, Katz SM, Meir-Kriesche HU, Van Buren CT. Immunosuppressive effects and safety of a sirolimus/cyclosporine combination regimen for renal transplantation. *Transplantation* 1998;66:1040–1046.
166. Nashan B. Review of the proliferation inhibitor everolimus. *Expert Opin Investig Drug* 2002;11:1842–1850.
167. Kirchner GI, Winkler M, Mueller L, Vidal C, Jacobsen W, Franzke A, Wagner S, Blick S, Manns MP, Sewing KF. Pharmacokinetics of SDZ RAD and cyclosporin including their metabolites in seven kidney graft patients after the first dose of SDZ RAD. *Br J Clin Pharmacol* 2000;50:449–454.
168. Innofluor® Certican® Assay System (package insert). Seradyn Inc, Indianapolis, IN, 2003.
169. Lehmkuhl H, Ross H, Eisen H, Valantine H. Everolimus (Certican) in heart transplantation: optimizing renal function through minimizing cyclosporine exposure. *Transplant Proc* 2005;37:4145–4149.
170. Salm P, Warnholtz C, Boyd J, Arabshahi L, Marbach P, Taylor PJ. Evaluation of a fluorescent polarization immunoassay for whole blood everolimus determination using samples from renal transplant recipients. *Clin Biochem* 2006;39:732–738.
171. Baldelli S, Murgia S, Merlini S, Zenoni S, Perico N, Remuzzi G, Cattaneo D. High-performance liquid chromatography with ultraviolet detection for therapeutic drug monitoring of everolimus. *J Chromatogr B* 2005;816:99–105.
172. Salm P, Taylor PJ, Lynch SV, Pillans PI. Quantification and stability of everolimus (SDZ RAD) in human blood by high-performance liquid chromatography-electrospray tandem mass spectrometry. *J Chromatogr B Anal Technol Biomed Life Sci* 2002;772:283–290.
173. Quinn CM, Bugeja VC, Gallagher JA, Whittaker PA. The effect of mycophenolic acid on the cell cycle of *Candida abicans*. *Mycopathologia* 1990;111:165–168.
174. Lee WA, Gu L, Miksztal AR, Chu N, Leung K, Nelson PH. Bioavailability improvement of mycophenolic acid through amino ester derivatization. *Pharm Res* 1990;7:161–166.
175. Franklin TJ, Cook JM. The inhibition of nucleic acid synthesis by mycophenolic acid. *Biochem J* 1969;113:515–524.
176. Wu JC. Mycophenolate mofetil: molecular mechanisms of action. *Perspect Drug Discov Design* 1994;2:185–204.
177. Eugui EM, Allison A. Immunosuppressive activity of mycophenolate mofetil. *Ann NY Acad Sci* 1993;685:309–329.
178. Allison AC, Eugui EM. Purine metabolism and immunosuppressive effects of mycophenolate mofetil (MMF). *Clin Transplant* 1996;10:77–84.
179. Bullingham R, Monroe S, Nicholls A, Hale M. Pharmacokinetics and bioavailability of mycophenolate mofetil in healthy subjects after single-dose oral and intravenous administration. *J Clin Pharmacol* 1996;36:315–324.
180. Bullingham RE, Nicholls AJ, Kamm BR. Clinical pharmacokinetics of mycophenolate mofetil. *Clin Pharmacokinet* 1998;34:429–455.
181. Nowak I, Shaw LM. Mycophenolic acid binding to human serum albumin: characterization and relation to pharmacodynamics. *Clin Chem* 1995;41:1011–1017.
182. Korecka M, Nikolic D, van Breemen RB, Shaw LM. Inhibition of inosine monophosphate dehydrogenase by mycophenolic acid glucuronide is attributable to the presence of trace quantities of mycophenolic acid. *Clin Chem* 1999;45:1047–1050.
183. Shipkova M, Armstrong VW, Wieland E, Niedmann PD, Schutz E, Brenner-Weiss G, Voihsel M, Braun F, Oellerich M. Identification of glucoside and carboxyl-linked glucuronide conjugates of

mycophenolic acid in plasma of transplant recipients treated with mycophenolate mofetil. *Br J Pharmacol* 1999;126:1075–1082.
184. Wieland E, Shipkova M, Schellhaas U, Schutz E, Niedmann PD, Armstrong VW, Oellerich M. Induction of cytokine release by acyl glucuronide of mycophenolic acid: a link to side effects? *Clin Biochem* 2000;33:107–113.
185. Shaw LM, Korecka M, van Breeman R, Nowak I, Brayman KL. Analysis, pharmacokinetics and therapeutic drug monitoring of mycophenolic acid. *Clin Biochem* 1998;31:323–328.
186. Kaplan B, Meier-Kriesche HU, Friedman G, Mulgaonkar S, Gruber S, Korecka M, Brayman KL, Shaw LM. The effect of renal insufficiency on mycophenolic acid protein binding. *J Clin Pharmacol* 1999;39:715–720.
187. Holt DW. Monitoring mycophenolic acid. *Ann Clin Biochem* 2002;39:173–183.
188. Mourad M, Malaise J, Chaib Eddour D, De Meyer M, Konig J, Schepers R, Squifflet JP, Wallemacq P. Pharmacokinetic basis for the efficient and safe use of low-dose mycophenolate mofetil in combination with tacrolimus in kidney transplantation. *Clin Chem* 2001;47:1241–1248.
189. Sollinger HW. Mycophenolates in transplantation. *Clin Transplant* 2004;18:485–492.
190. Vidal E, Cantarell C, Capdevila L, Monforte V, Roman A, Pou L. Mycophenolate mofetil pharmacokinetics in transplant patients receiving cyclosporine or tacrolimus in combination therapy. *Pharmacol Toxicol* 2000;87:182–184.
191. Shipkova M, Armstrong VW, Kuypers D, Perner F, Fabrizi V, Holzer H, Wieland E, Oellerich M; MMF Creeping Creatinine Study Group. *Ther Drug Monit* 2001;23:717–721.
192. Schmidt LE, Rasmussen A, Norrelykke MR, Poulsen HE, Hansen BA. The effect of selective bowel decontamination on the pharmacokinetics of mycophenolate mofetil in liver transplant recipients. *Liver Transplant* 2001;7:739–742.
193. Undre NA, van Hooff J, Christiaans M, Vanrenterghem Y, Donck J, Heeman U, Kohnle M, Zanker B, Land W, Morales JM, Andres A, Schafer A, Stevenson P. Pharmacokinetics of FK 506 and mycophenolic acid after the administration of a FK 506-based regimen in combination with mycophenolate mofetil in kidney transplantation. *Transplant Proc* 1998;30:1299–1302.
194. Vietri M, Pietrabissa A, Mosca F, Pacifici GM. Mycophenolic acid glucuronidation and its inhibition by non-steroidal anti-inflammatory drugs in human liver and kidney. *Eur J Clin Pharmacol* 2000;56:659–664.
195. Kato R, Ooi K, Ikura-Mori M, Tsuchishita Y, Hashimoto H, Yoshimura H, Uenishi K, Kawai M, Tanaka K, Ueno K. Impairment of mycophenolate mofetil absorption by calcium polycarbophil. *J Clin Pharmacol* 2002;42:1275–1280.
196. Shaw LM, Holt DW, Oellerich M, Meiser B, van Gelder T. Current issues in therapeutic drug monitoring of mycophenolic acid: report of a roundtable discussion. *Ther Drug Monit* 2001;23:305–315.
197. Tsina I, Chu F, Hama K, Kaloostian M, Tam YL, Tarnowski T, Wong B. Manual and automated (robotic) high-performance liquid chromatography methods for the determination of mycophenolic acid and its glucuronide conjugate in human plasma. *J Chromatogr B* 1996;675:119–129.
198. Streit F, Shipkova M, Armstrong VW, Oellerich M. Validation of a rapid and sensitive liquid chromatography-tandem mass spectrometry method for free and total mycophenolic acid. *Clin Chem* 2004;50:152–159.
199. Saunders DA. Simple method for the quantitation of mycophenolic acid in human plasma. *J Chromatogr B Biomed Sci Appl* 1997;704:379–382.
200. Tsina I, Kaloostian M, Lee R, Tarnowski T, Wong B. High-performance liquid chromatographic method for the determination of mycophenolate mofetil in human plasma. *J Chromatogr B* 1996;681:347–353.
201. Shipkova M, Armstrong VW, Kiehl MG, Niedmann PD, Schutz E, Oellerich M, Wieland E. Quantification of mycophenolic acid in plasma samples collected during and immediately after intravenous administration of mycophenolate mofetil. *Clin Chem* 2001;47:1485–1488.
202. Mahalati K, Kahan BD. Pharmacological surrogates of allograft outcome. *Ann Transplant* 2005;5:14–23.
203. Filler G, Mai I. Limited sampling strategy for mycophenolic acid area under the curve. *Ther Drug Monit* 2000;22:169–173.

204. Pawinski T, Hale M, Korecka M, Fitzsimmons WE, Shaw LM. Limited sampling strategy for the estimation of mycophenolic acid area under the curve in adult renal transplant patients with concomitant tacrolimus. *Clin Chem* 2002;48:1497–1504.
205. Le Guellec C, Buchler M, Giraudeau B, Le Meur Y, Gakoue JE, Lebranchu Y, Marquet P, Paintaud G. Simultaneous estimation of cyclosporin and mycophenolic acid areas under the curve in stable renal transplant patients using a limited sampling strategy. *Eur J Clin Pharmacol* 2002;57:805–811.
206. van Gleder T, Shaw LM. The rationale for and limitations of therapeutic drug monitoring for mycophenolate mofetil in transplantation. *Transplantation* 2005;80(Suppl 2):S244–S253.
207. Brunet M, Cirera I, Martorell J, Vidal E, Millan O, Jimenez O, Rojo I, Londono MC, Rimola A. Sequential determination of pharmacokinetics and pharmacodynamics of mycophenolic acid in liver transplant patients treated with mycophenolate mofetil. *Transplantation* 2006;81:541–546.
208. van Gelder T, Meur YL, Shaw LM, Oellerich M, DeNofrio D, Holt C, Holt DW, Kaplan B, Kuypers D, Meiser B, Toenshoff B, Mamelok RD. Therapeutic drug monitoring of mycophenolate mofetil in transplantation. *Ther Drug Monit* 2006;28:145–154.
209. Teshima D, Kitagawa N, Otsubo K, Makino K, Itoh Y, Oishi R. Simple determination of mycophenolic acid in human serum by column-switching high-performance liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002;780:21–26.
210. Sparidans RW, Hoetelmans RM, Beijnen JH. Liquid chromatographic assay for simultaneous determination of abacavir and mycophenolic acid in human plasma using dual spectrophotometric detection. *J Chromatogr B Biomed Sci Appl* 2001;750:155–161.
211. Renner UD, Thiede C, Bornhauser M, Ehninger G, Thiede HM. Determination of mycophenolic acid and mycophenolate mofetil by high-performance liquid chromatography using post column derivatization. *Anal Chem* 2001;73:41–46.
212. Jain A, Venkataramanan R, Hamad IS, Zuckerman S, Zhang S, Lever J, Warty VS, Fung JJ. Pharmacokinetics of mycophenolic acid after mycophenolate mofetil administration in liver transplant patients treated with tacrolimus. *J Clin Pharmacol* 2001;41:268–276.
213. Weber LT, Shipkova M, Armstrong VW, Wagner N, Schutz E, Mehls O, Zimmerhackl LB, Oellerich M, Tonshoff B. The pharmacokinetic-pharmacodynamic relationship for total and free mycophenolic acid in pediatric renal transplant recipients: a report of the German Study Group on Mycophenolate Mofetil Therapy. *J Am Soc Nephrol* 2002;13:759–768.
214. Gross SP, Driscoll JA, McClafferty TA, Edwards RL, Chun A. Performance of the mycophenolic acid method on the Dade Behring Dimension® Clinical Chemistry System. *Clin Chem* 2006;52(Suppl):A63 (Abstract).
215. Luo W, Nimmagadda S, Ruzicka R, Tsai A, Loor R. Development of application protocols for CEDIA® mycophenolic acid assay on the Hitachi 911, Olympus AU640, and MGC 240 Clinical Chemistry Analyzers. *Clin Chem* 2006;52(Suppl):A67 (Abstract).
216. Domke I, Engelmayer J, Langmann T, Liebisch G, Streit F, Luthe H, Dorn A, Schmitz G, Oellerich M. Measurement of total and free mycophenolic acid with new enzyme receptor methods on COBAS INTEGRA Systems. *Clin Chem* 2005;51(Suppl):A148 (Abstract).
217. Shipkova M, Schutz E, Armstrong VW, Niedmann PD, Weiland E, Oellerich M. Overestimation of mycophenolic acid by EMIT correlates with MPA metabolite. *Transplant Proc* 1999;31: 1135–1137.
218. Weber LT, Shipkova M, Armstrong VW, Wagner N, Schutz E, Mehls O, Zimmerhackl LB, Oellerich M, Tonshoff B. Comparison of the EMIT immunoassay with HPLC for therapeutic drug monitoring of mycophenolic acid in pediatric renal-transplant recipients on mycophenolate mofetil therapy. *Clin Chem* 2002;48:517–525.
219. Beal JL, Jones CE, Taylor PJ, Tett SE. Evaluation of an immunoassay (EMIT) for mycophenolic acid in plasma from renal transplant recipients compared with a high-performance liquid chromatography assay. *Ther Drug Monit* 1998;20:685–690.
220. Schutz E, Shipkova M, Armstrong VW, Niedmann PD, Weber L, Tonshoff B, Pethig K, Wahlers T, Braun F, Ringe B, Oellerich M. Therapeutic drug monitoring of mycophenolic acid: comparison of HPLC and immunoassay reveals new MPA metabolites. *Transplant Proc* 1998;30:1185–1187.
221. Westley IS, Sallustio BC, Morris RG. Validation of a high-performance liquid chromatography method for the measurement of mycophenolic acid and its glucuronide metabolites in plasma. *Clin Biochem* 2005;38:824–829.

222. Premaud A, Rousseau A, Le Meur Y, Lachatre G, Marquet P. Comparison of liquid chromatography-tandem mass spectrometry with a commercial enzyme-multiplied immunoassay for the determination of plasma MPA in renal transplant recipients and consequences for therapeutic drug monitoring. *Ther Drug Monit* 2004;26:609–619.
223. Schutz E, Shipkova M, Armstrong VW, Wieland E, Oellerich M. Identification of a pharmacologically active metabolite of mycophenolic acid in plasma of transplant recipients treated with mycophenolate mofetil. *Clin Chem* 1999;45:419–422.
224. Oellerich M, Shipkova M, Schutz E, Wieland E, Weber L, Tonshoff B, Armstrong VW. Pharmacokinetic and metabolic investigations of mycophenolic acid in pediatric patients after renal transplantation: implications for therapeutic drug monitoring. German Study Group on Mycophenolate Mofetil Therapy in Pediatric Renal Transplant Recipients. *Ther Drug Monit* 2000;22:20–26.
225. Shaw LM, Korecka M, Aradhye S, Grossman R, Bayer L, Innes C, Cucciara A, Barker C, Naji A, Nicholls A, Brayman K. Mycophenolic acid area under the curve values in African American and Caucasian renal transplant patients are comparable. *J Clin Pharmacol* 2000;40:624–633.
226. Mandla R, Line PD, Midtvedt K, Bergan S. Automated determination of free mycophenolic acid and its glucuronide in plasma from renal allograft recipients. *Ther Drug Monit* 2003;25:407–414.