ABSTRACT
Immunosuppressant drugs have contributed significantly to the success of organ transplantation. Therapeutic drug monitoring is an integral part of transplant protocols. Immunosuppressants require therapeutic drug monitoring because of their narrow therapeutic index and significant variability in blood concentrations between individuals. The variability in blood concentrations can be because of factors like drug-nutrient interactions, gender influence and polymorphism. Drug monitoring is widely practised especially for cyclosporine, tacrolimus, sirolimus and mycophenolic acid. The accuracy and specificity of the drug measurements are fundamental to the clinical interpretation of the concentration data. The issues surrounding the measurement and interpretation of immunosuppressant drug levels such as body fluids used for drug estimation, protein binding, sampling time and method for assay of the drug are discussed in detail in the article.

KEY WORDS: Cyclosporine, mycophenolic acid, sirolimus, tacrolimus, therapeutic drug monitoring

Individualizing drug therapy to obtain an optimum balance between therapeutic efficacy and occurrence of adverse events is one of the main goals for the physician. Intra- and interpatient variability makes this difficult to achieve. This variability leads to variation in drug concentration at the site of action, creating the need for individualization of the dose for each patient. In case of organ transplantation, cyclosporine became the first “critical dose” immunosuppressant drug that required a routine measurement. Since that time, there has been an expanding range of new immunosuppressant drugs for the prophylaxis of rejection in solid organ transplant recipients.

Tacrolimus, sirolimus, everolimus and mycophenolic acid are also registered immunosuppressants. Many of these drugs require measurement of concentration with subsequent dosage adjustment. With the introduction of each of these drugs, new challenges have arisen for the laboratory scientist performing therapeutic drug monitoring (TDM). The measurement of low drug concentrations seen in biological fluids during drug treatment became possible by adopting new analytical technology in the early 1960s. The first half of this article is devoted to the need for TDM of immunosuppressants while the latter half revolves around the analytical and clinical aspects of four most commonly used immunosuppressant drugs.

Why TDM for Immunosuppressants?
The most commonly used immunosuppressants require TDM because of their narrow therapeutic index and significant variability in blood concentrations between individuals. In transplant recipients, both supratherapeutic and subtherapeutic drug concentrations can have devastating results. For example, the therapeutic index of tacrolimus is narrow with a targeted therapeutic range of 5-20 ng/ml. At subtherapeutic drug concentrations, the transplant recipient is at risk for allograft rejection. At supratherapeutic drug concentrations, the patient is at risk for over-immunosuppression which can potentially lead to infection or drug-specific side effects. It is known that neurological and gastrointestinal side effects occur more frequently at higher concentrations of tacrolimus.

Immunosuppressants display significant interindividual variability in plasma drug concentrations, which creates the demand for TDM when such drugs are used. It is appropriate to look into the multitude of factors that contribute to the interindividual variability. Some of the factors discussed in this article are drug-nutrient interactions, drug-disease interactions, renal insufficiency, inflammation and infection, gender, age, polymorphism and liver mass.

Drug-nutrient interactions are becoming very widely appreciated. The metabolism of drugs sometimes also depends on the type of diet taken by the patients. For example, widely consumed grapefruit juice affects the metabolism of many drugs. Several studies have demonstrated that grapefruit juice can increase plasma concentration of cyclosporine (CsA) by
Inhibiting CYP3A-mediated metabolism and by increasing drug absorption via inhibition of P-glycoprotein efflux transporters.\textsuperscript{13,14} Hence, drug concentration needs to be monitored if the patient is consuming grape fruit juice and CsA concomitantly.

Similarly, drug-disease interactions can also contribute to interindividual variability in plasma concentration of immunosuppressants. For example, renal insufficiency can result in an altered free fraction of mycophenolic acid (MPA) due to the reduction in protein binding.\textsuperscript{8} Moreover, inflammation and infection are generally associated with a decline in cytochrome P450 enzyme activity. Hence, the quantum of drugs metabolized by this enzyme will increase CsA concentrations.\textsuperscript{7}

Gender also influences drug concentration. For example, the rate of MPA metabolism is higher in male kidney transplant recipients. This is secondary to a difference in glucuronidation between the two sexes.\textsuperscript{9} Likewise, age can also contribute to interindividual difference in immunosuppressant plasma concentration. For instance, pediatric transplant recipients require higher doses of CsA to maintain blood concentrations equal to those found in adults.\textsuperscript{9} Early studies on the effect of age with cyclosporine demonstrated that there was no difference in the pharmacokinetics of oral CsA with age.\textsuperscript{9} However, it is possible that this apparent lack of difference with CsA may mask a complex interplay of age-related changes.

Polymorphism has demonstrated functional consequences of many drug metabolizing enzymes. For example, CsA is a known substrate for CYP3A4/5 and P-glycoprotein. CYP3A5 is one of the main CYP3A enzymes and its expression is clearly polymorphic and shows ethnic dependence. High levels of metabolically active CYP3A5 are expressed by 45-73% of African-American but by only 5-15% of Caucasians and Japanese.

CYP3A5*3C polymorphism significantly affects the area under the curve (AUC) of oral CsA in healthy subjects as high CYP3A5 activity may increase first-pass metabolism in African Americans as compared to Caucasians.\textsuperscript{10} Several studies have demonstrated that liver mass correlates with body mass. Liver mass itself is a primary determinant in the metabolism of drugs with low extraction (or high bioavailability). While many immunosuppressants display low-to-moderate bioavailability, there is a great interindividual variability in their bioavailability, suggesting that body weight and/or liver mass may be an important source of interindividual variability in drug concentration.\textsuperscript{11} All these factors contribute to the variability of immunosuppressant concentrations which has to be maintained within therapeutic range in order to achieve the optimal benefit of drug therapy, rendering TDM necessary for these drugs.

### Drug-Specific Scenarios

In this section, issues like body fluid used for drug estimation, protein binding, sampling time and method for assay of the drug (and its metabolites) are summarized [Table 1] for cyclosporine, mycophenolic acid, tacrolimus and sirolimus.

**Cyclosporine**

Cyclosporine is a cyclic peptide immunosuppressant used to prevent graft rejection in solid organ transplant patients. CsA has been used in patients since 1978 and has proven to be effective in improving the long-term survival of patients with renal and other solid organ transplants.\textsuperscript{12} Earlier, both blood and plasma were used to measure CsA concentration. Currently, the sample matrix issue has been resolved by the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDM-CT) by considering whole blood as the matrix for estimation of CsA.\textsuperscript{13} An alternative method using saliva as the matrix for therapeutic monitoring of CsA in patients has been reported by Mendonza et al.\textsuperscript{14} This can be used in patients with difficult venous access and in pediatrics and adult patients suffering from gingival hyperplasia. The relevance of saliva concentration monitoring in CsA TDM requires further investigation. Finally, the correlation between saliva and total as well as unbound concentrations of CsA in plasma needs to be established.

CsA is more than 99% bound to blood cells and plasma proteins, leaving a very small fraction of unbound (free) drug. The pharmacological action of a highly protein-bound drug is dependent on the drug concentration at the receptor site, which is directly related to the concentration of unbound drug in plasma.\textsuperscript{14} There are now at least seven different assay systems used for the TDM of total CsA, viz., HPLC-UV, HPLC-MS, immunoassays (enzyme, enzyme donor, radiolabeled and fluorescence).\textsuperscript{15} Although immunoassays are predominantly used throughout the world, HPLC-UV is considered as the gold standard for CsA measurement.\textsuperscript{15} Although the trough CsA concentration is well within the analytical range of HPLC-MS, there are few reported HPLC-MS methods. HPLC-MS has been used where sample requirements are limited, such as biopsy or blood samples from pediatric patients.\textsuperscript{22}

### Table 1

<table>
<thead>
<tr>
<th>Feature</th>
<th>Tacrolimus</th>
<th>Sirolimus</th>
<th>Mycophenolate mofetil</th>
<th>Cyclosporine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral bioavailability</td>
<td>4-93%</td>
<td>-15%</td>
<td>94%</td>
<td>10-46%</td>
</tr>
<tr>
<td>Time to peak concentration (hours)</td>
<td>0.5-6</td>
<td>0.8-3</td>
<td>6-12</td>
<td>1.5-2</td>
</tr>
<tr>
<td>Plasma protein binding</td>
<td>88%</td>
<td>92%</td>
<td>97%</td>
<td>91-95%</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Intestinal and hepatic CYP3A4</td>
<td>Intestinal and hepatic CYP3A4</td>
<td>Hepatic glucuronidation</td>
<td>CYP3A4/5</td>
</tr>
<tr>
<td>Preferred analytical method</td>
<td>MEIA</td>
<td>HPLC-MS and HPLC</td>
<td>HPLC</td>
<td>HPLC-UV</td>
</tr>
<tr>
<td>Matrix for concentration measurement</td>
<td>Whole blood</td>
<td>Whole blood</td>
<td>Plasma</td>
<td>Plasma</td>
</tr>
<tr>
<td>Therapeutic trough concentration range</td>
<td>5-20 g/L</td>
<td>5-15 g/L</td>
<td>1-3.5 mg/L</td>
<td>150-400 mcg/L</td>
</tr>
</tbody>
</table>
A key development that has implications for both analytical methodology and approaches to monitoring CsA has been the introduction of the microemulsion formulation of the drug. [10] The microemulsion produces a more consistent concentration profile during the absorption phase with most of the intraindividual and intersubject variability occurring during the first four hours after dosing. [13] The currently available evidence suggests the use of measurement of CsA at two hours after dosing (C2) as a remedy for the variability of exposure during the first four hours after dosing. [10,19] The measurement of CsA in C2 samples does present some methodological problems to many laboratories using immunoassay techniques and possibly those using HPLC. A survey conducted by IFCC / IATDM-GT had shown that respondents report target concentrations of C2 as high as 2100 mcg/L, a concentration higher than the top calibrator of the immunoassay. [13] As a result, sample dilution becomes necessary. New control procedures are required for laboratories adopting this monitoring strategy. In light of this problem, Keevil et al. [20] reported an assay using HPLC-MS / MS that has a wide analytical range (1-5000 µg/L). The method required simple protein precipitation and had an injection-to-injection cycle time of 1.5 minutes, which is suitable for TDM.

Mycophenolic acid

Mycophenolic acid is the active immunosuppressant metabolite of the prodrug mycophenolate mofetil (MMF). MMF has been shown to be effective in reducing the rate of acute rejection. It is widely used in kidney transplantation and is indicated for heart transplantation. [21] MMF was approved for use in renal transplant patients in 1995, heart transplantation in 1998 and liver transplantation in 2000 as an alternative to azathioprine. MMF also has been used in association with tacrolimus and sirolimus and is gaining importance as a primary component in long-term immunosuppressant regimens. [19] The pharmacokinetics of MP shows large intraindividual and interindividual variability.

Plasma is the preferred matrix for MP measurement. In plasma samples, MP concentration remains constant for as long as eight hours at room temperature, for at least 96 hours at 4°C and for at least 11 months at -20°C. The recommended anticoagulant for the plasma samples is ethylene diamine tetraacetic acid (EDTA). [21] MP is extensively bound to albumin with a range of protein binding of 97-99% in patients with normal renal and liver function. In stable transplant patients, the MP free fraction ranges from 1-3%. [22] Factors that alter protein binding can affect the relationship between total and free concentrations. As significant changes in the free fraction of MP occur as a result of factors such as changes in albumin concentration, displacement of bound MP from albumin, renal failure or hyperbilirubinemia, there has been interest in the measurement of the unbound concentration of MP in plasma. Under circumstances of altered binding, the interpretation of total MP plasma concentrations must take into account the unbound plasma concentration of MP. [21]

Although a relatively simple HPLC method is available to measure drug concentrations, many centers opt to use an immunoassay. The enzyme-multiplied immunoassays technique (EMIT) is available for commercial assays. There is a recurring assay bias against chromatographic techniques with an estimated positive bias averaging about 20% although a higher bias has been noted in samples collected to 2-4 hours oral dosing. The principal reason for this bias appears to be cross-reactivity with a recently identified metabolite of MPA, the acyl glucuronide, usually known as MPAG. This metabolite should not be confused with the phenolic glucuronide. MPAG does not cross react with the antibody used in the EMIT assay. Therefore, EMIT is the preferred method for estimation. Plasma samples should be stored carefully as MPAG may be unstable in plasma, breaking down to MPA.

The time commitment for laboratory staff would be considerably reduced in the case of the EMIT-MPA assay compared with the HPLC assay. [23,24] In case of altered binding of MPA, the unbound concentration needs to be monitored.

Sometimes, the unbound MPA concentration is below the sensitivity of the EMIT assay and difficult to measure by conventional HPLC techniques. This problem was overcome by one center by developing an HPLC assay with MS detection for the measurement of unbound MPA [25] while another group opted to modify the EMIT assay to allow the unbound concentration to be measured. [26] Clinical interpretation for effective TDM is based on the monitoring of free MPA concentrations but as mentioned above, measurement of this parameter may not be possible for all laboratories because of constraints in assay sensitivity.

The trough plasma concentration monitoring of MPA and its metabolites may not provide a useful clinical tool for guiding MMF dose adjustments to avoid drug-related toxicity. [27] The AUC for MPA is the most reliable predictor of risk for acute rejection. [28] In general, AUC data have been associated more significantly with clinical efficacy than trough concentration data. [29] A full MPA AUC typically requires at least eight blood samples during a 12 hour dose interval. In clinical practice, this is impractical; therefore, abbreviated sampling schemes involving the collection of three to five plasma samples are under investigation. In clinical studies, good correlations have been noted between estimates of 12 hour AUCs using samples collected at three, four or five time points and full 12-hour AUC determinations. The measurement of AUC does incur some logistic and cost problems. [21]

Tacrolimus

Tacrolimus is an immunosuppressant agent that has emerged as a valuable therapeutic alternative to cyclosporine following solid organ transplantation. Tacrolimus has a narrow therapeutic window, displays large interindividual and intra-individual pharmacokinetics variability and is susceptible to cytochrome P450-mediated drug interactions. Thus, it is recommended that dosing of this agent be guided by routine TDM of whole blood trough concentrations. [29] Several research groups support monitoring tacrolimus concentration in plasma [30-32] but other results support the use of whole blood. [33,34] However, only a few studies have reported a significant correlation between tacrolimus blood concentration and the incidence of graft rejection. [34,35]

Optimizing tacrolimus TDM requires a detailed understanding of the drug distribution in recipients to identify a potentially better predictor of clinical outcome. [36] Warty et al. [37] studied the distribution of tacrolimus in 13 transplant recipients and reported that in plasma, 64 ± 8.0% of tacrolimus binds to lipoprotein-deficient plasma (LPDP), 21 ± 8% associates with...
high density lipoprotein (HDL), 3 ± 3% associates with low density lipoprotein (LDL) and 11 ± 3% associates with very low density lipoprotein (VLDL). Piekoszewski et al. have reported plasma protein binding of tacrolimus to be 72 ± 3.6% in a group of eight liver transplant recipients. The above two studies show that distribution and protein binding of tacrolimus vary significantly leading to changes in its unbound concentrations.

The conflicting evidence of a relationship between trough blood tacrolimus concentration and clinical outcome complicates TDM of tacrolimus. The trough concentration (achieved 12 hours after dose administration) monitoring is still the mainstay of tacrolimus measurement, based on the belief that this measurement is a good reflection of the total drug exposure. Recent data question the predictive value of a single trough concentration and suggest that a measurement made at an additional time point during the absorption phase would be useful. If the samples collected during the absorption phase are to be analyzed, the suitability of the commonly used methods needs to be considered, both in terms of calibration range and the effects of tacrolimus metabolites on performance of the assays.

A recent study by Venketaramanan used the enzyme-linked immunosorbent assay (ELISA) to measure tacrolimus concentrations in samples. The trough concentration of tacrolimus alone was not able to discriminate between graft rejection and other causes of liver dysfunction. However, the risk of acute rejection at a tacrolimus concentration of 5 ng/ml was approximately twice that at tacrolimus concentrations and was ultimately related to tacrolimus-induced nephrotoxicity. A number of analytical methods including radioreceptor monitoring, enzyme immunoassays, high performance liquid chromatography tandem mass spectrometry (HPLC/MS) and bioassays, have been developed for the measurement of tacrolimus concentrations.

Tacrolimus immunoassays (ELISA and microparticle enzyme immunoassay, MEIA) overestimate results in patient samples because the antibody cross-reacts with metabolites of the parent drug. An alternative to immunoassays for the measurement of tacrolimus is liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS is specific for the measurement of the parent drug and thus avoids the problem of metabolite interference. It is important to note that there is evidence to suggest that ELISA measurements of tacrolimus were not automatically interchangeable with measurements obtained by LC-MS/MS. The initial cost and thus lack of availability of HPLC-mass spectrometers in the many laboratories, limits the widespread use of the technique.

Sirolimus

It is a new macrolide immunosuppressant derived from Streptomyces hygroscopicus. Sirolimus is poorly absorbed from the gastrointestinal tract and the oral bioavailability is approximately 15%. Clinical studies have shown that monitoring blood sirolimus levels is necessary for successful patient management. Whole blood has been established as the matrix for routine monitoring. The drug is sequestered into erythrocytes to such an extent (approx. 95%) that measurements in plasma would be virtually impossible by the published technique. Although the drug has been shown to be stable in blood samples at temperatures up to 30°C, instability of the drug in blood samples kept at temperatures above 35°C has been noted. Thus, when samples are transported for long distances for analysis in central laboratories, refrigerated containers should be used. The peak concentrations are reached in approximately 1.4 ± 1.2 hours. Studies have shown a correlation between the trough concentration and AUC. Thus, sirolimus trough concentrations may be useful for guiding therapy. The frequency of monitoring is however less often that the other drugs considered in this review because it has a relatively long half life of about 60 hours. Currently, there is no commercial immunoassay method for sirolimus. The two major techniques for the quantification of circulating sirolimus levels are HPLC-UV and HPLC-MS. The current HPLC-UV method requires large sample volumes which are unfavorable for pediatric patients and tend to involve elaborate and labor-intensive extractions resulting in lengthy turnaround times. While extraction for HPLC-MS methods tend to be simpler which also have shorter sample analysis times, the equipment necessary for such methods is expensive and not available to most clinical laboratories.

A synergistic effect between sirolimus and cyclosporine is reported. Both sirolimus and CsA are metabolized by CYP3A4. If these two drugs are taken concomitantly, the trough sirolimus concentration is significantly higher than if sirolimus is administered four hours after CsA. Finally, the interpretation of sirolimus concentration data is based almost entirely on its use with CsA. Also, the current target concentrations for the use of sirolimus in combination with tacrolimus after liver transplantation is based on those used for sirolimus in combination with CsA. Additionally, although one of the adverse effects related to sirolimus blood concentrations is hyperlipidemia, there are no reports of pharmacokinetic interaction between sirolimus and statins.

Conclusions

The appropriate use of TDM may minimize the risk of rejection after transplantation. The monitoring of immunosuppressant drugs is still an evolving field. The value of monitoring can be enhanced by attention to both the choice of monitoring strategy and adherence to accurate sample timing. Improvement in bioanalytics will allow more sensitive and selective measurements of these drugs. The growth in the use of HPLC with MS detection holds the possibility of increased selectivity for the measurement of these compounds as well as allowing the simultaneous measurement of several co-prescribed immunosuppressant drugs. As a result, it will be possible to investigate the relationship between selective drug measurements and clinical outcome and to more easily assess the impact of genetic factors on drug metabolism.

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