Low Hematocrit and Serum Albumin Concentrations Underlie the Overestimation of Tacrolimus Concentrations by Microparticle Enzyme Immunoassay versus Liquid Chromatography–Tandem Mass Spectrometry

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Background: Rapid liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods are used increasingly for tacrolimus (TRL) monitoring but show a negative difference with respect to a microparticle immunoassay (MEIA). This report examines possible reasons for this difference between methods.

Methods: We collected 1156 samples from 277 adult and 121 pediatric recipients of liver, renal, and bone marrow grafts or hepatocyte or pancreatic islet cell implants. TRL was measured by MEIA and LC-MS/MS, and hematologic and biochemical data were collected when available.

Results: LC-MS/MS was significantly more precise ($P < 0.02$) than the MEIA with increased sensitivity. The MEIA had a median difference of 16.2% vs LC-MS/MS overall, and this was significantly affected by patient cohort ($P < 0.001$). The difference was greater in adult or pediatric liver graft recipients while they were inpatients rather than outpatients (31.8% and 14.0% vs 7.5% and 6.5%, respectively). The difference was also greater in bone marrow than kidney graft recipients (32.8% vs 15.8%, respectively). Multiple linear regression analysis showed significant inverse relationships of this difference with hematocrit (packed cell volume) and plasma albumin ($P < 0.001$) in the total cohort and a positive relationship with plasma bilirubin in a subgroup of pediatric liver graft recipients.

Conclusions: Patients with a low packed cell volume and plasma albumin are likely to show artificially high concentrations of TRL when measured by MEIA. The increased risk of underimmunosuppression must be considered should doses be reduced to lower these seemingly high TRL concentrations.

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Tacrolimus (TRL) is a potent calcineurin inhibitor that is increasingly used for primary immunosuppression after liver transplantation because of its perceived advantages over cyclosporine (1, 2). Recent clinical practice has been to reduce the TRL dosage and side-effects by use of adjunctive therapy with mycophenolate mofetil, sirolimus, or anti-interleukin-2 receptor monoclonal antibodies. Thus there has been an increased demand for therapeutic monitoring of TRL (3) coupled with a requirement for increased analytical sensitivity.

Therapeutic monitoring of TRL by microparticle enzyme immunoassay (MEIA) has been the mainstay for monitoring of this agent for the last decade. However, the MEIA has a relatively high limit of detection (LOD) (4)
and shows particularly poor precision at lower TRL concentrations with a lower limit of quantification (LLOQ) of 3.1 μg/L (5). This has led to a report (6) suggesting caution in the interpretation of TRL concentrations <9 μg/L. Certainly accurate and precise measurement below 5.0 μg/L is problematic, and the prevalence of low concentrations in stable liver graft recipients has increased because of wider use of TRL-sparing regimens (e.g., to 38.6% of our results in adult liver outpatients in the last 12 months). Recent developments in liquid chromatography–tandem mass spectrometry (LC-MS/MS) technology may address this need, delivering assays for TRL with rapid turnaround times and improvements in accuracy, precision, and sensitivity in the lower therapeutic range.

Several reports in the literature have described LC-MS/MS methods for measurement of TRL in whole blood and compared them with MEIA (7–9). These studies showed a 10–18% difference for MEIA values with respect to LC-MS/MS, with no examination of the possible causes of this difference. The present study compares an established LC-MS/MS method (9) with MEIA in a large number of samples from a diverse range of patients. It attempts to explain the differences between the two methods in relation to the hematocrit, which has been noted to adversely affect the extraction of TRL (for MEIA) from whole blood (10, 11) and markers of hepatic and renal function (thought to modulate TRL clearance).

Materials and Methods

PATIENTS AND SAMPLES

Patient samples were allocated into one of nine groups: adult liver recipients while inpatients (ALI) and outpatients (ALO); pediatric liver and hepatocyte recipients while inpatients (PLI) and outpatients (PLO); renal recipients, both in- and outpatients; bone marrow recipients and hematology patients (BM); pharmacokinetic profile samples; miscellaneous, including pancreatic islet recipients and heart recipients; and samples from patients receiving cyclosporine, not TRL. When available, hematocrit [packed cell volume (PCV)], international normalized ratio of prothrombin time, aspartate transaminase, alkaline phosphatase, γ-glutamyltranspeptidase, phosphate, and creatinine results were collected because these have either known or hypothesized relationships to TRL elimination or binding in blood. The numbers of patients and samples in each group, the details of the two phases in which the comparisons were performed, and the summary statistics on the routine hematologic and biochemical data are given in the Tables 1 and 2, respectively, of the online Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue3/.

ANALYTICAL METHODS

The MEIA was performed on the IMx analyzer according to the manufacturer’s instructions (Tacrolimus MEIA-II; Abbott Diagnostics). The LC-MS/MS assay was performed with a Waters 2795 HPLC system and a Micromass Quattro Micro mass spectrometer (Waters Ltd.) according to the method of Keevil et al. (9). Calibrators were prepared from blank, calibrator, and quality-control pools of whole blood supplied by ChromSystems to provide an eight-point calibration curve with concentrations of 0, 0.68, 3.4, 6.7, 13.4, 18.0, 27.0, and 36.0 μg/L. The full curve was run daily and used for all subsequent analyses. Quality-control samples were provided by Recipe and Abbott Diagnostics. In addition, samples from the International Proficiency Testing Scheme for TRL (PT) (12) were analyzed and the MEIA TRL calibrators were also analyzed by LC-MS/MS to further assess assay performance. The LOD of the assays were calculated as 3 SD of 10 replicates of the zero calibrator, and the LLOQ was calculated as the lowest concentration at which the interassay variability and accuracy of the assay were ≤20%.

Patient samples (derived from the sources shown in Table 1 of the online Data Supplement) were analyzed by MEIA and LC-MS/MS within 3 days of each other. To evaluate whether analytical error underlay a marked discrepancy in assay results, we selected several patient samples at random and reanalyzed them by MEIA (n = 92) and LC-MS/MS (n = 142) for separate determinations of interassay variance. Pooled patient samples used to assess intra- and interassay precision were prepared by collecting samples with TRL concentrations (MEIA) of <4.0, 4.0–8.0, 8.0–12.0, and 12.0–20.0 μg/L to produce total volumes of 30 mL each. The concentration of TRL in each pool was confirmed by MEIA.

STATISTICS

Data were analyzed by use of Analyze-It for Excel (Ver. 1.48; Smart Software) for method comparisons and by SPSS (Ver. 11.5; SPSS Inc) for comparison of data. Of the 1156 samples analyzed by both methods, 58 (5.0%) had TRL concentrations <2.0 μg/L (the cutoff value routinely used in this laboratory) when measured by MEIA and were excluded from the statistical analyses.

Results

ASSAY PERFORMANCE OF THE MEIA AND LC-MS/MS ASSAYS

For the LC-MS/MS assay, the LOD was 0.05 μg/L and the LLOQ was 1.0 μg/L. For the MEIA, the LOD was 1.58 μg/L and the LLOQ was 3.0 μg/L.

Analysis of TRL-enriched samples issued by the PT scheme gave a mean (SD) recovery of 104.5 (8.6)% by LC-MS/MS, but the MEIA showed significantly higher recovery (P = 0.001) of 135.3 (16.4)%. The MEIA-II calibrators assayed by LC-MS/MS gave a median (range) recovery of 102 (95–105)%. Analysis of PT scheme patient pooled samples showed no significant difference between the methods for this group of samples. None of the results
for the samples was outside ±2 SD of the respective group mean values calculated by the PT scheme organizers.

Interassay precision data obtained with quality-control and pooled patient samples (Table 1) showed higher precision with the LC-MS/MS. Again, duplicate analyses showed that the LC-MS/MS was significantly more precise (P <0.001, Mann–Whitney) with a variance of 4.1(0–38)% vs 13 (0–58)%.

**DIFFERENCES BETWEEN THE MEIA AND LC-MS/MS ASSAYS**

For the complete sample cohort (n = 1098), MEIA showed a median (range) difference of 16.2% (−110.3% to 200.0%) relative to LC-MS/MS. Analysis of the data by Passing and Bablok regression gave a slope of 1.134 (95% confidence interval, 1.104–1.167) and an intercept of 0.291 (0.067–0.448). The difference between methods varied among the patient groups (P <0.001, one-way ANOVA) as shown in Table 2. Post hoc analysis (Tukey) showed a smaller difference for the ALO group than the ALI and BM groups (P <0.001), a smaller difference for the PLO than the BM or PLI cohorts (P <0.001 and 0.02, respectively), and a smaller difference for the renal transplant than the ALI and BM groups (P <0.001). Difference plots for adult liver, pediatric liver, and renal recipients and for bone marrow graft/hematology patients are shown in panels A through D respectively, of Fig. 1. These data suggest an effect of the time post transplantation and the organ engrafted on the difference between methods.

**EFFECT OF HEMATOLOGIC AND BIOCHEMICAL VARIABLES ON ALL SAMPLES**

Multiple stepwise linear regression with intermethod difference as the dependent variable gave an optimal relationship of PCV, albumin, creatinine, and phosphate as predictor variables (P <0.001, <0.001, <0.039, and <0.042, respectively) with the adjusted r² being 0.343 (P <0.001). Bivariate analysis showed that the difference between methods correlated inversely with the PCV (Spearman r = −0.53; P <0.001), and stratification of the PCV results (Fig. 2) showed an increasingly large difference at a PCV of ~0.360. Similarly, for plasma albumin concentrations, the difference correlated inversely with plasma albumin (Spearman r = −0.46; P <0.001), increasing within the reference interval (Fig. 3).

There was a significant correlation between the intermethod difference and creatinine in both the adult (r = 0.11; P = 0.015; n = 551) and pediatric groups (r = 0.146; P = 0.05; n = 185), which were subdivided to control for

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Table 1. Interassay precision for the pools of patient and commercial control samples.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>MEIA Intercept (µg/L)</th>
<th>MEIA Slope (µg/L)</th>
<th>LC-MS/MS Intercept (µg/L)</th>
<th>LC-MS/MS Slope (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled patient blood</td>
<td>3.03 (0.84)</td>
<td>2.08 (0.32)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abbott QC Low</td>
<td>4.72 (0.85)</td>
<td>4.82 (0.24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abbott QC Medium</td>
<td>11.29 (1.12)</td>
<td>10.49 (0.51)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abbott QC High</td>
<td>22.46 (2.05)</td>
<td>22.21 (1.05)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Summary data from MEIA vs LC-MS/MS regression and difference plots.

<table>
<thead>
<tr>
<th>Patient cohort</th>
<th>Passing and Bablok Difference</th>
<th>Interquartile range</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALI</td>
<td>0.675 (−0.210 to 0.035)</td>
<td>1.250 (1.147–1.345)</td>
</tr>
<tr>
<td>ALO</td>
<td>−0.603 (−1.060 to −0.126)</td>
<td>1.190 (1.105–1.279)</td>
</tr>
<tr>
<td>BM</td>
<td>1.137 (0.433–1.920)</td>
<td>1.133 (0.957–1.304)</td>
</tr>
<tr>
<td>PLI</td>
<td>0.280 (−0.240 to 1.100)</td>
<td>1.104 (1.000–1.200)</td>
</tr>
<tr>
<td>PLO</td>
<td>−0.567 (−1.155 to −0.032)</td>
<td>1.143 (1.067–1.221)</td>
</tr>
<tr>
<td>Renal</td>
<td>−0.185 (−1.000 to 0.562)</td>
<td>1.206 (1.095–1.333)</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>0.973 (−2.513 to 0.424)</td>
<td>1.226 (0.932–1.550)</td>
</tr>
<tr>
<td>PK</td>
<td>0.636 (0.286–0.911)</td>
<td>1.091 (1.037–1.143)</td>
</tr>
</tbody>
</table>

a The results of Passing and Bablok regression analysis are shown as the mean (95% confidence interval), and the differences between methods for each of the patient groups are shown as the median (range).
b Renal transplant recipients, in- or outpatient.
c Includes samples from pancreatic islet recipients.
d PK, pharmacokinetic profile samples (collected from adult liver recipients).
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the effect of body mass on serum creatinine. However, in the adults the slope of the correlation line approached 0, whereas in the pediatric group, 10 of the 11 samples with a high creatinine (>100 μmol/L) came from one patient. There was no significant bivariate correlation between intermethod difference and plasma phosphate (Spearman

Fig. 1. Bland–Altman difference plots for MEIA vs LC-MS/MS in four patient populations. Results are presented for samples from adult liver transplant recipients (A), pediatric liver transplant recipients (B), bone marrow recipients and hematology patients (C), and renal transplant recipients (D). In all cases: Difference = [(LC-MS/MS result – MEIA result)/LC-MS/MS] × 100. In A and B, the median difference for inpatients is indicated by the dotted line (with individual points as •) and the difference for the outpatient group by the solid line (with individual points as ○). The median differences between methods for the in- and outpatients are 31.7% and 7.7%, respectively, in adults and 14% and 6.1%, respectively, in the children. In C and D, the median difference is indicated by the dashed line and is 32.7% and 16.0%, respectively.

Fig. 2. Increase in difference between assays with decreasing PCV values. Data are presented grouped by incremental PCV values of 0.01 L/L. × indicate outliers.
There were no significant effects of any serologic marker in the pediatric cohort should be interpreted cautiously. Between interassay differences and increases in bilirubin concentrations. Therefore, the relationship between interassay differences and increases in bilirubin in the pediatric cohort should be interpreted cautiously. There were no significant effects of any serologic marker in the BM, renal transplant, or pharmacokinetic profile sample cohorts.

**Discussion**

The higher TRL MEIA results compared with LC-MS/MS results were strongly associated with the PCV and/or the serum albumin concentration. In establishing this association, we have extended previous findings of differences between the two assay methods that have been interpreted without the benefit of additional clinical or biochemical data (7–9, 13, 14). These differences are important for the management of transplant recipients because nonequivalence of assay results may complicate the setting of TRL dosages.

Low PCV values are known to increase the concentration of TRL measured by MEIA, possibly by affecting the extraction of TRL from blood cells (10) or by a cross-reaction of non-TRL components in the extracted sample and the anti-tacrolimus antibody used in the MEIA (11, 15). Additional explanations may be an increasing hepatic extraction of TRL from blood with decreasing PCV (16) or with low concentrations of TRL-binding proteins in blood (e.g., α1-acid glycoprotein and albumin). However, albumin in buffer had no effect on hepatic TRL extraction in studies of perfused rabbit livers (16). The net effect of this proposed increase in extraction efficiency will be an increase in TRL metabolism, with likely increases in metabolite concentrations in blood, particularly in cholestasis. This is suggested by the effect of high bilirubin concentrations on the interassay difference in the PLI group and may be masked in the ALI group by the use of artificial liver support, which decreases the concentrations of bilirubin (and other compounds) in plasma. TRL is tightly bound to blood cells and plasma proteins (17), and if the PCV in particular is decreased (16), the unbound fraction of TRL will increase. The likely consequences will be a marked effect on extraction efficiency, both in vivo (affecting metabolism) and in vitro (affecting assay extraction efficiency), and a disproportionate increase in TRL and its metabolites.

A relationship between TRL metabolites and the difference between MEIA and LC-MS/MS results has been proposed in heart and lung (7, 9) and liver and kidney recipients (8, 13, 14). TRL has at least 15 metabolites (18), but only 1 of these, M2 (31-O-demethyl tacrolimus), shows substantial immunosuppressive activity (19, 20) comparable to the parent compound. The MEIA has been shown to cross-react with TRL metabolites M2, M3, and M5 at cross-reactivities of 54–67% of that of TRL (Abbott MEIA II assay insert). Although there are few studies of TRL metabolites in blood in a wide range of patients in the immediate posttransplantation or long-term stable populations (8, 21–24), there does appear to be an increase in metabolite concentrations during periods of impaired liver function (24) and after 2 weeks post transplantation (21). The latter effect is confirmed by an increase in interassay differences from 6% to 40% between days 6 and 10 post transplantation, with no corresponding marked change in PCV or albumin concentration observed in one patient in the ALI group with intensive posttransplantation monitoring.

All of these data are consistent with the smaller interassay difference observed in the outpatient groups characterized by relatively higher PCVs and plasma albumin concentrations (Table 2 of the online Data Supplement). There may be an additional contribution from a decrease in the total amount of CYP3A-inducing medication taken by these patients (e.g., steroids) compared with the inpa-
tient population. An alternative explanation might be ion suppression of TRL or the internal standard in the LC-MS/MS assay. Such suppression may be greatest in patients with poor hepatic or renal function because of the concentrations of potentially interfering compounds. However, our own, albeit limited, assessment of ion suppression and the known inaccuracy of the MEIA with enriched samples suggest this is not the case, as does the parallel observations of comparable differences in at least five other studies using dissimilar LC-MS/MS methodologies (7–9, 13, 14).

Further resolution of the basis of these differences between LC-MS/MS and MEIA results will require more detailed studies on the effect of the PCV and plasma bilirubin and albumin concentrations on TRL metabolite concentrations, extraction efficiency, and ion suppression. The effects of these variables on interassay differences for patients receiving TRL for indications other than liver transplantation also require investigation, given the low percentage of samples in the BM group with biochemical and hematologic data and the low spread of PCV values in the renal group (Table 2 of the online Data Supplement).

A final consideration must be differences between the magnitude of the difference in this and other published series, reported as 10–18% (7–9, 13, 14), compared with the patient samples circulated in the Tacrolimus International Proficiency Testing Scheme (median ~6%). A possible explanation is that metabolite concentrations in the stored pooled samples (median difference, 6.9% and 5.6% in liver and renal pools, respectively; n ≥10) are lower than those found in fresh samples from corresponding patients (median = 7.5% and 15.8%, respectively, in this study) because of instability. Another possibility is that the PT pools are prepared largely from outpatient samples and therefore have near-normal PCV and albumin values. The additional possibility, that MEIA routinely overestimates TRL concentrations, is suggested from consistent overestimates by the assay of TRL concentrations in enriched PT samples, and we have confirmed this with parallel observations using independent quality-control samples. However, analysis by LC-MS/MS of the MEIA calibrators supplied by Abbott gave recoveries of between 95% and 105%; thus, the complexity of these findings requires further clarification.

We found the LC-MS/MS assay to be significantly more precise than the MEIA over the clinically relevant concentration range, with CVs ranging from 4.7% to 15% for the LC-MS/MS compared with 9.1–28% for the MEIA (Table 1). This confirms the findings of Ghoshal and Soldin (6), which led them to suggest that TRL concentrations <9.0 μg/L should be treated with caution. In our series of 989 samples monitored routinely (median MEIA TRL concentration = 6.6 μg/L; interquartile range, 4.2–9.3 μg/L), 72.7% had concentrations below this value, and this high proportion emphasizes the reason for concern in relation to current therapeutic and management practices.

In summary, we have reported that the MEIA-II for TRL gives results that are a median of 16.2% higher than those obtained by LC-MS/MS, confirming several reports in the literature. We have been able to demonstrate that this difference is related to the PCV and plasma albumin, with decreases in both variables increasing the difference between the assays. In patients with normal or near-normal PCV and plasma albumin concentrations within the reference interval, the customary small difference between the results (<7%) is unlikely to affect clinical management. However, patients with marked decreases in PCV and/or plasma albumin will show proportionately lower TRL concentrations when samples are measured by LC-MS/MS, and this may also apply during severe cholestatic episodes. The precise cause of this larger difference is unclear at present, but the increased values seen with the MEIA could lead to the false impression of adequate immunosuppression in patients with a low PCV or albumin (attributable, for example, to a poorly functioning or rejecting liver graft or graft-vs-host disease in bone marrow transplant patients). The introduction of LC-MS/MS to this laboratory for measurement of TRL has led to a marked improvement in assay performance. It has enabled the delivery of TRL concentrations, seemingly without interference from metabolites and with increased sensitivity and precision, in a timely manner. An audit of effective therapeutic ranges for TRL in the transplant populations is now required to evaluate the clinical impact of this improved analytical technique.

References

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