Measurement of Whole Blood Tacrolimus Level by High Performance Liquid Chromatography Tandem Mass Spectrometry in Renal Transplant Recipients — A Single Center Perspective

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Background and Methods: Measurement of whole blood tacrolimus level by high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) was compared with that measured by microparticle enzyme immunoassay (MEIA) in 30 renal transplant recipients.

Results: Whole blood tacrolimus concentrations measured by HPLC-MS/MS were significantly lower than those measured by MEIA, with a median difference (interquartile range, IQR) of −0.40 μg/L (2.03 μg/L) (p < 0.0005). MEIA overestimated tacrolimus concentrations by a median (IQR) of 5.04% (19.5%). There was good correlation between the two methods (r = 0.94; p < 0.0005). The Passing-Bablok regression equation was: HPLC-MS/MS (μg/L) = 0.96 (95% confidence interval, CI, 0.91–1.00) × MEIA (μg/L) − 0.02 (95% CI, −0.40–0.46). Bland-Altman analysis showed that the 95% limits of agreement were 2.98 to −4.10 μg/L. The 12-hour area under the concentration curve (AUC₁₂) of tacrolimus derived using the two-point sampling equation with tacrolimus concentrations measured by HPLC-MS/MS was compared with that measured by MEIA. The AUC₁₂ values calculated by the two methods were highly correlated (r = 0.90; p < 0.0005). The mean difference between the AUC₁₂ values was 3.4 ± 11.6 hr.μg/L, and the mean percentage difference was 2.6 ± 11.4%, both of which were not statistically significant.

Conclusion: For tacrolimus concentrations within the recommended therapeutic range, the concentration measured by HPLC-MS/MS was statistically significantly lower than that measured by MEIA, but the difference was not clinically significant. Introduction of the more specific HPLC-MS/MS method does not require adjustment in the recommended tacrolimus trough concentration or the AUC₁₂ level estimated by our abbreviated regression equation. [Hong Kong J Nephrol 2005;7(2):65–9]

Key words: drug concentration, HPLC-MS/MS, MEIA, kidney transplant, tacrolimus
INTRODUCTION

Tacrolimus is a potent immunosuppressive agent used in solid organ transplantation. It has a relatively narrow therapeutic index [1]. The correlation of dosage to its blood concentration is poor as a result of variability in pharmacokinetic parameters among patients [2]. A consensus has been made that therapeutic monitoring of whole blood tacrolimus concentration at steady state is required in view of its dose-related efficacy and toxicity, narrow therapeutic index, possible cytochrome P450 mediated drug interactions, and considerable interpatient variability in its pharmacokinetics [3]. Microparticle enzyme immunoassay (MEIA) has been commonly used for the measurement of tacrolimus concentrations in blood as the instrument required is not very expensive and can be found routinely in clinical laboratories [4]. However, MEIA uses an anti-tacrolimus monoclonal antibody that recognizes not only the parent drug but also several of its metabolites. Previous studies have demonstrated that this may lead to overestimation of drug concentration when compared with a method that is specific for the parent drug [4,5].

High performance liquid chromatography (HPLC) tandem mass spectrometry (MS/MS) is a sensitive and specific method for measuring whole blood tacrolimus concentrations [6]. HPLC-MS/MS has recently been introduced into hospitals in Hong Kong to replace the less specific MEIA. However, our past clinical experience with tacrolimus in renal transplant recipients has been based on tacrolimus levels measured by MEIA. To determine if the change in assaying method would affect our recommended drug dosages for renal transplant recipients, we compared the results obtained by the new HPLC-MS/MS method against the previously established MEIA assay results in the clinical setting of a group of stable renal transplant recipients in our center. We examined whether the change in measurement methods would affect the clinical management of these patients, especially regarding whether or not there is a need to develop an assay-specific target blood tacrolimus concentration range in the maintenance immunosuppressive therapy of renal transplant recipients.

METHODS

Thirty renal transplant recipients receiving tacrolimus were recruited into the study. Their standard immunosuppressive regimen included tacrolimus, prednisolone (7.5 mg/day) and azathioprine (1.5 mg/kg). To compare the analytical performance of the two assay methods, 134 blood samples from these patients were collected by venipuncture and sent to the laboratory in ethylenediaminetetraacetic acid (EDTA) tubes. The whole blood tacrolimus concentrations of each blood sample were measured by both MEIA and HPLC-MS/MS.

To compare the effects of the two analytical methods on clinical management, 50 pairs of 2-hour post-dose (C2) and 4-hour post-dose (C4) whole blood tacrolimus concentrations from the recruited patients were used. Estimation of the 12-hour area under the concentration curve (AUC$_{12}$) for tacrolimus was done by using a two-point sampling method with the equation, AUC$_{12} = 16.2 + 2.4 \times C2 + 5.9 \times C4$, that was previously validated by our group using tacrolimus concentrations as measured by MEIA [7]. The AUC$_{12}$ derived from tacrolimus concentrations measured by HPLC-MS/MS using the same equation was then compared with the AUC$_{12}$ derived from tacrolimus concentrations measured by MEIA.

MEIA

MEIA was performed on an IMx System analyzer according to the manufacturer’s instructions (IMx Tacrolimus II assay; Abbott Diagnostics, Abbott Park, IL, USA). In short, a whole blood sample was extracted with a precipitation reagent and centrifuged. Tacrolimus in the supernatant competed with tacrolimus-alkaline phosphatase conjugate for antibodies coated on microparticles. An aliquot of the reaction mixture containing the tacrolimus or conjugate bound microparticles was transferred to a glass fiber matrix; the microparticles bind irreversibly to the glass fiber matrix. The matrix was washed to remove unbound materials. A fluorogenic substrate for alkaline phosphatase was added to the matrix and the fluorescent product was measured by the optical assembly. The rate of fluorescence production is inversely related to the concentration of tacrolimus in the test sample. Between-run coefficients of variation were 4.2% at 19.7 g/L and 9.7% at 6.2 g/L. The limit of detection was 1.5 g/L.

HPLC-MS/MS

The HPLC-MS/MS method was performed on the Agilent 1100 HPLC system (Agilent Technologies Inc, Palo Alto, CA, USA) equipped with the Sciex API 2000 MS/MS detector (Applied Biosystems, Foster City, CA,
USA). For sample preparation, 40 μL of calibrators, quality control or patient samples (EDTA whole blood) were vortex-mixed for 30 seconds with 140 μL of a protein precipitation solution containing ascomycin and zinc sulfate in methanol. After centrifugation for 5 minutes at 17,000g, the supernatants were dispensed into disposable sample vials and then placed into the auto-sampler for injection into the system. To separate tacrolimus and ascomycin from matrix interference prior to MS/MS analysis, 40 μL of supernatant was injected into a DASH™ HyPURITY C18 column (20 mm × 2.1 mm; 5 μm) (Thermo Electron Corp, Bellefonte, PA, USA) maintained at 50°C inside the column oven. The column was washed for 30 seconds (isocratic flow rate, 900 μL/min) with a mixture of methanol and 0.1% formic acid in 2 mM ammonium acetate (50:50, by volume). Both tacrolimus and ascomycin were eluted into the detector. MS/MS analysis was performed in multiple reactions monitoring mode using the following transactions: m/z809.5→756.5 for ascomycin, and m/z821.6→768.5 for tacrolimus, with a dwell time of 40 ms/channel. The column was reconditioned with the 50% methanol solution for 1 minute before the next injection. The injection-to-injection cycle time was 4 minutes.

System control and data acquisition were performed using Analyst 1.2 software (Applied Biosystems/MDS Sciex) for automated data processing. Tacrolimus/ascomycin peak area ratios for the calibrators, quality control samples and patient samples were calculated. The calibration curve was constructed using linear least squares regression with 1/x weighting. The between-batch coefficients of variation for HPLC-MS/MS were 3.2–5.2% for three levels of quality control samples (mean concentrations: 4.3, 10.0 and 22.6 μg/L). The mean recovery was 96.4%. The detection limit was 0.5 μg/L. Ninety percent of the patients had an absolute difference of less than 3.1 μg/L. Thus, in our center, blood tacrolimus concentrations measured by HPLC-MS/MS were found to be significantly lower than that measured by MEIA, with a median difference of −0.40 (2.03) μg/L (p < 0.0005). Percentage difference (%diff) was calculated as the difference between the two methods as a percentage of the HPLC-MS/MS value. The median %diff was 5.04% (19.5%). The relationship between tacrolimus concentrations as measured by MEIA and HPLC-MS/MS is shown in Figure 1. There was a good correlation between the two methods (r = 0.94; p < 0.0005). The Passing-Bablok regression equation was: HPLC-MS/MS (μg/L) = 0.96 (95% confidence interval, CI, 0.91–1.00) × MEIA (μg/L) − 0.02 (95% CI, −0.40–0.46). A Bland-Altman analysis was also performed and the 95% limits of agreement were calculated [7]. The differences between the tacrolimus concentrations measured by HPLC-MS/MS and MEIA were plotted against their average values (Figure 2). The 95% limits of agreement were 2.98 to −4.10 μg/L. Ninety percent of the patients had an absolute difference of less than 3.1 μg/L. Thus, in our center, blood tacrolimus concentrations measured by

**Results**

Of the 30 stable renal transplant recipients recruited, 16 (53%) were male. The mean age was 42.6 ± 11.4 years. All patients had normal liver function. Whole blood tacrolimus concentrations were measured at a median of 13.5 months (IQR, 0.1–88.9 months) post-transplant as part of routine clinical care. The number of samples taken from each patient ranged from 1 to 14.

For the evaluation of the analytical performance of the two assays, the median blood tacrolimus concentrations measured by HPLC-MS/MS and MEIA were 9.75 (7.08) μg/L and 10.30 (8.08) μg/L, respectively. Blood tacrolimus concentrations measured by HPLC-MS/MS were found to be significantly lower than that measured by MEIA, with a median difference of −0.40 (2.03) μg/L (p < 0.0005). Percentage difference (%diff) was calculated as the difference between the two methods as a percentage of the HPLC-MS/MS value. The median %diff was 5.04% (19.5%). The relationship between tacrolimus concentrations as measured by MEIA and HPLC-MS/MS is shown in Figure 1. There was a good correlation between the two methods (r = 0.94; p < 0.0005). The Passing-Bablok regression equation was: HPLC-MS/MS (μg/L) = 0.96 (95% confidence interval, CI, 0.91–1.00) × MEIA (μg/L) − 0.02 (95% CI, −0.40–0.46). A Bland-Altman analysis was also performed and the 95% limits of agreement were calculated [7]. The differences between the tacrolimus concentrations measured by HPLC-MS/MS and MEIA were plotted against their average values (Figure 2). The 95% limits of agreement were 2.98 to −4.10 μg/L. Ninety percent of the patients had an absolute difference of less than 3.1 μg/L. Thus, in our center, blood tacrolimus concentrations measured by

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**Statistical analysis**

MedCalc statistical package version 7 (MedCalc Software, Mariakerke, Belgium) was used for data analysis. The Kolmogorov-Smirnov test was used for Gaussian distribution testing. Data were expressed as mean ± standard deviation or median (interquartile range, IQR) where appropriate. The Wilcoxon test was used to test for significance of difference between the two assays. To analyze the agreement between the two assays, Passing-Bablok regression analysis, the Bland-Altman method [8], and Spearman’s rank correlation were used. A p value of less than 0.05 was considered to be statistically significant.

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Figure 1. Passing-Bablok regression plot of tacrolimus concentrations as measured by MEIA and HPLC-MS/MS in renal transplant recipients.
the new HPLC-MS/MS tended to be lower than the previously used MEIA.

To evaluate the effects of the two assays on clinical management, the AUC$_{12}$ values calculated by our previously validated equation using tacrolimus concentrations measured by the two methods were compared in 50 paired samples. The medians of AUC$_{12}$ for tacrolimus measured by HPLC-MS/MS and MEIA were 86.9 (35.0) hr.$\mu$g/L and 92.2 (41.4) hr.$\mu$g/L, respectively. The AUC$_{12}$ values derived from tacrolimus concentrations measured by the two methods were highly correlated ($r = 0.90; p < 0.0005$). The mean difference between the AUC$_{12}$ values was 3.4 ± 11.6 hr.$\mu$g/L, which was not statistically significant ($p = 0.059$). The mean %diff calculated as the mean of the difference between the two methods as a percentage of AUC$_{12}$-MEIA was 2.6 ± 11.4%, which was not statistically significant ($p = 0.107$).

**DISCUSSION**

HPLC-MS/MS is a sensitive and specific method for measuring tacrolimus concentration, but its use has been limited by availability and high instrumental cost. Immunoassays such as MEIA provide a simple and convenient alternative for measuring tacrolimus concentration [4]. However, these assays have the disadvantage of the anti-tacrolimus monoclonal antibody exhibiting substantial cross-reactivity with tacrolimus metabolites, in particular 31-O-demethyl (M-II), 15-O-demethyl (M-III) and 15,31-O-didemethyl (M-V) [9]. Several groups have reported higher tacrolimus concentrations in patient samples measured by immunoassays compared with specific methods such as HPLC-MS/MS [4, 5, 10]. This discrepancy could be caused by the presence of cross-reacting tacrolimus metabolites in the patient specimens that may or may not be immunosuppressive. In our study, we also confirmed the overestimation of whole blood tacrolimus concentration by MEIA. The median difference between the two assays was 0.40 $\mu$g/L, corresponding to a median overestimation of 5.04% by MEIA. However, the correlation between the two assays was excellent ($r = 0.94$).

The reported overestimation of tacrolimus concentrations by immunoassays in renal transplant recipients in other studies has ranged from 18% to 48% [4, 10], which is much greater than our current finding. It has been reported that for cyclosporine drug level monitoring, the discrepancy between HPLC-MS/MS and immunoassays is greater when trough level samples are used instead of C2 level samples [11]. The reason might be due to the different proportions of parent cyclosporine and metabolites at these two time points. At the trough level, the relative concentration between the parent drug and its cross-reacting metabolites is higher when compared with that at C2, thus resulting in a larger discrepancy [11]. We believe that our smaller observed discrepancy between the two assaying methods compared with previously published results is due to the fact that the majority of our samples were collected at C2 or C4, when there is a lower relative concentration between the parent drug and its cross-reacting metabolites.

The specific HPLC-MS/MS assay of tacrolimus concentration has been recently introduced in Hong Kong to replace the immunoassay methods. The change in assay method for measuring tacrolimus drug concentration might affect our recommended drug concentration for our renal transplant recipients. In this study, we demonstrated that the median difference in tacrolimus concentration measured by the two methods was only 0.4 $\mu$g/L, with a %diff of 5.04%. Although the difference was statistically significant, it was not clinically significant and did not affect our daily clinical management. We believe that a change in the recommended drug concentration for maintenance tacrolimus therapy will not be required. However, clinicians should be aware that for tacrolimus concentrations lying at the extremes of distribution, the discrepancy between the two assays would be much greater and might be of clinical significance. More aggressive dosage adjustment of tacrolimus to minimize tacrolimus-related adverse effects might be required, especially at the time of early post-kidney transplant.

The usefulness of using tacrolimus trough levels in differentiating graft rejection episodes from nephrotoxicity has been questioned [12]. The use of AUC for tacrolimus has been suggested as a more
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precise model for tacrolimus monitoring after renal transplant [13]. A dosing regimen with a target AUC_{12} of approximately 100 μg/L has been advised in renal transplant recipients to minimize adverse events [13]. Traditionally, AUC is determined from six or more concentration-time data points. Its routine clinical use is limited by the need for multiple blood samples and drug measurements, which causes inconvenience to both patients and medical personnel. Instead, our group has derived and validated a two-point sampling method using C2 and C4 tacrolimus concentrations measured by immunoassay to estimate the AUC_{12} as the tacrolimus monitoring strategy for our renal transplant recipients [7]. In our center, we have been using this abbreviated regression equation to guide our tacrolimus dosing, aiming for AUC_{12} of 100 hr.μg/L at 3 months post-kidney transplant, with good outcome. However, the change in the tacrolimus assay method may affect our dosing recommendation using the abbreviated regression equation. In this study, we estimated the AUC_{12} of 50 paired samples by the equation using tacrolimus concentrations measured by HPLC-MS/MS and MEIA respectively. We found that the AUC_{12} calculated with tacrolimus levels measured by the two methods were highly correlated ($r = 0.90$; $p < 0.0005$), and the mean difference between them was statistically insignificant. We believe that for maintenance therapy in renal transplant recipients, clinicians should continue to titrate the dosage of tacrolimus using the same abbreviated equation calculated with tacrolimus concentrations as measured by HPLC-MS/MS, targeting at AUC_{12} of approximately 100 hr.μg/L at 3 months post-transplant. However, further validation of the equation by using tacrolimus concentrations measured by HPLC-MS/MS will be required in future.

In conclusion, blood tacrolimus concentrations measured by HPLC-MS/MS were significantly lower than those measured by MEIA. Apparently, for tacrolimus concentrations within the therapeutic range, the magnitude of the difference between tacrolimus concentrations measured by HPLC-MS/MS and MEIA II was not clinically significant. The introduction of the more specific HPLC-MS/MS assay method does not require adjustment in the recommended tacrolimus trough concentration or the AUC_{12} level as estimated by our abbreviated regression equation for maintenance immunosuppressive therapy. However, care should be taken when interpreting tacrolimus concentrations at the extremes of distribution to avoid tacrolimus-related adverse effects. Further longitudinal study will be required to determine the clinical importance of the observed differences between the new and old assay methods in our renal transplant population.

REFERENCES