Switching From HPLC/UV to MEIA for Whole Blood Sirolimus Quantitation: Comparison of Methods

Luigi Alberto Pini,* Daniela Galesi, Daria Brovia, Alfio Bertolini, Diego Pinetti, Valentina Ruggieri, Stefania Pisa, Brunella Poppi, and Carmela Nives Castellana
Division of Toxicology and Clinical Pharmacology, University of Modena and Reggio Emilia,
Policlinico of Modena, Modena, Italy

Sirolimus is a immunosuppressive agent for renal transplant recipients. Monitoring of whole blood sirolimus concentration is necessary in order to improve clinical outcomes. An increasing number of clinical laboratories (4–14% during 2005) are using microparticle enzyme immunoassay (MEIA) for sirolimus quantitation but previous reports indicated a high variability, with a mean difference of 17% for MEIA method vs. high-performance liquid chromatography/ultraviolet (HPLC/UV). This study was aimed at comparing the reliability of MEIA with the HPLC/UV method. Blood samples from transplant patients were processed using both HPLC/UV and MEIA assays. Comparison and Bland-Altman plots, as well as regression analysis and paired *t*-test were used to compare results

of the assays. Concentrations were stratified into three groups and used to investigate whether any observed difference between methods could be influenced by sirolimus concentration. Regression analysis yielded a coefficient of correlation *R* of 0.9756, the line of best fit being $y = 0.9832x + 0.1976$. The statistical analysis showed no difference between the two sets of experimental data. The average percentage difference between the two methods was found to be $-0.2 \pm 19.2\%$. On the basis of our present results, the tested MEIA assay is able to quantify sirolimus concentration with a clinically acceptable imprecision, similar to that of HPLC/UV method. *J. Clin. Lab. Anal.* 20:239–244, 2006.

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Key words: sirolimus; rapamycin; high-performance liquid chromatography; microparticle enzyme immunoassay; therapeutic drug monitoring

INTRODUCTION

Sirolimus (rapamycin) (Rapamune; Wyeth-Ayerst Laboratories, Philadelphia, PA), a macrolide antibiotic originally isolated around 1970 in Rapa Nui, Easter Island, from a soil sample, is produced by *Streptomyces hygroscopicus* (1). The drug was found to exert potent antiproliferation and immunosuppressive activities. It has been shown to have a synergistic immunosuppressive effect when combined with cyclosporine in a rat model and on human blood lymphocytes in vitro (2,3). Clinical studies in renal transplant recipients have shown that a cyclosporine plus sirolimus-based immunosuppressive regimen decreases the incidence of acute rejection episodes. Moreover, this drug has a limited toxicity, and is in particular almost devoid of nephrotoxicity, neurotoxicity, and hepatotoxicity. In 1999, sirolimus was approved by the U.S. Food and Drug Administration (FDA) for renal transplantation. Later,

sirolimus has been tested and used both in liver and in bowel transplantation (4–6). Studies of blood distribution of sirolimus showed that a large percentage of the drug is found within erythrocytes (about 95%) and only 3% in plasma (7). Thus, whole blood is the matrix of choice to determinate circulating sirolimus concentrations (8). Sirolimus is metabolized by cytochrome P-450 3A4 enzymes into several demethylated and hydroxylated metabolites, that display an immunosuppressive activity of 10–30% with respect to the parent drug. A strong correlation has been reported between toxicity

*Correspondence to: Luigi Alberto Pini, Division of Toxicology and Clinical Pharmacology, University of Modena and Reggio Emilia, Policlinico of Modena, Largo del Pozzo 71, 41100 Modena, Italy.
E-mail: pinila@unimore.it

Received 16 March 2006; Accepted 28 June 2006

DOI 10.1002/jcla.20148

Published online in Wiley InterScience (www.interscience.wiley.com).

(major adverse events reported were hyperlipidemia and myelosuppression) and sirolimus concentrations above 15 ng/mL (9). Like other immunosuppressive drugs, sirolimus displays a narrow therapeutic range, characterized by a large inter- and intraindividual variability that is relevant to underdosing (transplant rejection) or overdosing (infections and side effects). Therapeutic drug monitoring (TDM) of whole blood sirolimus concentration is thus necessary in order to optimize the treatment and improve clinical outcomes.

Different chromatographic methods for the quantitation of sirolimus in whole blood have been developed and require extraction of sirolimus with solid-phase (SPE) or liquid-liquid (LLE) techniques, followed by chromatographic separation with either high-performance liquid chromatography (HPLC)–mass spectrometry (MS) detection or HPLC/ultraviolet (HPLC/UV) detection. This last technique is generally accepted for the validation of quantitative analysis of immunosuppressive drugs (10), and largely used in Italian transplantation programs. These chromatographic methods are often demanding, since they are long-time consuming and need complex extraction procedures; a long chromatographic time is often necessary to resolve the peak of sirolimus in a complex matrix as whole blood extract is. For these reasons, we investigated the possible use of reliable but less time-consuming, semi-automated method for the therapeutic drug monitoring quantitation of immunosuppressant drugs for clinical use. At the date there are several commercially available immunoassays, such as fluorescence polarization immunoassay (FPIA), microparticle enzyme immunoassay (MEIA), enzyme immunoassay (EIA) and immunophilin-binding assay (RRA). These assays represent a compromise between sensitivity, specificity and the need of fast sample preparation and high sample turnover. A preliminary study suggested a MEIA overestimation of $42.5 \pm 16.9\%$ compared to HPLC-MS. This observed positive bias was attributed to the cross reactivity of MEIA antibody to sirolimus metabolites, an effect that should decrease with increasing sirolimus blood concentration (11). Similar results were obtained by Jones et al. (12), who showed mean positive biases of 21% for MEIA in measuring sirolimus in pooled patients samples.

The present study was aimed to compare the reliability of a MEIA commercialized method (Abbott Diagnostics, Chicago, IL; kit code 5C91-21) with an HPLC/UV method. In September 2004, 57 out of 117 laboratories (49%), involved in a multicenter survey program (International Proficiency Testing Scheme), were using HPLC/UV for sirolimus monitoring and only four (3%) were using MEIA. In about 1 year, MEIA switched to 14 out of 127 (11%). This tendency

has to be examined in order to relate about the reliability and continuity of clinical data provided by clinical laboratories operating with either MEIA and/or HPLC.

MATERIALS AND METHODS

Study Design

A total of 269 consecutive blood samples from transplant patients, receiving sirolimus as part of routine immunosuppressive therapy, were used for this study. Whole blood (4 mL) was sampled into Vacutainer tubes containing ethylene diamine tetraacetic acid (EDTA), during steady-state and 12 or 24 hr after the last drug administration. At arrival in our laboratory, the sample was split into two aliquots and stored at -20°C until use. In order to compare the assays, all of the 269 blinded samples were processed using both techniques, by different operators. The results of both assays were compared using visual methods, such as comparison plot and Bland-Altman plot, and by regression analysis and paired *t*-test. External proficiency testing samples were assayed with both HPLC/UV and MEIA and results are presented here as a tool for comparison. Calibration samples of each assay were reciprocally processed in triplicate and results were used to evaluate the cross performance of the methods. Experimental sirolimus concentration range was found to be wide thus we decided to stratify our data into three groups (rank 1: < 10 ng/mL; rank 2: 11–20 ng/mL; rank 3: > 21 ng/mL) according to the mean concentration obtained by both assays. Cross-statistical *t*-test between ranks was used to investigate whether any observed difference between methods could be influenced by sirolimus concentration. This stratification has been chosen in order to compare to previous work by Salm et al. (11) and thus to compare our results to those obtained by other research groups.

HPLC Assay

The method we used has been previously described by Maleki et al. (13), and we set it up with few modifications. Sirolimus and 32-desmethoxyrapamycin, the internal standard, were provided by Wyeth-Ayerst Research Laboratories (Princeton, NY); all reagents were analytical grade and/or HPLC grade. Sirolimus and internal standard stock solutions were prepared in methanol 50%. The extraction procedure started from 0.5 mL of whole blood added to an internal standard. After the addition of 1 mL of 5% zinc sulfate and 1 mL acetone, the sample was vortex-mixed and centrifuged. The upper layer was then separated and basified with 200 μL of 100 mM sodium hydroxide, and the analytes

were extracted with 2 mL of 1-chlorobutane. The organic phase was evaporated at 37°C and the residue reconstituted with 160 µL of mobile phase. After a last washout extraction step with 0.5 mL of hexane, a 90 µL aliquot of the reconstituted sample was injected. The liquid chromatographic instrument was a Beckman-Coulter System Gold model. The analytical column was an Alltima C18 150 × 2.1 mm inner diameter (ID), 5 µm (Alltech Italia Srl, Milan, Italy) preceded by a guard column of the same kind. The analysis was performed in isocratic mode, with a mobile phase consisting of 60% acetonitrile in water flowing at 0.5 mL/min. The eluate was monitored at 278 nm, and peak height ratio (analyte vs. internal standard) was used for quantitation. Calibration standards for chromatographic analysis were prepared from whole blood added of internal standard (50 ng/mL) and sirolimus in order to obtain analyte concentrations of 0.0, 3.0, 5.0, 10.0, 25.0, 50.0 ng/mL. The limit of quantitation (LOQ) for the HPLC method was 3.0 ng/mL. Intraday and interday imprecision of our HPLC assay was tested by processing, during three consecutive sessions, five samples at three different concentrations (covering the whole calibration range, being 7.5, 15.0, and 30.0 ng/mL, respectively). Those samples were prepared in-house on sirolimus-free whole blood spiked with known analyte concentrations. The performance was evaluated using the average coefficient of variation expressed as percent (CV%) for each sirolimus control level. The intraday performance of the assay at 7.5, 15.0, and 30.0 ng/mL was 10.8%, 7.2%, and 13.0%, while the interday was 13.3%, 11.4%, and 12.7%, respectively. Average analytical recovery of sirolimus was found to be $70.2 \pm 8.5\%$. After its setting, and in-house testing, our HPLC assay entered the "Pre-Study Proficiency Testing for the Measurement of Sirolimus by HPLC" developed by Holt et al. (14). This prestudy program required the quantitation of 78 unknown sirolimus whole blood samples for testing the precision and accuracy of the assay. For acceptance, the overall CV% should have been less than or equal to 20% for samples with sirolimus concentrations above 7.5 ng/mL and less than or equal to 25% for samples with concentration below this level by reference to defined values determined using HPLC/MS/MS (14).

MEIA Assay

The microparticle enzyme immunoassay method used was developed by Abbott Diagnostics and performed on an IMx clinical analyzer. The automated IMx sequence is preceded by a pretreatment step: the whole blood sample is extracted with a precipitation reagent (zinc sulfate solution in methanol and ethylene glycol) and

centrifuged. The supernatant is then decanted into the sample well and processed. Whole blood Calibrators (0.0, 3.0, 6.0, 12.0, 20.0, and 30.0 ng/mL) and control samples (5.0, 11.0, and 22.0 ng/mL) for the immuno-enzymatic analysis were included in the kit. The LOQ for this assay is 2.5 ng/mL and intraday precision of the assay at 5.0, 11.0, and 22.0 ng/mL was 8.6%, 5.1%, and 4.9%, while the interday was 5.4%, 5.6%, and 6.2%, respectively (data from Abbott Diagnostic). The overall imprecision of MEIA assay, assessed by our laboratory and calculated again as CV% values for the three control levels provided in the kit, was found to be larger, being 10.8%, 8.4% and 10.4% at 5.0, 11.0, and 22.0 ng/mL, respectively.

Our HPLC/UV and MEIA assays fulfilled all the criteria of precision and accuracy for acceptance in a multicenter laboratories survey program: the "International Proficiency Testing Scheme" for sirolimus. This quality control protocol tests the assay performance with three samples on a monthly basis. The test includes pooled samples from patient receiving sirolimus and blood samples to which sirolimus has been added at known concentrations. The 3-monthly results are checked to see if any result is outside 3 standard deviations (3SD) of the method mean (from all laboratories participating this quality control program). If any result is, on the basis that the analysis is under suspect, all three results are excluded from the calculation of the method's mean. Then another pool of control samples is analyzed from the suspected laboratory and again the performance is evaluated (14).

RESULTS

Both our HPLC/UV and MEIA assays satisfy the requirements of the International quality control program and present quite the same average bias with respect to the monthly overall laboratories' method mean, being $3.1 \pm 12.1\%$ and $3.1 \pm 11.4\%$, respectively. In our laboratory, HPLC/UV sirolimus quantitation required 2 days to work-up an entire analysis session (about 40–60 blood samples per week, for an annual amount of 3,000 samples), while MEIA took about 2–3 hr to process the same amount of samples.

Figure 1 plots the results for each sample of MEIA against that of HPLC/UV (comparison plot). The range of whole blood sirolimus concentration measured by HPLC/UV was 3.0–34.0 ng/mL and by MEIA was 2.5–33.4 ng/mL. Regression analysis of the data yielded a coefficient of correlation R of 0.9756 and an equation for the line of best fit of $y = 0.9832x + 0.1976$. The standard error for the estimate from the linear regression was 1.5 ng/mL. Intercept doesn't differ significantly from zero ($P = 0.62$) and the equation can be recalculated.

Fig. 1. Comparison plot of sirolimus concentrations in 269 blood samples from transplant patients, as measured by HPLC/UV and MEIA. The line of best fit is represented by the solid line while the dotted line shows the line of identity. Concentrations are expressed in ng/mL.

Fig. 2. Bland-Altman plot: difference between MEIA and HPLC assay plotted against the sample mean concentration value. Concentrations are expressed in ng/mL.

lated for passing through the origin of axes ($y = x$), and slope doesn't differ significantly from 1 ($P = 0.90$). Curvature, evaluated by Mandel test, was not statistically significant.

Figure 2 plots the difference between results of tested assays and the average result for each sample, the Bland-Altman plot. On the basis of these visual representations of results comparison we carried out a paired t -test

($\alpha = 0.01$) on the two sets of data which showed no statistical difference. The average difference between results was 0.1 ± 1.5 ng/mL with an average percentage difference with respect to the sample mean concentrations of $-0.2 \pm 19.2\%$.

In Table 1, our data were divided into the three ranks. For each concentration range the resulting average difference is shown along with the relative percent value

TABLE 1. For each concentration rank the resulting average difference and average percentage difference between HPLC/UV and MEIA results along with their standard deviations are shown along with the number of data for each group (N)

Rank by sirolimus concentrations (ng/mL)		Average difference (ng/mL)	Average % difference
Rank 1 (N = 176)	<10	0.0 ± 1.4	-1.1 ± 21.9
Rank 2 (N = 62)	11–20	0.5 ± 1.8	2.8 ± 12.5
Rank 3 (N = 31)	>21	-0.1 ± 1.8	-0.1 ± 7.0

TABLE 2. Economic cost (€) of MEIA and HPLC/UV methods for a single sample

	MEIA	HPLC/UV
Staff cost (33€/hr)	0.83	22.2
Equipment	In free use	35,000.00 ^a
Equipment maintenance	0.1	1.92
Reagents	25.07	2.48
Total	25.93	27.39

^aNot computed.

with respect to the sample's mean concentration. Parametric statistical significance between groups was performed using a paired *t*-test ($\alpha = 0.01$) that showed no statistical difference.

HPLC calibrators were processed three times during successive MEIA sessions, and average bias, for known added sirolimus concentrations of 3.0, 5.0, 10.0, 25.0, and 50.0 ng/mL, was 11.5%, 10.9%, 8.6%, 8.6%, and 8.4%, respectively. The same cross evaluation on MEIA calibration samples, processed in three consecutive HPLC/UV sessions, provided an average percent variability, for sirolimus concentrations of 3.0, 6.0, 12.0, 20.0, and 30.0 ng/mL, of 12.1%, 13.2%, 11.0%, 9.8%, and 8.4%, respectively. Blank calibration samples always gave a “below limit of quantitation” response when tested with the other assay.

Finally, we evaluated the economic costs of the assays, that are reported in Table 2. The cost is referred to one sample complete of administrative and mailing charges and was computed on the basis of about 3,000 samples performed per year. The time spent by staff as the instruments use was calculated in standard conditions. Values are expressed for single sample and the currency is expressed in Euros.

DISCUSSION

HPLC-MS is the reference method for therapeutic drug monitoring. However, a large percentage of clinical laboratories operate with the HPLC/UV assay (49% for sirolimus monitoring). Since its release, in later 2004, the

MEIA sirolimus assay represents an attractive alternative to chromatographic analysis, and an increasing number of laboratories (from 4–11% in less than a year) set up with, or turned to, this immunoassay. The principal aim of this study was to make a comparison between the MEIA method and a classic HPLC/UV method used in monitoring sirolimus therapy. International Proficiency Testing Scheme evaluation established almost same performances for our HPLC/UV and MEIA assays, their average biases being $3.1 \pm 12.1\%$ and $3.1 \pm 11.4\%$, respectively.

Other reports suggested a high variability, with an over estimation of sirolimus ranging around 21% for MEIA method versus HPLC-MS, and a mean difference of 17% vs. HPLC/UV (12). Immunoassays are affected by cross-reactivity which could lead to a positive bias when compared to more specific methods. Sample hematocrit is reported to influence the overestimation of MEIA vs. HPLC. In our study we did not find this overestimation. In fact our results, as visualized in the comparison plot (Fig. 1) are spread out evenly around the line of identity ($y = x$) represented by the dotted line and this is clearly a sign of methods yielding the same results. The regression analysis of data yielded an equation for the line of best fit whose intercept could be considered not statistically different from 0 as its slope not different from 1. By this first evaluation we can exclude the influence of both constant and proportional systematic error. The tested assays are to be considered equivalent for their precision. Moreover the Bland-Altman plot (Fig. 2) does not show a trend, either in magnitude or in range. We found an overall average difference of 0.1 ± 1.5 ng/mL (the average percentage difference with respect the sample mean concentrations being $-0.2 \pm 19.2\%$), which includes 0 and we found this difference to be not statistically different from 0. Again the two methods are equivalent and no bias has been detected.

The extraction procedure for our chromatographic analysis share the same initial precipitation step (using zinc sulfate) with the MEIA sample pretreatment procedure. Thus, the influence of hematocrit on both assays should be similar and its contribution to relative differences could be probably neglected. Moreover, the tested ranks were not statistically different, which means that we did not notice any influence of sirolimus concentration on assays' relative response. Also the imprecision (CV%) data for our assays were acceptable for clinical use. In all samples we found a good correlation between both methods, and we did not find the overestimation of samples with MEIA method, possibly due to the metabolites' cross-reactivity, as hypothesized by Holt et al. (15).

Finally, the evaluation of economic cost for the two methods indicates similar costs for a medium-size laboratory performing about 3,000 samples per year. This evaluation demands that both assays be processed by the same operator. A laboratory performing a higher number of samples should be advantaged by using HPLC methods, because of the low cost of reagents and of the possibility of an automatic samples preparation, whereas laboratories performing a lower number of samples per year should use the MEIA method. In fact, the influence of calibration samples and system equilibration time for HPLC/UV is highly time spending and this cost will be stratified over a single sample's cost.

On the basis of our results, the tested MEIA assay is able to quantify sirolimus whole blood concentration with a clinically acceptable imprecision, similar to that of a widespread HPLC/UV method. In fact we did not find any overevaluation of samples, possibly linked to metabolite cross reactivity as suggested by Salm et al. (11), and we found a good correlation both at lower and higher concentrations. The technical simplicity of the immunoassay test and its turnaround time makes this assay attractive in comparison with the HPLC method and make the possibility of a widespread drug monitoring for this drug in patients with kidney or other transplants. MEIA provides a simple and rapid method for the measurement of sirolimus in blood and thus may be suitable for high-throughput routine monitoring.

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