A Fast and Simple High-Performance Liquid Chromatography/Mass Spectrometry Method for Simultaneous Measurement of Whole Blood Tacrolimus and Sirolimus

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**Context.**—Combined immunosuppressant therapy using tacrolimus and sirolimus has demonstrable benefits. Simultaneous chromatographic monitoring of whole blood tacrolimus and sirolimus is useful for reducing reagent consumption and turnaround time. We report here a simple and rapid method using high-performance liquid chromatography/mass spectrometry for simultaneous measurement of whole blood tacrolimus and sirolimus.

**Objective.**—To develop and validate a high-performance liquid chromatography/mass spectrometry method that is suitable for clinical laboratories and that is simple, rapid, and cost-effective.

**Design.**—Whole blood (80 μL) was mixed with zinc sulfate solution, followed by protein precipitation with acetonitrile containing the internal standards. After brief centrifugation, the supernatant (20 μL) was injected onto a C18 guard column. The drug and the internal standard ammonium adducts were monitored by multiple reaction monitoring. One-point calibration at levels of 200 ng/mL (249 nM) tacrolimus and 100 ng/mL (109 nM) sirolimus was prepared by adding tacrolimus and sirolimus to immunosuppressant-free whole blood.

**Results.**—The assay took 2.5 minutes per sample injection. The total imprecision was between 2.46% and 7.04% for tacrolimus and between 5.22% and 8.30% for sirolimus across the concentrations tested. No carryover was observed, and recoveries were 92% to 98% for tacrolimus and 100% for sirolimus at all levels tested. The tacrolimus was linear from 0.52 to 155.5 ng/mL (0.65–193.4 nM), and sirolimus was linear from 0.47 to 94.8 ng/mL (0.51–103.7 nM). Biases of correlations with commercial methods were within 7%.

**Conclusions.**—This improved method is simple, fast, cost-effective, and suitable for clinical laboratories. It has been implemented for routine clinical monitoring of post-transplantation immunosuppressant therapy.

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Tacrolimus is a macrolide immunosuppressant used for solid organ transplantation that inhibits cell-mediated and humoral immune responses. It is claimed to be more potent than cyclosporine A, with lower rejection rates and potentially lower toxicity. Sirolimus is also a macrolide lactone that inhibits both generation of the costimulatory signal during G0 to G1 activation of lymphocytes as well as the posttransduction events after cytokine stimulation during G1. Therapeutic drug monitoring of both tacrolimus and sirolimus is indicated because of the narrow therapeutic ranges, complex metabolism, variable pharmacokinetics, and potential drug interactions. Because of the distinct immunosuppressant mechanisms of tacrolimus and sirolimus, synergistic effects of the 2 drugs in combination have been well documented. The doses of tacrolimus and sirolimus in combination must be carefully adjusted during the management of posttransplantation patients, and the doses of sirolimus and tacrolimus have been found not to correlate well with the corresponding areas under the concentration curves in pediatric transplant patients. Because of the complex pharmacokinetics of this drug combination, close monitoring of both tacrolimus and sirolimus is essential in posttransplantation patient care.

Although a number of immunochemical methods are available for monitoring tacrolimus and sirolimus, high-performance liquid chromatography/tandem mass spectrometry (HPLC-MSMS) is considered the method of choice because of the assay specificity and simple sample preparation. In addition, HPLC-MSMS can be used for simultaneous determination of both immunosuppressants to improve throughput and efficiency. Published HPLC-MSMS methods for simultaneous determination of tacrolimus, sirolimus, or other immunosuppressants have 1 or more of the following features: multipoint calibration, solid-phase extraction (either offline or online), and analytic chromatography. Recently, an
HPLC-MSMS method has been developed for determining sirolimus levels with simplified sample preparation (no solid-phase extraction) and HPLC using only a C18 guard column.²⁴⁻²⁶ To simplify the assays of tacrolimus and sirolimus, 1-point calibration has proven to be sufficient for HPLC-MSMS determination of tacrolimus and sirolimus separately.²⁷,²⁸ Because of the increased frequency of combined therapy using tacrolimus and sirolimus, it is important to systematically evaluate an optimally simplified method measuring both drugs simultaneously. Here, we report an HPLC-MSMS method—simplified by all the feasible elements just mentioned—to determine whole blood levels of tacrolimus and sirolimus simultaneously. The assay uses structurally appropriate internal standards (ascomycin for tacrolimus, desmethoxyrapamycin for sirolimus) with simple sample preparation (protein precipitation and centrifugation), an economical HPLC method (guard column only), and rapid throughput (2.5 minutes instrument time for each sample).

**MATERIALS AND METHODS**

The tacrolimus (98.3% purity by the manufacture) and ascomycin (internal standard) were purchased from LC Laboratories (Woburn, Mass) and Calbiochem (San Diego, Calif), respectively, and sirolimus (96.7% purity by the manufacture) and desmethoxyrapamycin (internal standard) were purchased from Supelco (Bellefonte, Pa). All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ). Methanol, acetonitrile, and water were chromatography grade. Ammonium acetate and formic acid were American Chemical Society grade, and the zinc sulfate was United States Pharmacopeia grade. The assay was performed on a Quattro Micro tandem mass spectrometer fitted with a Z-spray ion source (Waters Micromass, Beverly, Mass). The mass spectrometer was operated in electrospray positive ionization mode and coupled to a Waters 2795 Alliance HT HPLC system. The operation and data processing were controlled by MassLynx NT 4.0 software (Waters Micromass).

Whole blood (with EDTA, 80 µL) and zinc sulfate solution (0.2 M, 40 µL) were mixed well to lyse the cells; this was followed by vortexing with 200 µL of an acetonitrile solution of internal standards consisting of 50 ng/mL (63 nM) ascomycin and 20 ng/mL (23 nM) desmethoxyrapamycin. After centrifugation at 9015g for 1 minute in a MicroPrep centrifuge (StatSpin, Norwood, Mass), the supernatant (20 µL) was injected onto a C18 guard column (5 µm, 4.0 mm length × 3.0 mm inside diameter, Phenomenex, Torrance, Calif) maintained at 50°C, eluted at 0.6 mL/min of 50% methanol (time, 0–0.4 minute) and 100% methanol (0.4–1.8 minutes) with 2 mM ammonium acetate and 0.1% formic acid. The instrument was re-equilibrated with 50% methanol (1.8–2.5 minutes), at which point it was ready for the next sample injection. The instrument was optimized for the application by tuning each compound by infusing a 10-ng/L solution at 10 µL/min while teeing with HPLC mobile phase at a flow of 0.6 mL/min. Multiple reaction monitoring was used to monitor the drugs and the internal standards: m/z (mass/charge) 821.5→768.5 for the ammonium adduct ion of tacrolimus, m/z 809.5→756.5 for the ammonium adduct ion of ascomycin, m/z 931.6→864.6 for the ammonium adduct ion of sirolimus, and m/z 901.6→834.6 for the ammonium adduct ion of desmethoxyrapamycin. One-point calibration at levels of 200 ng/mL (249 nm) tacrolimus and 100 ng/mL (109 nM) sirolimus and 3 quality control specimens (low, medium, and high concentrations of tacrolimus and sirolimus) were prepared by adding tacrolimus and sirolimus to immunosuppressant-free outdated whole blood obtained from the blood bank. Quantification was achieved by standard curves based on the ratios of peak areas of the drugs/internal standards.

The design of the evaluation was partially derived from the NCCLS EP10-A2 protocol for initial evaluation of laboratory methods.²⁹ The EP10-A2 protocol is a tightly structured experimental design that involves measuring 3 levels of reference material in 5 runs of 9 samples (3 at each level) for a minimum of 5 days. It estimates 2 components of variance (between- and within-run) as well as linearity, carryover, and drift. The protocol used for this evaluation used the same 9-sample run design but was expanded to 40 runs that took place during 20 days. This protocol also included a 3-component analysis of variance (within-run, between-run, and between-sample preparation; each sample preparation was run twice in consecutive runs). In each run, the sequence of specimens is designed so that 1 of each high-, medium-, and low-level specimen is preceded immediately by 1 specimen at each level. To detect carryover, the mean values of each level specimen, high, medium, and low levels specimen that immediately followed a

| Validation Data From Statistical Protocol* |
|-----------------|-----------------|-----------------|
| Tacrolimus      | Mid             | High            |
| Theoretical value, ng/mL | 1.9             | 46.7            | 97.3             |
| Recovery, % (95% CI)      | 92.1 (90.9–93.3) | 97.4 (97.0–97.9) | 97.5 (97.3–97.7) |
| Coefficient of variation | 4.54 (3.82–5.27) | 0.29 (0.24–0.33) | 0               |
| Between prep, % (95% CI)   | 2.55 (2.26–2.84) | 1.96 (1.74–2.18) | 1.37 (1.22–1.53) |
| Between run, % (95% CI)    | 4.73 (4.42–5.50) | 1.73 (1.61–1.84) | 2.04 (1.91–2.18) |
| Estimated carryover, %     | -0.01 (P = .37)  | -0.46 (P = .03)  | -0.10 (P = .43)  |
| Total analytic error       | 0.35 (18.56)     | 3.17 (6.79%)     | 6.26 (6.44%)     |
| Sirolimus              | Mid             | High            |
| Theoretical value, ng/mL | 1.9             | 24.0            | 76.9             |
| Recovery, % (95% CI)      | 100.0 (98.5–101.5) | 100.0 (99.1–101.0) | 100.1 (99.8–100.4) |
| Coefficient of variation | 0               | 3.03 (2.69–3.38) | 0.96 (0.81–1.12) |
| Between prep, % (95% CI)   | 4.87 (4.32–5.42) | 4.33 (4.04–4.61) | 5.04 (4.71–5.37) |
| Between run, % (95% CI)    | 6.72 (6.28–7.16) | 4.30 (4.94–5.63) | 5.22 (4.88–5.56) |
| Estimated carryover, %     | -0.04 (P = .20)  | -0.34 (P = .17)  | 0.46 (P = .33)   |
| Total analytic error       | 0.31 (16.28%)    | 2.49 (10.37%)    | 7.88 (10.25%)    |

* CI indicates confidence interval. To convert concentrations from conventional units to SI units: tacrolimus (ng/mL) × 1.2438 = tacrolimus (nM); sirolimus (ng/mL) × 1.0939 = sirolimus (nM).
low specimen were compared with the same respective means of those following a high-level specimen using a 1-sided \( t \) test, and all 3 levels were examined in aggregate using a 1-sided paired \( t \) test. The observed data points were fitted to a line using least-squares fit (Deming regression). Nonlinearity was tested with an \( F \) test, using the ratio of the sum-squared mean deviation from the fitted line divided by the "pure" error sum-squared scatter about the mean at each level. Finally, total analytic error (95% confidence limit) was calculated versus weighed-in theoretical values at each sample level. The statistical analysis was programmed into an Excel spreadsheet (Microsoft, Redmond, Wash). In addition, serial dilutions of known-value samples prepared with drug-free blood were performed and measured in triplicate to determine the analytic limits of quantification and linearity. Correlations with commercial methods, IMx (Abbott Laboratories) and LC-MSMS (Mayo Medical Laboratories) were performed with all clinical specimens and quality control samples.

Figure 1. Linearity result of tacrolimus (A) and sirolimus (B) by serial dilution – mean ± SD (ng/mL).

Figure 2. Method comparison: high-performance liquid chromatography/tandem mass spectrometry (HPLC-MSMS) compared with Abbott IMx for tacrolimus with all 68 clinical specimens (A) and with exclusion of the outlier (B). C, HPLC-MSMS compared with Mayo Medical Laboratories’ LC-MSMS for sirolimus with 22 clinical specimens, 3 quality controls, and the calibrator.
ries, Abbott Park, Ill) for tacrolimus and LC-MSMS (Mayo Medical Laboratories, Rochester, Minn) for sirolimus, were performed using de-identified patient specimens. The significance level for all the statistical analyses was defined as $P < .05$.

**RESULTS**

Recovery and precision data for both tacrolimus and sirolimus are shown in the Table. Analytic recovery was essentially quantitative across the measured range for both drugs, although tacrolimus showed statistically significant negative bias ($P < .001$) at all 3 levels, probably because of the imprecision of the particular calibrators or of the quality controls prepared in house using different stock solutions. The total imprecision was between 2.46% and 7.04% for tacrolimus and between 5.22% and 8.30% for sirolimus across the concentrations tested. The between-sample preparation component was in general the smallest component of variability (for sirolimus, it was barely discernible). There was no significant carryover except for midlevel tacrolimus samples, where a significant ($P = .03$) negative carryover ($-0.46\%$) probably represents a statistical artifact. Based on the combined data, carryover was not significant for either drug (paired $t$ test; $P = .28$ and .50 for tacrolimus and sirolimus, respectively). Linearity studies by serial dilution showed tacrolimus to be linear from 0.52 to 135.5 ng/mL (0.65–193.4 nM) and sirolimus to be linear from 0.47 to 94.8 ng/mL (0.51–103.7 nM), as shown in Figure 1, A and B. The limits of quantification were not investigated lower than the lowest concentrations of the linearity study (0.52 ng/mL for tacrolimus and 0.47 ng/mL for sirolimus) based on clinical needs. The clinical robustness of this method was confirmed by correlation studies using de-identified patient samples. Tacrolimus was correlated with an in-house IMx immunoassay (Abbott Laboratories), and sirolimus was correlated with a commercial HPLC-MSMS test offered by a reference laboratory (Mayo Medical Laboratories). There was virtually no bias between the HPLC-MSMS and the IMx methods (Figure 2, A), and exclusion of the data point with the highest concentration showed an underestimate of $\sim 5\%$ compared to IMx (Figure 2, B). As shown in Figure 2, C, the bias for sirolimus was less than 7% using the Mayo method as the standard.

**COMMENT**

The HPLC-MSMS assays of immunosuppressants are quickly recognized as the method of choice. Compared to standard immunoassays, HPLC-MSMS is cheap, fast, and specific, and it additionally allows simultaneous measurement of 2 or more drugs. It is our intention to report an optimally simplified HPLC-MSMS method for simultaneous measurement of tacrolimus and sirolimus to help clinical laboratories that are considering startup of HPLC-MSMS. Generally, the HPLC-MSMS returns lower results of the immunosuppressants than immunoassays, presumably because there is less interference by cross-reacting metabolites of the parent drugs. Lensmeyer and Poquetter found that patient results from HPLC-MSMS were 91.2% of IMx results, Cogill et al found a mean of 15.6%, and Armstrong et al showed 10% to 16% lower values by HPLC-MSMS than IMx depending on the patient populations. The results of tacrolimus by HPLC-MSMS were not significantly different from those of IMx in our study, probably because of our pediatric population (Figure 2, A). When we excluded the data point with the highest concentration as an outlier, the HPLC-MSMS showed $\sim 5\%$ lower results than the IMx method (Figure 2, B).

Clinical studies have established the benefits (lower rejection rates and potentially lower toxicity) of the combination therapy of tacrolimus and sirolimus in solid organ transplantation. In our facility, the use of combined tacrolimus and sirolimus has increased rapidly, and the use of cyclosporine has been decreased throughout the past years. Simultaneous monitoring of the 2 drugs is more efficient and economical than monitoring them separately when the combination therapy is used. Previously described HPLC-MSMS methods for simultaneous measurement of tacrolimus and sirolimus, sometimes with other immunosuppressants, use multipoint calibration (frequently with 6 or 8 calibrators in each batch), online or offline solid-phase extraction, and analytic chromatography. Because single-point calibration has been demonstrated to provide performance equivalent to multipoint calibration for tacrolimus and sirolimus separately, we employed single-point calibration in our method for simultaneous measurement of both drugs. In addition, we used a simplified sample preparation by protein precipitation only, followed by high-speed 1-minute centrifugation. We used a C18 guard column that functioned as an online cleanup, retaining the compounds of interest while washing off the more polar compounds. All these steps result in an assay that is highly optimized for efficiency (turnaround time of 2.5 minutes per injection, with only 1 calibrator and 3 control samples), cost-effectiveness (reduced labor and material costs), as well as excellent medical quality. Our data demonstrate not only the superiority of this method of sample preparation, but also high consistency of analytic recovery. The method has proven robust in routine operation and has been in service in our clinical laboratory for more than 18 months, with more than 3700 tacrolimus and 1000 sirolimus results reported.

**References**


