Rapid Liquid Chromatography–Tandem Mass Spectrometry Routine Method for Simultaneous Determination of Sirolimus, Everolimus, Tacrolimus, and Cyclosporin A in Whole Blood, Frank Streit, Victor William Armstrong,* and Michael Oellerich (Department of Clinical Chemistry, George-August University Goettingen, 37075 Goettingen, Germany; * address correspondence to this author at: Abteilung Klinische Chemie, Zentrum Innere Medizin, Georg-August-Universität Göttingen, Robert-Koch-Straße 40, 37075 Göttingen; fax 49-551-398551, e-mail varmstro@med.uni-goettingen.de)

With the introduction of the novel immunosuppressive agents sirolimus and everolimus, new, potentially more effective immunosuppressive regimens are undergoing clinical evaluation. Whereas the calcineurin inhibitors cyclosporin A (CsA) and tacrolimus suppress early activation of T lymphocytes through inhibition of cytokines such as interleukin 2, the primary target of sirolimus and everolimus is mammalian target of rapamycin (mTOR), a specific cell-cycle regulatory protein. The inhibition of mTOR leads to suppression of cytokine-driven T-lymphocyte proliferation (1). Because of their distinct modes of action, a calcineurin inhibitor and an mTOR inhibitor act synergistically to block acute allograft rejection (2–4). Current evidence (5) suggests that drug monitoring is necessary, not only for the calcineurin inhibitors, but also for the mTOR inhibitors. In contrast to the calcineurin inhibitors, however, there are currently no commercially available immunoassays for the latter two drugs. There is a need, therefore, for assays that can simultaneously quantify both the calcineurin and the mTOR inhibitors. Furthermore, a major shortcoming of the commercial immunoassays for CsA and tacrolimus is their cross-reactivity with unpredictable amounts of both active and inactive metabolites of the parent drugs in patient samples. In the case of CsA, no immunoassay has completely fulfilled the criteria recommended by a consensus panel (6).

New developments in immunosuppression protocols require that assays for calcineurin inhibitors provide broader dynamic ranges. On the one hand, C2 monitoring has been proposed for optimizing dosage of the CsA microemulsion (7), thereby necessitating quantification of CsA at concentrations up to 2500 µg/L; the lack of validated dilution protocols can be a problem for C2 monitoring (8). On the other hand, lower target ranges for CsA (5) and tacrolimus (9) are being investigated for long-term maintenance therapy to minimize the adverse side effects, in particular nephrotoxicity, of these drugs. Current immunoassays exhibit high coefficients of variation at drug concentrations below the present therapeutic ranges.

These facts stimulated us to develop a rapid, sensitive liquid chromatography–tandem mass spectrometry (LC-MS/MS) method with a broad dynamic range for the simultaneous determination of sirolimus, everolimus, tacrolimus, and CsA in whole blood.

Sirolimus was a kind gift of Wyeth-Ayerst Research (Princeton, NJ). CsA, cyclosporin D (CsD), and everolimus were kind gifts of Novartis (Basel, Switzerland), and tacrolimus was a kind gift of Fujisawa (Osaka, Japan). HPLC-grade methanol and analytical grade ammonium acetate and ZnSO4 were obtained from Merck. Ascomycin was purchased from Sigma-Aldrich. The calibrators and in-house controls were prepared in drug-free whole blood from stock solutions of the respective drugs in methanol. The final calibrator concentrations for sirolimus, everolimus, and tacrolimus were 0.25, 1.0, 2.5, 5.0, 10.0, 20.0, 50.0, and 100 µg/L; for CsA, the concentrations were 5.0, 25, 50, 100, 250, 500, 1000, and 2500 µg/L. The concentrations of the in-house controls were 3, 12, and 30 µg/L for sirolimus, everolimus, and tacrolimus and 60, 200, 600, and 2000 µg/L for CsA. Controls were aliquoted and kept frozen at −20 °C until use. Two internal standards were selected on the basis of the similarity of their chemical structures to those of the analytes (i.e., ascomycin for the determination of sirolimus, everolimus, and tacrolimus and CsD for the determination of CsA).

For sample preparation, 100 µL of calibrator, quality-control, or patient sample (EDTA blood) was vortex-mixed for 30 s with 200 µL of a mixture of methanol and 0.3 mol/L ZnSO4 (70:30, by volume) containing the internal standards ascomycin (50 µg/L) and CsD (250 µg/L) in 1.5-mL polypropylene tubes. After centrifugation for 5 min at 4000g, the supernatants were decanted; after recentrifugation for 1 min at 4000g, they were then placed in a series 200 autosampler (Perkin-Elmer) for injection into the LC-MS/MS system.

The column was an Aqua Perfect (150 mm × 3.0 mm; 5-µm) C18-reversed phase column (MZ-Analysetechnik) maintained at 65 °C with a Du Pont column oven. The LC-MS/MS system further consisted of a Series 200 binary pump from Perkin-Elmer, an M480 pump (Dionex), and a 10-port Rheodyne valve. Supernatants (150 µL) were injected onto the column with the series 200 autoinjector fitted with a 200-µL sample loop. The column was washed for 1 min (flow rate, 1500 µL/min) with methanol and 30 mmol/L ammonium acetate solution (80:20, by volume), followed by a 2.5-min elution step (flow rate, 800 µL/min) with methanol and 30 mmol/L ammonium acetate solution (97:3, by volume), and a 0.5-min step with methanol and 30 mmol/L ammonium
acetate solution (80:20, by volume) in preparation for the next injection.

For detection, a Sciex API 2000 triple quadrupole mass spectrometer with a turbo-ion spray (heated electrospray) interface from PE Applied Biosystems was used. The analytes that eluted from the HPLC column were introduced splitless into the turbo-ion spray source (heated to 450 °C). High-purity argon was used as the collision gas. Ionization was achieved in the positive ion mode by the following: an ionization voltage of 5700 V and an orifice voltage of 50 V; a collision energy of 19 eV for the ammonium adduct ions and an orifice voltage of 150 V; a collision energy of 65 eV for the protonated ions; and a heater-probe temperature of 450 °C. The first quadrupole was set to select the ammonium adducts [M+NH4]+ of sirolimus (m/z 931.5), everolimus (m/z 975.5), tacrolimus (m/z 821.5), ascomycin (m/z 809.5), and the protonated ions [M+H]+ of CsA (m/z 1202.8) and CsD (m/z 1216.8). The second quadrupole was used as a collision chamber, and the third quadrupole was then used to select the characteristic product ions of sirolimus (m/z 864.5), everolimus (m/z 908.5), tacrolimus (m/z 768.5), CsA (m/z 425.4), ascomycin (m/z 756.5), and CsD (m/z 425.4). The elution times for sirolimus, everolimus, tacrolimus, ascomycin, CsA, and CsD were 2.7, 2.7, 2.6, 2.6, 3.0, and 3.2 min, respectively.

A PowerMac computer with PE Sciex Sample Control (Ver. 1.4) software was used to control the LC-MS/MS and to record the output signals from the detector. Integration of peak areas, calculation of peak area ratios, calculation of the calibration curve, and calculation of the drug concentrations were performed with the FE Sciex TurboQuan™ (Ver. 1.0) software.

To investigate potential ion suppression effects attributable to the matrix, we performed the following experiments. Ten different immunosuppressant-free EDTA-anticoagulated whole-blood samples were treated with a solution of methanol and 0.3 mol/L ZnSO4 (70:30 by volume) as described above. The supernatants were then enriched with the four immunosuppressive drugs and the two internal standards to final nominal concentrations of 10 μg/L (sirolimus, everolimus, tacrolimus, and ascomycin) or 100 μg/L (CsA and CsD). A reference solution comprising 100 μL of physiologic sodium chloride solution (9 g/L) and 0.3 mol/L ZnSO4 (70:30 by volume) was also enriched with the immunosuppressive drugs and internal standards to the same nominal concentrations. The 10 supernatants and 10 preparations from the reference solution were injected into the analytical column, and the peak areas obtained from the supernatants were compared with the corresponding peak areas produced by the reference solutions. The ratios for the mean peak areas in matrix to those in the reference solution were as follows: sirolimus, 1.02; everolimus, 1.06; tacrolimus, 0.96; ascomycin, 1.00; CsA, 1.03; CsD, 1.03. Thus, no ion-suppression occurred.

The lower limits of quantification were set at drug concentrations for which an acceptable precision (CV <15%; n = 20) could still be obtained: 0.25 μg/L for sirolimus, everolimus, and tacrolimus, and 5 μg/L for CsA. The assay was linear over the working range between 0.25 and 100 μg/L (r > 0.999) for sirolimus, everolimus, and tacrolimus, and between 5 and 2500 μg/L (r > 0.999) for CsA. Performance characteristics were tested at several concentrations of sirolimus, tacrolimus, everolimus, and CsA added to EDTA-anticoagulated blood from healthy volunteers (Table 1). The measured values for the in-house control samples were within 5% of the nominal values. The within-run and between-run imprecisions were acceptable (<10%) for all four drugs. Long-term statistical evaluation of the daily in-house control data during routine measurement of sirolimus and everolimus yielded mean monthly CVs of 6.9% (3 μg/L), 6.1% (12 μg/L), and 6.9% (30 μg/L) for sirolimus and 8.3% (3 μg/L), 6.6% (12 μg/L), and 8.1% (30 μg/L) for everolimus. As might be expected, the imprecision of the method during routine application was somewhat greater than that observed during the initial evaluation.

To determine absolute recoveries, whole-blood samples were enriched with the drugs and internal standards to nominal concentrations of 10 μg/L (sirolimus, everolimus, tacrolimus, and ascomycin) or 100 μg/L (CsA and CsD). After the extraction step with a solution of methanol and 0.3 mol/L ZnSO4 (70:30 by volume), as described above, the supernatants were injected into the LC-MS/MS instrument. The peak areas obtained from the extracted blood samples were compared with the peak areas obtained on injection of equivalent amounts of the compounds in a reference solution comprising 100 μL of

<table>
<thead>
<tr>
<th>Drug</th>
<th>Analytical recovery, % (n = 5)</th>
<th>CV, %</th>
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<tr>
<td></td>
<td>3.0 μg/L</td>
<td>12.0 μg/L</td>
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<td>Within run (n = 20)</td>
<td>Between run (n = 5)</td>
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<tr>
<td>Tacrolimus</td>
<td>98.5</td>
<td>101.7</td>
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<td></td>
<td>4.5</td>
<td>3.9</td>
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<tr>
<td>Sirolimus</td>
<td>102.1</td>
<td>104.7</td>
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<td></td>
<td>5.0</td>
<td>4.5</td>
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<tr>
<td>Everolimus</td>
<td>99.0</td>
<td>96.9</td>
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<td></td>
<td>5.4</td>
<td>4.3</td>
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<td></td>
<td>60 μg/L</td>
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<td></td>
<td>3.0</td>
<td>3.1</td>
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<td>CsA</td>
<td>98.5</td>
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physiologic sodium chloride solution (9 g/L) and 200 µL of a mixture of methanol and 0.3 mol/L ZnSO₄ (70:30 by volume). Mean absolute recoveries were as follows: sirolimus, 72%; everolimus, 86%; tacrolimus, 89%; ascomycin, 93%; CsA, 71%; and CsD, 65%.

To further validate the accuracy of our method, samples from an external international proficiency-testing scheme for CsA, tacrolimus, and sirolimus (10) were analyzed by the LC-MS/MS procedure, and the results were compared with the respective HPLC-based median values from participating laboratories. As can be seen in Fig. 1, the results obtained by our LC-MS/MS method were in good agreement with those documented in the international proficiency-testing scheme. All external quality-control results were within the acceptance criteria of the proficiency-testing scheme. In context with the corresponding therapeutic ranges, even the outlier points (>95% confidence interval) would not be expected to adversely affect clinical decisions regarding dosing. This method has now been in use for almost 2 years, providing an extensive internal and external routine service for patients on sirolimus and everolimus and reporting results within 24 h of receipt of the samples. In a collective of 746 predose samples from kidney transplant recipients receiving sirolimus as part of their immunosuppressive protocol, the median concentration for sirolimus was 10.5 µg/L. The LC columns showed in general no deterioration in performance for at least 500 samples.

The tandem mass spectrometric approach excludes interference attributable to the main hydroxylated and/or demethylated metabolites of the four immunosuppressive drugs, or other commonly administered drugs. To further investigate the potential effects of other drugs, we have regularly examined the mass spectra of samples from transplant recipients on different immunosuppressive protocols to determine whether interfering peaks could be observed in the MS/MS responses of the immunosuppressive drugs that were not being administered to the patients. For example, when spectra from patients on a combination of sirolimus and CsA were examined, the MS/MS responses for the transitions m/z 821.5-768.5 (tacrolimus) and m/z 975.5-908.5 (everolimus) were below the respective lower limits of quantification. We have not yet observed any interfering peaks for the four immunosuppressive drugs in samples from solid organ transplant recipients.

The LC-MS/MS method described here for measurement of sirolimus, everolimus, tacrolimus, and CsA in blood is rapid, reproducible, and specific and requires only a small sample volume (100 µL). Several LC-MS and LC-MS/MS methods have been published for the simultaneous measurement of two or more of these immunosuppressive drugs (11–15). However, only one previously described LC-MS method measured all four drugs in a single run (12). The present procedure offers substantial advantages over available methods with a comparable limit of quantification. The analysis time (4 min) is as short as that for other methods with a more laborious offline extraction. Because samples are injected directly on the analytical column and then eluted, problems from the use of a complex switching system can be avoided. The coadministered drugs can be monitored simultaneously in the same analytical run, and 30 samples can be processed in 2 h by a single technician. Although capital costs for the LC-MS/MS equipment are high, direct operating costs are lower than those for commercial immunoassays. On the basis of our current expenditure for the routine measurement of CsA and tacrolimus using commercially available immunoassays, we have calculated that there would be a saving of ~40% per test in direct and technician costs if these measurements were transferred to
Because of its ease and rapidity, this method is ideally suited for the routine monitoring of these four major immunosuppressive drugs in clinical practice. Laboratories using such tests must, however, have sufficient expertise with operating LC-MS/MS systems. Furthermore, it should be considered that when switching from the commonly used immunoassays for CsA and tacrolimus to this LC-MS/MS method, the therapeutic ranges will need to be revised. As noted, a major disadvantage of the immunoassays is their cross-reactivity with variable concentrations of immunosuppressive drug metabolites present in the blood samples from transplant recipients. Because it has been shown that inactive metabolites make a major contribution to this bias (16–18), the clinical application of the LC-MS/MS method could, without appropriate adjustment of the therapeutic range, lead to a potential overdose. On the other hand, the LC-MS/MS method will provide the possibility for more accurate individualized patient dosing based on the parent drug. Rigorous reevaluation of the current therapeutic ranges for tacrolimus and CsA in cooperation with local transplant centers will therefore be necessary if a change to this highly specific method is planned.

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References


A Positive Drug Test in the Pain Management Patient: Deception or Herbal Cross-Reactivity? Kelly Hickey,1 Rania Sellem,1 James Shields,1 Alfred Mc Kee,2 and James H. Nichols1 (1 Department of Pathology and 2 Pain Management Center, Baystate Health System, Springfield, MA 01199; * address correspondence to this author at: Clinical Chemistry, Department of Pathology, Baystate Medical Center, 759 Chestnut St., Springfield, MA 01199; fax 413-794-5893, e-mail: james.nichols@bhs.org)

Our laboratory was contacted by the Pain Management Center regarding a patient who tested positive for cocaine metabolite in urine, but denied abuse. Because this population is receiving prescriptions for controlled narcotics for pain, the physicians need to determine patient compliance and rule out abuse of street drugs for continued participation in the program. For this patient, the laboratory was consulted to distinguish whether the urine positivity was attributable to herbal medication cross-reactivity or whether the patient was deceiving the clinic physicians.

The patient was a 47-year-old female with a history of Wegener granulomatosis and vasculitis. She had undergone extensive surgery, including resection of the frontal and nasal sinus cavity and septum, and was receiving aggressive analgesic management, including opioid analgesics for head pain related to her condition. It is the policy of the Pain Management Center to test all patients on a random basis three to four times a year for medication compliance and to exclude abuse of street drugs. The patient tested positive once before this episode for urine cocaine metabolite.

On October 31, 2001, the patient’s urine tested positive for cocaine metabolite (qualitative, >300 μg/L) by fluo-