Sensitive, specific quantitative analysis of tacrolimus (FK506) in blood by liquid chromatography–electrospray tandem mass spectrometry

PAUL J. TAYLOR,1* ALUN JONES,2 GLENDA A. BALDSON,3 STEVE V. LYNCH,3 ROSS L.G. NORRIS,1 and SUSAN M. POND1,4

The capacity of liquid chromatography–tandem mass spectrometry (LC-MS) to detect and define individual components in a complex mixture has been utilized to develop a quantitative assay of the potent immunosuppressant drug, tacrolimus. Trough blood concentrations were measured in 175 samples obtained over several weeks after liver transplantation from seven patients. The assay was linear over the range of 0.2 to 100 μg/L. Imprecision was <8%, and accuracy was 99–101%. The turnaround time for a batch of 20 samples was 2.5 h. No interference from any of the other drugs being administered to the patients was evident. An ELISA also performed on the same samples overestimated the concentrations substantially, as indicated by a plot of the difference between the results for the two methods vs their mean. The favorable characteristics of the LC-MS2 assay, especially its sensitivity and specificity, will facilitate detailed pharmacokinetic studies of tacrolimus, particularly under circumstances in which metabolism is perturbed by either hepatic dysfunction or drug interactions.

INDEXING TERMS: immunosuppressant drugs • monitoring therapy • liver transplantation

Tacrolimus, a lipophilic 23-membered macrolide (CAS 104987-11-3; molecular mass 804 Da) isolated from the fermentation broth of the fungus Streptomyces tsukubaensis [1, 2], is marketed in several countries for prophylaxis of rejection in patients undergoing solid organ transplantation. In contrast to cyclosporine, tacrolimus is 50–100 times more potent as an immunosuppressant; on a mg/kg basis, doses of tacrolimus are correspondingly much lower [3–6]. Like cyclosporine, tacrolimus has a specific immunosuppressant action on T-lymphocytes, a narrow therapeutic index, and variable kinetics within and between subjects; is subject to a range of metabolic drug interactions involving cytochrome P450, and produces adverse effects such as nephrotoxicity and neurotoxicity [2–9]. Thus, frequent monitoring of tacrolimus concentrations to achieve optimal therapeutic efficacy and minimal toxicity is mandatory. The distribution of tacrolimus between whole blood and plasma is >20:1, and this ratio is concentration-, hematocrit-, and temperature-dependent [10, 11]. Therefore, measurement of tacrolimus is now generally undertaken in blood rather than plasma [12–15], again similar to cyclosporine.

Most therapeutic drug monitoring of tacrolimus has relied on immunoassay-type assays, e.g., enzyme-linked immunoassay (ELISA) and microparticle enzyme immunoassay with a murine monoclonal antibody [16–21]. However, the antibody cross-reacts with some of the metabolites of the drug [22, 23]. Tacrolimus is metabolized so extensively that <5% of the dose is excreted as parent drug in urine or bile [7, 24, 25]. Christians et al. isolated nine metabolites of the drug produced by human liver microsomes, two of which were shown to have significant immunosuppressive activity [26]. Iwasaki et al. characterized and isolated several hepatic microsomal metabolites of tacrolimus from phenobarbital- or dexamethasone-induced rats; three metabolites had immunosuppressive activity of the same order of magnitude as tacrolimus, and three cross-reacted with the antibody used in the ELISA [22, 23]. If these metabolites are in the circulation in measurable concentrations in humans, this cross-reactivity would preclude determination of the correct pharmacokinetic parameters of the drug and therapeutic range of blood concentrations. To what extent the estimation of
concentration is inaccurate will be known only when results from the immunoassays are compared with those from a specific assay for the parent drug [27].

Considerable effort has been put into development of specific methodology to measure tacrolimus quantitatively. Difficulties arise because of the low circulating concentrations of the drug and its lack of chromophores or intrinsic fluorescence [28]. Two groups have collected the tacrolimus fraction eluted from an HPLC column and then used ELISA for quantitative analysis [29–31]. Takada et al. combined an HPLC assay with derivatization of tacrolimus and detection by chemiluminescence [32]. Christians et al. took advantage of the recent advances in the field of mass spectrometry (MS) to develop an HPLC-MS assay for tacrolimus and some of its metabolites in blood and urine [15]. This assay is sensitive and specific but has the disadvantages of long and complicated sample preparation. Gonschori et al. used this assay to measure the blood concentrations of tacrolimus in seven liver transplant patients and compared these results with those measured by immunoassay [33]; they reported substantial agreement between the two methodologies.

We report here the rapid, sensitive, and specific quantitative analysis of tacrolimus in blood by liquid chromatography–tandem mass spectrometry (LC-MS2). We studied samples collected longitudinally from seven liver transplant recipients to compare the results obtained by LC-MS2 and ELISA. We also assessed the stability of the drug in blood stored at various temperatures.

**Materials and Methods**

**Patients’ Samples**
The study was approved by the Princess Alexandra Hospital Research Ethics and The University of Queensland Human Ethics Committees. Informed consent was obtained for all patients—three pediatric and four adult liver transplant recipients. Just before a dose of tacrolimus was due, a blood sample for therapeutic drug monitoring was withdrawn by venipuncture into a Vacutainer Tube® collection tube (Becton Dickinson, Rutherford, NJ) containing EDTA. The patients were receiving oral tacrolimus twice daily, beginning on day three posttransplant. In the pediatric group, no. 1, a 7-month-old, 6-kg infant, received a tacrolimus dosage range (mg/day) of 1.5–2.0; no. 2, a 32-month-old weighing 14 kg, received 3.0–5.5; no. 3, a 12-month-old weighing 8 kg, received 1.5 to 3.0. In the adult group, no. 1, a 49-year-old, 83-kg patient, received 9.0–12.0 mg/day; no. 2, a 45-year-old, 66-kg patient, received 10.0–12.0; no. 3, a 55-year-old, 55-kg patient, received 6.0–8.0; no. 4, a 22-year-old, 84-kg patient, received 10.0–20.0.

**Materials**
Tacrolimus, FR900520 (a tacrolimus analog), and the murine monoclonal anti-tacrolimus antibody were donated by Fujisawa Pharmaceutical Co. (Osaka, Japan). HPLC-grade methanol and acetonitrile (Merck, Darmstadt, Germany), glacial acetic acid (Mallinckrodt, Clayton, VIC, Australia), ammonium acetate (Sigma Chemical Co., St. Louis, MO) and C18 solid-phase extraction cartridges (Waters, Millford, MA) were purchased from their respective suppliers. Reagent-grade deionized water (MilliQ; Millipore Corp., Millford, MA) was used.

From a tacrolimus stock solution (1.0 g/L), we prepared a series of calibrators (0.2, 1.0, 2.5, 10.0, 20.0, 50.0, and 100.0 μg/L) and controls (0.3 and 80.0 μg/L) in blood obtained from a patient undergoing venesection for homochromatosis. These were used for both the LC-MS2 assay and the ELISA.

**Analytical Procedures**

**LC-MS2 analysis.** A stock solution of internal standard (100.0 mg/L) was prepared by dissolving FR900520 in methanol. A precipitation reagent (25.0 μg/L) was prepared by mixing 250 μL of internal standard stock solution with 700 mL of acetonitrile and 300 mL of water. We placed 1 mL of calibrator, control, or patient’s sample in 12-mL polypropylene centrifuge tubes, added 2 mL of precipitation reagent (containing internal standard), vortex-mixed for 1 min, and then centrifuged the mixture for 5 min at 850g. To C18 solid-phase extraction cartridges preconditioned with 10 mL of methanol followed by 10 mL of water we applied the supernate and withdrew the solvent under reduced pressure. The cartridges were then washed sequentially with 10 mL of water, 10 mL of methanol/water (1/1 by vol), and 4 mL of heptane. A full suction was applied to the cartridge for 15 min. The eluates were eluted with 2 mL of heptane/isopropyl alcohol (1/1 by vol) and evaporated to dryness under air at 60°C.

The samples were reconstituted with 140 μL of mobile phase and injected into the LC system, which consisted of a 140B solvent-delivery system (Applied Biosystems, Ramsey, NJ) with a ISS 200 autoinjector (Perkin-Elmer, Danbury, CT). Chromatography was performed with a C18 Brownlee column, 2.1 mm × 30 mm (Brownlee Labs., Santa Clara, CA), at ambient temperature and a mobile phase of 80/20 (by vol) methanol/ water (1/1 by vol), and 4 mL of heptane. A full suction was applied to the cartridge for 15 min. The analytes were eluted with 2 mL of heptane/isopropyl alcohol (1/1 by vol) and evaporated to dryness under air at 60°C.

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An API III triple quadrupole instrument (PE-SCIEX, Thornhill, Toronto, Canada) was used for quantitative mass spectrometric detection. An ion-spray (pneumatically assisted electrospray) interface was used in positive ionization mode. The orifice potential was set to 35 V to promote the presence of the tacrolimus ammonium ion adduct. The interface heater was set at 45°C. Multiple reactant-ion monitoring (MRM) was used with argon as the collision gas at a thickness of 300 × 10–12 molecules cm–2. The mass spectrometer was programmed to allow the ammonium adducts [M+NH4]4+ of tacrolimus (m/z 821.5) and the internal standard (m/z 809.5) to pass through the first quadrupole (Q1) and into the collision cell (Q2). The product ions for tacrolimus (m/z 768.4) and internal standard (m/z 756.4) were monitored through the third quadrupole (Q3). For data acquisition, the dwell time was 800 ms at a scan speed of 3.20 s. Peak-area ratios obtained from MRM of tacrolimus (m/z 821.5→768.4) and internal standard (m/z 809.5→756.4) were used for quantification. Calibration curves

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5 Nonstandard abbreviations: MS, mass spectrometry; LC-MS2, liquid chromatography–tandem mass spectrometry; and MRM, multiple reactant-ion monitoring.
were constructed by using weighted \((1/x^2)\) linear least-squares regression. Data were collected and manipulated on a Macintosh computer operating on RAD and MACQUAN software (PE-SCIEX).

ELISA. Calibrators, controls, or patients' samples, 25 μL, were pretreated with 500 μL of methanol in 1.5-mL centrifuge tubes. The mixtures were vortex-mixed and centrifuged, and 400-μL aliquots of supernates were transferred to 8-mL glass culture tubes and evaporated to dryness under air at 40 °C. Tacrolimus concentrations were analyzed by the modified ELISA method of D'Ambrosio et al. [34].

Stability of tacrolimus in blood. We studied the stability of tacrolimus in blood stored over an interval of 28 days. Blood was supplemented with tacrolimus to give a nominal concentration of 8 μg/L and then subdivided into three groups of samples, each of which was stored at 40, 4, or −20 °C. Samples from these groups were assayed in six replicates by LC-MS². Blood stored at 40 or 4 °C was assayed on days 1, 2, 3, 4, 5, 6, 7, 15, 21, and 28. Blood stored at −20 °C was assayed on days 1, 7, 15, 21, and 28. All three groups were compared with the results for six replicates assayed on day 0.

STATISTICAL ANALYSES
To compare the tacrolimus concentrations obtained by LC-MS² and ELISA, we used the statistical procedure recommended by Bland and Altman [35]. Imprecision of both assays was determined over 4 days by the method of Krouwer et al. [36]. Analysis of the blood stability data was by repeated measures analysis of variance.

Results
LC-MS² assay. To produce intense molecular ions for mass spectrometric monitoring of tacrolimus and the internal standard, we added ammonium acetate (40 mmol/L) to the mobile

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Fig. 2. Typical chromatograms, obtained from blood extracts, with multiple reactant monitoring for (A) tacrolimus-supplemented blood (20.0 μg/L), (B) blank blood, and (C) a patient's sample containing 10.6 μg/L tacrolimus.
phase. This produced a charged species adduct [M+NH₄]⁺ of the neutral tacrolimus and the internal standard. Full-scan positive-ion spectra for both tacrolimus and the internal standard produced precursor ions of m/z 821.5 and m/z 809.5, respectively. Fragmentation of these precursor ions was monitored by using instrument settings that gave maximum intensity to one major product ion (Fig. 1). The precursor and the major product ion pair for each analyte were selected to optimize maximum sensitivity and specificity for MRM quantitative analysis (tacrolimus m/z 821.5→768.4 and internal standard m/z 809.5→756.4). Typical chromatograms of extracts from drug-supplemented blood, blank blood, and a patient’s sample are shown in Fig. 2.

The assay was linear over the range 0.2 to 100 µg/L (r = 0.999). Imprecision and accuracy were determined at three concentrations across the linear range. Imprecision was <8% (total CV) and accuracy was between 99% and 101% (Table 1). Recoveries were 54.3% ± 13.5% for tacrolimus (n = 24) and 62.4% ± 12.9% for the internal standard (n = 30). No interference with the assay was observed in the samples despite the array of other drugs being administered concurrently (phe-

Table 1. Imprecision and accuracy of LC-MS² assay.

<table>
<thead>
<tr>
<th>Tacrolimus conc, µg/L</th>
<th>Added</th>
<th>Mean assayed</th>
<th>Within assay</th>
<th>Between assays</th>
<th>Total</th>
<th>Accuracy, %*</th>
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<tr>
<td>0.3</td>
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<td>1.8</td>
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<td>1.0</td>
<td>1.9</td>
<td>2.7</td>
<td>101.3</td>
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</tbody>
</table>

n = 16.

*(Mean assayed concentration/added concentration) X 100%.

Fig. 3. Longitudinal monitoring of trough blood concentrations of tacrolimus by LC-MS² (○) and ELISA (■) in three adult and three pediatric liver transplant recipients.
nytoin, vancomycin, tobramycin, ticarcillin, ganciclovir, acyclovir, ranitidine, azathioprine, methylprednisolone, omeprazole, diltiazem, amoxicillin, metronidazole, sulfasalazine). The analysis time for a batch of 20 samples was 2.5 h.

ELISA. The assay was linear over the range of 0.5 to 100 μg/L (r = 0.999). Imprecision and accuracy were determined at three concentrations across the linear range. Imprecision was <9% (total CV) and accuracy was between 92% and 107%. The analysis time for this assay was 8 h.

**Longitudinal study of blood concentrations in liver transplant patients.** A total of 175 blood samples collected at least 1 month posttransplant from the seven liver transplant recipients were analyzed by both methods. Results for three individual adult patients and three pediatric patients are presented in Fig. 3. None of these patients had an episode of rejection during the period of study shown or up to 10 months posttransplant. The fourth adult patient had a grand mal seizure on day 5 and was treated with phenytoin (200 mg twice daily) for 19 days; the data in Fig. 4 demonstrate the effect of phenytoin on the tacrolimus dose/concentration relationship. Apparently, phenytoin either reduces absorption or, more likely, enhances the metabolism of tacrolimus. This patient had no episodes of rejection or derangement of liver function. The concentrations obtained for all patients are shown in Fig. 5 (top). Fig. 5 (bottom) presents these same data as the difference in tacrolimus concentrations between the methods (ELISA minus LC-MS²) vs the mean concentration of both assays.

**Stability of tacrolimus in whole blood.** The stability of tacrolimus at three temperatures (-20, 4, and 40 °C) over a period of 28 days is shown in Fig. 6. Concentrations were unaffected by storage at -20 °C and 4 °C. Storage at 40 °C led to a gradual decrease in tacrolimus concentrations, which became significant at 3 days (4.3%, P <0.006) and by 28 days was 24.1%. Similar results at 4 °C were obtained over 14 days by Freeman et al. [37].

![Fig. 5. Comparison of tacrolimus concentrations measured by LC-MS² and ELISA in 175 blood samples collected from seven liver transplant recipients just before a dose of tacrolimus was due (trough): (top) correlation of measured concentrations; (bottom) difference between the ELISA and LC-MS² blood tacrolimus concentrations plotted vs the mean tacrolimus concentration.](image-url)

*(Top) The solid line represents the line of identity (i.e., slope = 1); y = 1.17x - 0.115 (Sₓₘ = 1.31). (Bottom) The solid line is the regression curve; difference = 0.187 mean + 0.401 (Sₓᵣ = 1.20). Dashed lines are the 95% confidence intervals for the differences.*

![Fig. 4. Trough blood concentrations of tacrolimus measured by LC-MS² (□) and ELISA (■) vs time after liver transplantation in a 22-year-old man.](image-url)

Phenytoin therapy (200 mg twice daily) was introduced on day 5 and ceased on day 25.
Fig. 6. The stability of whole-blood tacrolimus (8.0 μg/L) at three storage temperatures: (A) -20 °C, (B) 4 °C, and (C) 40 °C.

Discussion

Reliable therapeutic drug monitoring requires a specific, sensitive, accurate, precise, rapid, and robust analytical procedure. The LC-MS² assay reported here meets all of these criteria. The assay was linear from 0.2 to 100 μg/L and the CV was between 2.2% and 7.8% at the highest and lowest concentrations, respectively. The ability to induce fragmentation and perform successive mass spectrometry introduces great specificity and sensitivity to the assay, avoids interference by metabolites of the drug and other xeno- or endobiotics, and reduces the time required for sample preparation and cleanup [38, 39]. Because only 20 μL of the final 140-μL aliquot of mobile phase is injected, the initial volume of blood (1 mL) required for the assay may be reduced to 200 μL or less and still be analyzed with the same sensitivity, accuracy, and precision as reported. Given that sample preparation took ~1 h for a batch of 20 samples and the chromatography time was 4 min/sample, the turnaround time for the batch was 2.5 h. Analysis time can be further reduced by using a single-point calibration instead of seven points. Single-point calibration allowed for either batch or individual analysis of samples, which gives the flexibility required in a routine laboratory.

The favorable characteristics of the LC-MS² assay, particularly its sensitivity, will facilitate detailed pharmacokinetic studies of the drug, especially when metabolism is perturbed, as in patients with hepatic dysfunction or taking drugs that interfere with metabolism. It should also be possible to adapt the methodology to the identification and quantitative measurement of the metabolites of the drug and thus enable the required complete pharmacokinetic/pharmacodynamic studies [27, 40]. The cost of the instrumentation is the major drawback, although rapid technical advances are bringing mass spectrometers into the price range of most high-throughput analytical laboratories.

The ELISA also had acceptable calibration parameters and CVs over the whole range. Even though the results are preliminary and need to be extended to many more individual patients, tacrolimus concentrations overall appeared to be overestimated by ELISA. Fig. 5 indicates the magnitude and variability of the overestimation; this cannot be attributed to calibration bias because the same calibrators were used for each method. Therefore, as reported by others, the overestimation must be attributable to cross-reactivity of the murine monoclonal anti-tacrolimus antibody between tacrolimus and some of its metabolites [15, 22, 23]. A similar problem, albeit of greater magnitude, has been encountered with cyclosporine immunoassays [41].

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