Plasma vs Whole Blood for Therapeutic Drug Monitoring of Patients Receiving FK 506 for Immunosuppression

Michael Winkler,1 Burghardt Ringe, Jana Baumann, Martin Loss, Kurt Woniget, and Rudolf Pichlmayr

By retrospective analysis of 13 000 blood samples obtained from 248 patients receiving FK 506 therapy, we compared the suitability of plasma with that of whole blood as the matrix for therapeutic drug monitoring of FK 506. The plasma concentrations did not correlate with the concentrations in whole blood (r = 0.56). In contrast to plasma samples (analyzed by enzyme immunoassay), FK 506 was detectable in all whole-blood samples (analyzed by enzyme immunoassay/microparticle enzyme immunoassay). The inter- and intraindividual variations of FK 506 measurements were greater in plasma than in whole blood. Moreover, plasma concentrations correlated only poorly with clinical events. There was a tendency to greater plasma concentrations being measured during episodes of toxicity, but no clear difference was evident between stable course and rejection. In whole-blood specimens, a correlation between reduced or increased FK 506 concentrations and rejection or toxicity, respectively, was observed. The discriminatory power of whole-blood values was greater for the differentiation between toxicity and stable course than between rejection and stable course. We therefore recommend whole blood rather than plasma as the matrix for therapeutic monitoring of FK 506 concentrations.

Indexing Terms: sample treatment/organ transplantation/enzyme immunoassay

The macroline drug FK 506 is currently under clinical investigation for baseline and rescue immunosuppression in patients after solid organ transplantation (1–3). Compared with cyclosporine (CsA), FK 506 has similar but more potent immunosuppressant activity (1, 2).2 The clinical use of FK 506 can be associated with side effects such as hypertension, diabetogenicity, and nephro- or neurotoxicity (4, 5). These side effects are most frequent in patients with liver dysfunction (6–8) and were shown to be more closely associated with FK 506 concentrations (in plasma) than with dose (9, 10). Therefore, routine monitoring of plasma concentrations has become a prerequisite for FK 506 treatment (1, 7, 9). However, especially during the long-term course, FK 506 plasma concentrations can be very low, being unde

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2 Nonstandard abbreviations: TDM, therapeutic drug monitoring; EIA, enzyme immunoassay; MEIA, microparticle enzyme immunoassay; CsA, cyclosporine; pl-EIA, plasma EIA; wb-sl-EIA, whole-blood EIA after solid/liquid extraction; and wb-ll-EIA, whole-blood EIA after liquid/liquid extraction.

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germs such as cytomegalovirus or Pneumocystis carinii. Stable concentrations of FK 506 were those detected in blood or plasma at day 7 of therapy in liver-graft recipients under primary or rescue FK 506 treatment who showed no sign of rejection or FK 506 toxicity.

To characterize these clinical events (rejection, infection, and nephro- or neurotoxicity), we calculated the mean of the last three FK 506 plasma or whole-blood concentrations measured before onset of rejection or toxicity.

Calculation of predictive value of increased concentrations of FK 506. For analysis of the effect of high concentrations of FK 506 in plasma or whole blood on the clinical course of the patients, we used the following cutoff values: In patients with bilirubin <100 μmol/L, the cutoffs were <1.0 μg/L in plasma or <10.0 μg/L in whole blood; for bilirubin between 100 and 300 μmol/L, the cutoffs were 1.5 μg/L in plasma, 15.0 μg/L in whole blood; for bilirubin >300 μmol/L, the cutoffs were 2.0 and 20.0 μg/L, respectively. Only patients with three or more consecutive FK 506 plasma or whole-blood concentrations exceeding the cutoff values were considered for clinical evaluation. Clinical manifest toxicity was diagnosed if the criteria for FK 506 toxicity were fulfilled as outlined above. Besides neuro- and nephrotoxicity, two other clinical symptoms were judged as FK 506-mediated side effects: de novo hypertension necessitating antihypertensive therapy and de novo diabetes necessitating medication by oral antidiabetics or insulin.

Immunosuppression

FK 506 dosing. The initial dose of FK 506 used in kidney or kidney–pancreas transplant recipients was 0.20–0.30 mg/kg body wt. daily (taken orally twice a day). Patients treated for rescue immunosuppression during graft rejection were given a 1-day course of intravenous FK 506 (0.10–0.15 mg/kg) before initiation of oral therapy.

In liver-graft patients under primary FK 506 immunosuppression the oral starting dose varied between 0.05 and 0.20 mg/kg daily. Treatment protocol between September 1990 and April 1993 also included a short intravenous course (0.02–0.05 mg/kg) given as a 4-h bolus twice daily or as a continuous infusion for 24–36 h before oral administration of the drug. Since May 1993, however, intravenous FK 506 has not been given in patients under primary treatment.

Liver-graft patients switched from CsA to FK 506 to treat graft rejection received 0.20 mg/kg daily; most, before taking the oral dose, were given a short (24-h) intravenous course of FK 506 0.10 mg/kg. Patients switched to FK 506 for treatment of CsA toxicity or CsA malabsorption were given an oral dose of 0.10 mg/kg daily, with no intravenous FK 506.

Therapeutic drug monitoring (TDM) method. Between September 1990 and May 1993 we used a plasma EIA to monitor FK 506 concentrations. Since May 1993 we have used whole blood as the matrix for FK 506 TDM, determining whole-blood concentrations by MEIA (supplemented by EIA in samples with <5.0 μg/L FK 506).

Non-FK 506 immunosuppression. Beside FK 506 all patients under primary immunosuppression received a methyl–prednisolone bolus of 500 mg intravenously immediately after graft reperfusion. In the first 5 days after transplantation a maintenance daily steroid dose between 40 and 20 mg of prednisolone was given, followed by 20 mg daily from day 6 to day 21. The steroid dose was then gradually tapered to 5 mg per day. At 3 months posttransplant we stopped steroid therapy in selected patients. A subgroup of 50 liver- or kidney-graft patients under primary FK 506 were also treated with low-dose (1–2 mg/kg daily) azathioprine during the first 3 months posttransplant.

FK 506 Assays

Solid/liquid extraction, plasma EIA (pl-EIA). Plasma was separated at room temperature (after 2 h equilibration) from freshly drawn patients’ blood. After pretreating 100 μL of patients’ samples, calibrators (0.10–10.0 μg/L), or controls with 1 mL of 0.1 mol/L HCl, we loaded 1 mL of the pretreated sample onto C18 cartridges (Sep-Pak Plus C18; Waters, Milford, MA) that had been rinsed with 6 mL of methanol (Baker, Deventer, The Netherlands) and 6 mL of 0.7 mol/L acetic acid. Using a suction chromatography system (Vac-Elut; Analytichem, Munich, Germany) we washed samples with 6 mL of 0.7 mol/L acetic acid and then eluted them from the columns with 3 mL of methanol. The eluates were dried under a stream of nitrogen and reconstituted in FK 506–peroxidase (1:400 to 1:500 diluted) stock solution (Fujisawa, Osaka, Japan). The FK 506 concentration was analyzed in a modified ELISA as described (12), with a 2-h incubation at room temperature. The detection limit of the assay was 0.05 μg/L.

External controls were prepared from a 100 μg/L standard provided by the European Central Assay Laboratory (BCO, Breda, The Netherlands). In addition, in each test several (four to eight) control samples (poled patients’ plasma) with FK 506 ranging from 0.2 to 2.0 μg/L were measured in parallel. These control samples had been calibrated with the external control. If the control samples yielded values >30% higher or lower than the known “true” values, the assay was repeated. In addition, a regular quality assessment scheme was performed by participating in the European FK 506 round-robin test, in which known and unknown samples were shipped by the European Central FK 506 Assay Laboratory (BCO) to the participating centers at monthly intervals.

Solid/liquid extraction, whole-blood EIA (wb-sl-EIA). We pretreated 50-μL patients’ samples, calibrators (0.50–50 μg/L), or controls with 2 mL of a solution of ZnSO4/methanol (30/70 by vol). After centrifugation at 4000g for 20 min, the supernate was loaded onto C18 cartridges (Sep-Pak Plus) that had been rinsed with an equi-volume mixture of acetonitrile/water (both reagents chromatographic grade). The cartridges were then washed with 6 mL of 0.7 mol/L acetic acid and eluted...
with 3 mL of methanol. The dried eluates were resuspended in 250-fold diluted FK 506-peroxidase stock solution (Fujisawa) and the FK 506 concentration was determined as described above. External whole-blood controls were prepared as stated above, but at concentrations of 5.0 to 20.0 µg/L.

Liquid/liquid extraction, whole-blood EIA (wb-ll-EIA). This assay was performed at the Central European FK 506 Assay Laboratory (BCO) with samples frozen at −70°C and shipped to the laboratory. FK 506 was extracted from blood in a liquid/liquid extraction step in which the 10-µL patients' samples were shaken with 5.5 mL of CH$_2$Cl$_2$ (Merck) for 45 min, then centrifuged at 1000g for 5 min. The resulting lower layer was evaporated at 36°C under a stream of nitrogen, then analyzed by ELISA as described (12) but with overnight incubation at 4°C.

**MEIA.** The MEIA was performed strictly according to the instructions of the manufacturer, with FK 506 from whole blood after liquid/liquid extraction with zinc sulfate in methanol and ethylene glycol (13). Quality controls, e.g., internal control samples provided by the manufacturer as well as external samples sent from the European Reference Laboratory for the MEIA round robin test, were analyzed at regular intervals. The limit of detection of the MEIA was 5 µg/L. Whole-blood samples yielding a result <5 µg/L in the MEIA were remeasured with the wb-sl-EIA. For better comparability, the wb-sl-EIA and MEIA were cross-calibrated with each other.

**Results**

FK 506 concentrations were detected in 80 pairs of blood and plasma samples obtained from 25 different liver-graft recipients. Analyzing these samples by pl-EIA, wb-ll-EIA, wb-sl-EIA and MEIA showed that the plasma concentrations did not correlate with the whole-blood values ($r = 0.56$, see Fig. 1). Whole-blood concentrations measured on the same set of blood samples by both EIA and MEIA were significantly correlated: $r = 0.919$; $P = 0.006$ (paired $t$ test; see Fig. 2).

The time course of plasma and whole blood FK 506 concentrations was calculated in a subgroup of patients receiving long-term FK 506 treatment. For this analysis, we evaluated FK 506 detected in 11 000 different plasma or whole-blood samples. In a second subgroup of 1000 samples the plasma and whole-blood concentrations had been analyzed in parallel. As shown in Table 1 the mean FK 506 concentrations in plasma decreased from 0.8 µg/L at month 1 of therapy to 0.1 µg/L at month 24. In contrast, mean whole-blood concentrations remained relatively stable, between 6 and 9 µg/L. A significant proportion of the patients' plasma samples contained no detectable FK 506, the percentage of patients with FK 506 <0.05 µg/L increasing from 10% at month 1 of treatment to 25% at month 36. In contrast, FK 506 was detectable in all whole-blood samples by EIA/MEIA. The interindividual variation of FK 506 concentrations was also greater in plasma (SD: 80–160%) than in whole blood (SD: 25–80%).

**Table 1. FK 506 concentrations in plasma (EIA) or whole blood (MEIA/EIA) in stable patients after liver or kidney transplantation with FK 506 maintenance therapy.**

<table>
<thead>
<tr>
<th>Months of therapy</th>
<th>Plasma FK 506, µg/L*</th>
<th>% with undetectable plasma FK 506</th>
<th>Whole blood FK 506, µg/L*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Range</td>
</tr>
<tr>
<td>1</td>
<td>0.71</td>
<td>0.86</td>
<td>0–3.19</td>
</tr>
<tr>
<td>2</td>
<td>0.58</td>
<td>0.80</td>
<td>0–3.90</td>
</tr>
<tr>
<td>3</td>
<td>0.52</td>
<td>0.63</td>
<td>0–3.19</td>
</tr>
<tr>
<td>4</td>
<td>0.43</td>
<td>0.44</td>
<td>0–1.80</td>
</tr>
<tr>
<td>5</td>
<td>0.36</td>
<td>0.35</td>
<td>0–1.53</td>
</tr>
<tr>
<td>6</td>
<td>0.34</td>
<td>0.34</td>
<td>0–1.58</td>
</tr>
<tr>
<td>9</td>
<td>0.30</td>
<td>0.34</td>
<td>0–1.47</td>
</tr>
<tr>
<td>12</td>
<td>0.18</td>
<td>0.16</td>
<td>0–0.51</td>
</tr>
<tr>
<td>18</td>
<td>0.21</td>
<td>0.24</td>
<td>0–0.88</td>
</tr>
<tr>
<td>24</td>
<td>0.08</td>
<td>0.13</td>
<td>0–0.45</td>
</tr>
<tr>
<td>30</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>36</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* $n = 78$: 72 liver, 6 kidney.  
* $n = 124$: 112 liver, 12 kidney.
Plasma and whole-blood concentrations were retrospectively correlated with clinical events such as rejection or toxicity in the liver-graft recipients. The results (Fig. 3) showed that the plasma concentrations measured during different clinical episodes ranged between 0.05 and 50.0 μg/L, whereas the overall variation in whole-blood content ranged from 2.0 to 80.0 μg/L.

Plasma concentrations of FK 506 only poorly correlated with clinical events. There was a tendency to increased plasma values during episodes of toxicity, but no clear difference could be found between the concentrations detected during stable clinical course and those measured during graft rejection. Plotting plasma FK 506 vs bilirubin did not significantly increase the clinical correlation (Fig. 3, top).

Whole-blood specimens showed a correlation between decreased or increased FK 506 concentrations and rejection or toxicity, respectively. Depending on the patients' liver function (Fig. 3, bottom), FK 506 between 5.0 and 10.0 μg/L in patients without cholestasis and between 10.0 to 20.0 μg/L in patients with severe cholestasis avoided graft rejection or drug toxicity. The discriminatory power of whole-blood values was greater for the differentiation between toxicity and stable course than for the differentiation between rejection and stable course.

Figure 4 demonstrates the clinical course of a liver-graft recipient with early liver dysfunction during the first 2 weeks posttransplant. In this patient a massive accumulation of FK 506 in whole blood (wb-II-EIA) but not in plasma was observed. These high concentrations in whole blood correlated with clinical symptoms of FK 506 toxicity (e.g., neuro- and nephrotoxicity). After interruption of FK 506 therapy, kidney function stabilized and neurologic symptoms improved.

The predictive value of increased FK 506 in plasma or whole blood for clinical manifestations of FK 506 toxicity in liver-graft recipients ranged between 41% and 80% (Table 2). There was a tendency to somewhat higher values if whole blood was used as matrix, but this difference was not significant. In general, the predictive value of high FK 506 in plasma or whole blood was greater in the first postoperative month than later.

On May 1, 1993, the matrix for FK 506 TDM in our laboratory was changed from plasma to whole blood. The course of plasma and whole-blood concentrations

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**Fig. 3.** Concentrations of bilirubin and FK 506 (mean of three consecutive measurements) during different clinical episodes (rejection, toxicity) in liver-graft patients: FK 506 in plasma (top) and whole blood (bottom).

**Fig. 4.** Clinical course of a patient with early liver dysfunction after liver transplantation.

This patient showed signs of FK 506 nephro- and neurotoxicity from day 4 after transplantation. After interruption of FK 506 therapy, clinical signs of toxicity improved. During this period the whole-blood FK 506 showed a massive increase, whereas plasma values remained stable (1.0--2.0 μg/L). ALAT, alanine aminotransferase; i.v., intravenous; p.o., oral; ATG, anti-thymocyte globulin.
Table 2. Predictive value (%) of high FK 506 concentrations for clinical toxicity.*

<table>
<thead>
<tr>
<th>Bilirubin, μmol/L</th>
<th>Plasma FK 506</th>
<th>Whole-blood FK 506</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 100</td>
<td>45.4 (1.0)</td>
<td>52.1 (1.0)</td>
</tr>
<tr>
<td>100–300</td>
<td>66.7 (1.5)</td>
<td>58.3 (1.5)</td>
</tr>
<tr>
<td>&gt; 300</td>
<td>60.0 (2.0)</td>
<td>41.2 (2.0)</td>
</tr>
</tbody>
</table>

*Percentage of patients with FK 506 concentrations exceeding the cutoff values (μg/L, given in parentheses) who experienced clinically manifest FK 506 toxicity.

Fig. 5. Course of FK 506 plasma (left axis) and whole-blood (right axis) concentrations during the switch of analytical matrix for FK 506 TDM in patients receiving long-term maintenance immunosuppression with FK 506.

The liver-graft patients and 30% of the kidney-graft patients.

**Discussion**

TDM is generally accepted as a prerequisite for therapy with FK 506 (6, 7, 10, 14, 15). Among the various immunoassays available for routine FK 506 TDM, four assays utilize a plasma matrix (liquid/liquid and solid/liquid extraction EIA of FK 506 in plasma separated at 36°C or at ambient temperature); three utilize whole blood (liquid/liquid extraction EIA and IMx and solid/liquid extraction EIA). Thus far, only limited information has been available as to which matrix should be preferred for FK 506 TDM in the routine clinical laboratory (15–17).

Using plasma as matrix for TDM has several disadvantages over using whole blood. The distribution of FK 506 between plasma and blood cells depends on the hematocrit and the temperature of the sample (16–20). For example, the low sensitivity of the EIA that currently is the only assay suitable for detecting FK 506 in plasma can be partially overcome by using 36°C plasma separation, which leads to 20–50% higher results for FK 506 determination (15). However, some of our patients...
with plasma concentrations below the limit of detection presented with whole-blood concentrations >20 μg/L and clinically manifest toxicity. Therefore, it is unlikely that the 36°C plasma EIA might be able to differentiate these patients from patients without toxicity. Moreover, the interindividual variation (1000-fold) in FK 506 plasma concentrations is much higher than that in whole blood (40-fold), which makes difficult the definition of potential therapeutic windows. Finally, while the predictive value of increased plasma concentrations for FK 506 toxicity is comparable with that of whole blood, no lower limit for plasma values can be defined for avoidance of graft rejection. Thus, plasma concentrations cannot differentiate between stable course and underimmunosuppression (Fig. 3). In contrast, if liver function is taken into account (Fig. 3, bottom), the use of whole-blood FK 506 concentrations allows the definition of lower and upper target values in a way that therapeutic windows can be established. Based on these arguments, the matrix for FK 506 TDM should be whole blood.

In a recently published study ascertaining the preferred matrix for FK 506 TDM, Bäckman et al. (15) found plasma (36°C) to be somewhat superior to whole blood. In their study of blood or plasma samples obtained from a smaller group of liver-graft patients, both whole-blood and plasma concentrations were measured during episodes of FK 506 toxicity. After a year of treatment, however, only the yearly mean plasma concentrations correlated with an observed loss in glomerular filtration rate. Most of those patients were treated with relatively high doses of FK 506 (initial dose, 0.30 mg/kg daily in primary liver-graft recipients), which is also reflected by the high mean whole-blood concentration measured in stable liver-graft recipients in that study (15.2 ± 2.1 μg/L), well above the upper target for stable patients in our group. Plasma samples obtained from patients on such a high dose of FK 506 might yield detectable FK 506 in a higher percentage of patients than was observed in our study, thus eliminating one of the major pitfalls of plasma for TDM. However, patients on low-dose FK 506 still have a high percentage of plasma samples containing FK 506 below the limit of detection of the standard EIA.

Compared with the whole-blood EIA, the MEIA is less laborious and also less susceptible to technical failures. In addition, its precision is greater than that of the EIA (11, 13, 17). Therefore, we consider the MEIA the method of choice for clinical drug monitoring in whole blood. However, the detection limit of the whole-blood IMx, 5.0 μg/L, is too low for routine clinical use (11, 13), and samples containing <5.0 μg/L FK 506 should therefore be reassayed with a whole-blood EIA.

The ratio between FK 506 whole-blood concentration and FK 506 dose was higher in liver-transplant patients than in kidney-transplant patients. This effect was most pronounced during the early posttransplant course and might be due to accumulation of FK 506 metabolites (21, 22). FK 506 metabolites are known to cross-react with the monoclonal antibody used in all immunoassays to date (11, 16).

Although—compared with the parent drug—FK 506 metabolites exert only negligible immunosuppressive effects (16, 21), it is not clear whether at high concentrations the FK 506 metabolites might be toxic. Most episodes of FK 506 toxicity are observed in patients with liver dysfunction (5, 6, 8, 9): This might point to a role of FK 506 metabolites but could also be explained by parent drug accumulation in the blood of these patients. In a cholestatic patient a whole-blood FK 506 concentration (EIA/MEIA) of 15 μg/L usually does not correlate with clinical toxicity. In contrast, such a value might well be associated with some form of FK 506 toxicity in a stable long-term patient. This divergence argues against a toxic effect of FK 506 metabolites.

The degree of cross-reactivity of FK 506 metabolites with the monoclonal antibody utilized in the EIA as well as in the MEIA seems to be low: Using metabolites generated by human liver microsomes (11), we found that only one of four metabolites tested, didemethylhydroxyl-FK 506, cross-reacted with the antibody (by 10–15%). Moreover, in pharmacokinetic analysis of FK 506 blood concentrations in liver-graft recipients on day 5 after transplant, the results generated by MEIA were only slightly higher than those by HPLC-mass spectrometry (22). A relatively low cross-reactivity of FK 506 metabolites might explain the moderate influence of cholestasis on FK 506 concentrations (Fig. 3, bottom). In patients treated with CsA for immunosuppression, liver dysfunction (and subsequent accumulation of CsA metabolites) can lead to a 5- to 10-fold increase in the CsA concentrations measured by nonspecific CsA assays (23).

In the long term, liver-graft patients were treated with lower FK 506 doses and blood concentrations than the kidney-graft patients (Fig. 6). Nonetheless, no apparent difference in the frequency of graft rejection was observed between these two groups of patients. Most probably, because of microchimerism, which is more pronounced after liver than after kidney transplantation (24), liver grafts require less immunosuppression and are more resistant to immunologic destruction (25) than are kidney or heart transplants. Indeed, stable long-term liver-graft patients can be successfully weaned off immunosuppression without subsequent rejection (24), which is not possible in kidney or heart recipients. Thus, the observation of lower FK 506 dosing

<p>| Table 3. Recommended daily oral FK 506 dose and target blood concentrations. |</p>
<table>
<thead>
<tr>
<th>Transplanted organ</th>
<th>Weeks</th>
<th>Long-term</th>
</tr>
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<tbody>
<tr>
<td>Daily dose, mg/kg body wt.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.06–0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.20–0.30</td>
<td>0.20</td>
</tr>
<tr>
<td>Whole-blood target value, μg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>5–15</td>
<td>3–10</td>
</tr>
<tr>
<td>Kidney</td>
<td>10–20</td>
<td>5–10</td>
</tr>
</tbody>
</table>

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and target value requirements in liver-transplant patients might be the reflection of a lower total immunosuppressive load necessary for prevention of graft rejection in these patients.

In summary, our data clearly support whole blood as the preferable matrix for FK 506 TDM. The assay of choice currently seems to be the MEIA, although the detection limit of this assay should be improved. FK 506 concentrations measured by the MEIA are influenced by cross-reacting metabolites; their cross-reactivity, however, seem to be low. If liver function is taken into account, therapeutic windows for FK 506 TDM (Table 3) can be defined.

References