Specific and Sensitive Measurement of FK506 and Its Metabolites in Blood and Urine of Liver-Graft Recipients

Uwe Christians, Felix Braun, Michael Schmidt, Norbert Kosian, Hans-Martin Schieber, Ludger Ernst, Michael Winkler, Christiane Kruse, Annette Linck, and Karl-Friedrich Sewing

A specific and sensitive assay for quantifying the immunosuppressant FK506 and its metabolites in blood and urine was developed. 32-O-Acetyl FK506 was synthesized and used as internal standard. FK506 and its metabolites were purified from the samples by solid–liquid extraction and were injected into a high-performance liquid chromatographic (HPLC) system linked to a mass spectrometer (MS) by particle-beam interface. The FK506 derivatives were separated from interfering material by use of a 100 × 4 mm C8 analytical column and water/acetonitrile or water/methanol gradient elution; they were detected by negative chemical ionization with methane as reagent gas. The limit of detection was 25 pg in a standard solution, and the limit of quantification in blood was 250 pg (extracted from 1 mL of blood). The CV was 11.3% at 5 ng, and no interferences with other drugs were found.

Additional Keyphrases: immunosuppressive drugs · urine · chromatography, liquid · mass spectrometry

FK506 (Fujisawa, Osaka, Japan) is considered a valuable and potent immunosuppressant after organ transplantation (1) and in the therapy of immunological diseases (2, 3). FK506 is isolated from Streptomyces tsukubaensis (4) and has a macrolide structure (C49H69NO12) with a molecular mass of 803.5 Da (Figure 1). In solution (CDCl3), FK506 forms two rotamers in a ratio 2:1, the result of cis–trans isomerism of the C–N amide bonds. FK506 is soluble in alcohols, halogenated hydrocarbons, and ether. It is very sparingly soluble in aliphatic hydrocarbons and water. The molecule does not contain any chromophores, and its ultraviolet absorption maximum is 192 nm (5–7).

FK506 is metabolized by the intestinal and liver cytochrome P-450 system to at least nine metabolites (8). There is strong evidence that cytochrome P-450IIA4 is responsible for the FK506 metabolism (9). The reactions involved in FK506 metabolism are O-demethylation and hydroxylation. The metabolites formed retain biological activity. The immunosuppressive activity of the metabolites is ≤10% of that of FK506 (8, 10). All of these metabolism studies were performed in vitro with human liver microsomes. No data concerning the in vivo metabolism of FK506 are available, because of the lack of a specific assay sensitive enough to allow quantification in body fluids or tissues of patients. The structure of FK506 and its metabolic pathways are displayed in Figure 1.

Thus far, six different methods for quantifying FK506 have been described. The assay that was used to measure plasma concentrations of FK506 for clinical and pharmacokinetics studies (11–13) is an enzyme immunoassay, based on the use of a monoclonal antibody (14–16) that probably is cross-reactive with the FK506 metabolites, according to clinical observations (17). An automated whole-blood assay for the IMx analyzer (Abbott Labs, Abbott Park, IL) was developed on the basis of this antibody (18). HPLC/ultraviolet detection (UV)6 was used to isolate and quantify FK506 metabolites in

---

6 Nonstandard abbreviations: HPLC/UV, high-performance liquid chromatography with ultraviolet detection; MS, mass spectrometry; DCI, direct chemical ionization; FAB, fast atom bombardment; and NMR, nuclear magnetic resonance.
in-vitro studies (8, 10). The main disadvantage of the method is a detection limit of 10 µg/L, which is above the therapeutic range of the plasma and blood concentrations of FK506 usually expected in patients. To enhance the sensitivity of the HPLC assay, Friob et al. (19) collected fractions of FK506 and one metabolite after HPLC separation, which they quantified by enzyme immunoassay. Takada et al. (20) enhanced the sensitivity of their HPLC assay by derivatizing FK506 with dansyl hydrazine and using chemoluminescence detection. Zeevi et al. (21) developed a bioassay to monitor the immunosuppressive activity of plasma of FK506-treated patients.

None of these assays, however, allows the specific and appropriately sensitive quantification of FK506 and all of its metabolites, a measurement that is required to assess the pharmacokinetics of the drug and to investigate whether the metabolites are of clinical significance.

Materials and Methods

Apparatus

For HPLC/mass spectrometry (MS) analysis, we used a Model 1090IL liquid chromatograph connected by a particle-beam interface to an HP5989A mass spectrometer. Data were recorded and analyzed by an HP-UX ChemStation. HPLC/UV analysis was performed with a Model 1090A chromatograph equipped with a Model 1040 diode-array detector and an HP85B data processor and integrator unit (all from Hewlett-Packard, Waldbronn, FRG). For direct chemical ionization (DCI) and fast atom bombardment (FAB)-MS, we used a Finnigan MAT 8430MS (Finnigan MAT, Bremen, FRG); for structural analysis of the internal standard, we used a Bruker 400-MHz nuclear magnetic resonance (NMR) spectrometer (Bruker, Karlsruhe, FRG).

Chemicals and Reagents

The analytical HPLC columns were filled with Nucleosil® C8 (3-µm particles, 100-nm porewidth; Macherey Nagel, Düren, FRG). All solvents were of HPLC quality and purchased from Merck, Darmstadt, FRG. The Chromsystems extraction kit for cyclosporine and its metabolites was a gift from Chromsystems (Munich, FRG), and FK506 was from Fujisawa Pharmaceuticals. FK506 and its derivatives were stored in acetonitrile/water (75/25 by vol), pH 3.0, at 4°C. The solution was stable under these conditions for ≥4 weeks.

Synthesis and Structure of the Internal Standard

We incubated 5 mL of 1 g/L FK506 reagent in acetonitrile/water (70/30 by vol) with 5 mL of acetic anhydride at 75°C for 120 min. Acetic anhydride was evaporated at 60°C under a stream of nitrogen. The residue was dissolved in 1 mL of acetonitrile/water (75/25 by vol), pH 3.0, and injected into a preparative HPLC system, consisting of two sequentially linked 250 × 10 mm columns filled with Nucleosil. FK506 and its acetyl-derivatives were eluted by gradient elution. Water used in the mobile phase was adjusted to pH 3.0 with sulfuric acid. A linear gradient was run from 48% to 51% acetonitrile at a flow rate of 5.5 mL/min; the detection wavelength of the monitor was 205 nm. The oven temperature was 75°C, the injection volume 250 µL. Fractions were manually collected.

To identify the structures, we mixed the isolated fraction with an equal volume of dichloromethane. The sample was vortex-mixed and centrifuged (2 min, 2000 × g), and the dichloromethane layer was evaporated. For analysis by DCI-MS, we reconstituted the residue in methanol at −0.5 g/L. For NMR analysis, we dissolved the residue in 500 µL of CDCl3 (Merck) containing tetramethylsilane, 0.5 g/L, as the internal shift reference. The chromatograms and mass spectra are shown in Figure 2. The FK506 derivative with an HPLC retention time of 40.5 min (fraction 3, Figure 2) showed a mass spectrum with a molecular ion at 846 amu, which indicated an acetylation in one position. The structure of this acetyl-FK506 was derived from 400 MHz 1H-NMR experiments and comparison with FK506, including homonuclear decoupling and two-dimensional homonuclear shift correlation (H,H-COSY-90). Acetyl-FK506 showed a shifted ddd absorption (J = 11.4, 9.2, 4.8 Hz, all ± 0.2 Hz) at δ = 4.68, which was assigned to the hydrogen geminal to the acetylated hydroxyl group. In the COSY spectrum, this hydrogen displayed spin–spin coupling to a ddd [11.3, 9.4, 4.6 (± 0.2) Hz] at δ = 3.22. The derived partial structure
CH(OAc)-CH(OCH₃) is part of the cyclohexyl residue; i.e., the acetylated hydroxy group is situated at C-32. Like FK506, the acetyl derivative occurs in the form of two rotational isomers in a ratio of 2:1, due to cis-trans isomerism of the C–N amide bond.

For use as internal standard, 10 µL of the isolated fraction was injected into an analytical HPLC/UV system. FK506 and (or) the internal standard were eluted from the analytical columns (250 × 4 mm, C₈, 3-µm particles) by a linear acetonitrile/water (pH 3.0) gradient: at time 0 min, the mobile phase was 45% acetonitrile; at time 40 min, it was 55% acetonitrile. The flow rate was 0.7 mL/min, the oven temperature 75 °C, and the detection wavelength 205 nm. The internal standard was quantified by using the FK506 calibration curve. The concentration of the internal standard was adjusted with acetonitrile/water (75/25 by vol) to 1 µg/L.

Extraction of FK506 and Its Metabolites

We tested the extraction procedure described by Christians et al. (8, 10) and a commercially available extraction kit for cyclosporine and its metabolites (Chromsystems), both of which were based on solid-liquid extraction. For the former procedure, we added 10 µL of the internal standard solution (10 ng of 32-O-acetyl-FK506) and 2.1 mL of acetonitrile/water (70/30 by vol) to 1 mL of EDTA-anticoagulated blood or 1 mL of urine (8, 10). After vortex-mixing and centrifuging (2 min, 2000 × g) the samples, we aspirated the supernates through glass extraction columns filled with LiChroprep® (25–40 µm, C₈; Merck), which had previously been primed with 3 mL of acetonitrile and 3 mL of water (pH 3.0). The samples were washed with 3 mL of methanol/water (50/50 by vol) and 1 mL of hexane. FK506 and its metabolites were eluted by centrifuging 1.5 mL of dichloromethane through the columns. The dichloromethane was evaporated and the residues were dissolved in 250 µL of acetonitrile/water (75/25 by vol) and washed with 500 µL of hexane.

Using the Chromsystems kit, we added 10 µL of the internal standard solution to 1 mL of blood or urine. We followed the extraction process described for cyclosporine and its metabolites as described in the kit manual. The Chromsystems kit yielded considerably cleaner extracts, with fewer substances interfering with the HPLC/UV assay of FK506 and the internal standard (Figure 3). The absolute recoveries of 75–90% were not significantly different between the two extraction procedures. Therefore, we used the Chromsystems extraction procedure during the study.

Tests with the internal standard (32-O-acetyl-FK506), FK506, and an isolated metabolite (demethyl-FK506) showed no significantly different recoveries from one another.

HPLC/UV

The same columns and HPLC conditions as described for quantifying the internal standard by HPLC/UV were used, but with detection at 202 nm. For narrow-bore HPLC (100 × 2.1 mm, filled with 3-µm C₈ materi-

---

**Fig. 3.** Extraction of FK506 and its metabolites by using the Chromsystems extraction kit (A, B) or according to Christians et al. (8, 10) (C, D).

A, C: blood samples supplemented with 50 ng of FK506 and 50 ng of internal standard; B, D: blank blood samples. Arrows mark the retention times of FK506 and the internal standard. HPLC/UV: 250 × 4 mm (C₈, 3-µm particles) analytical column, 202 nm detection wavelength, acetonitrile/water (pH 3.0) gradient elution (see text), flow rate 0.7 mL/min, column temperature 75 °C.

al), the samples were evaporated by centrifugation under reduced pressure and redissolved in 20 µL of acetonitrile/water (70/30 by vol); 10 µL of this was injected into the HPLC system. The same gradient was run as described for normal-bore HPLC, but the flow rate was 0.25 mL/min.

**HPLC/MS of FK506 and Its Metabolites**

We injected 150 µL of the extract into the HPLC. FK506 and its metabolites were eluted from a 100 × 4 mm analytical column by using the following acetonitrile/water gradient: 0 min, 60% acetonitrile; 8 min, 80% acetonitrile; 8.1 min, 95% acetonitrile; and 12.5 min, 95% acetonitrile. Alternatively, we used the following methanol/water gradient: 0 min, 70% methanol; 8 min, 80% methanol; 8.1 min, 95% methanol; 14 min, 95% methanol. The column temperature was 40 °C, the flow rate 0.3 mL/min. The source temperature of the MS was 250 °C and the quadrupole temperature was 120 °C. For MS analysis we used chemical ionization with methane or butane (purity >99.5%; Messer, Griesheim, FRG) at 160 Pa and detected negative ions. The auto-
tune option was used and the multiplier current was set to 3000 V. The MS was run in the selected-ion mode focused on the following masses: 776 amu (double de-
methylated FK506 derivatives), 790 amu (demethylated FK506 derivatives), 792 amu (double demethylated and hydroxylated FK506 derivatives), 804 amu (FK506), 808 amu (demethylated, hydroxylated FK506 derivatives), 836 amu (double hydroxylated FK506 derivatives), and 846 amu (internal standard, 32-O-acetyl-FK506).

To quantify FK506 and its metabolites, we calculated the recovery of the internal standard. The peak areas of FK506 and its metabolites were corrected according to this recovery, and concentrations were calculated by using an FK506 calibration curve.

Identification of the FK506 Metabolites

FK506 metabolites were isolated as previously described (8, 10). Human liver microsomes, isolated by standard differential centrifugation techniques (22), were incubated with FK506 and an NADPH-regenerating system for 15 min at 37 °C. The reaction was stopped by adding acetonitrile, and FK506 and its metabolites were purified by solid–liquid extraction. The samples were injected into a preparative HPLC system consisting of two sequentially linked 250 x 10 mm columns packed with C8 (7-μm particles). The flow rate was 5.5 mL/min, the column temperature was 75 °C, and the signal was monitored at 205 nm. FK506 and its metabolites were separated by using a concave acetonitrile/water gradient: at analysis time 0 min, 37% acetonitrile; at 20 min, 51% acetonitrile; at 35 min, 85% acetonitrile. The elution was followed by a 5-min column washing step with 95% acetonitrile and reequilibration to the start conditions within 10 min. Fractions were manually collected. The metabolites were extracted from the isolated fractions by liquid–liquid extraction with an equal volume of dichloromethane. The dichloromethane layer was separated and evaporated. The residues were dissolved in 10 μL of methanol, and the structures of the metabolites were identified by DCI-MS, taking into account molecule ions and characteristic fragments. Butane was used as the reagent gas and negative ions were detected. The isolated metabolites were quantified by HPLC/UV, as described for the internal standard, with use of an FK506 calibration curve. These metabolites were used for identifying peaks and for preparing calibration curves in the HPLC/MS system.

Quality Assessment

Quality controls. EDTA-anticoagulated blood was supplemented with FK506, 5 or 25 μg/L, and 1-mL portions were transferred into 10-mL centrifuge tubes. Samples were incubated at 37 °C for 30 min and frozen at -20 °C until use.

Calibration controls. Each calibration curve comprised five data points at concentrations of 0, 1, 5, 10, and 50 μg/L with n = 5 per data point. We added the respective FK506 concentrations to blood samples, incubated them for 30 min at 37 °C, and froze these standards at -20 °C until use. In a set of 10 samples assayed, 2 samples were quality controls, and 2 samples were calibration controls. We measured 10% of the patients' samples twice.

Cross-validation with the FK506 enzyme immunoassay of plasma. For cross-validation with the enzyme immunoassay of FK506 in plasma, we selected without conscious bias 25 blood samples from 17 different liver graft patients (no more than two samples per patient) and measured FK506 by both methods.

Interferences of other drugs with the assays. To evaluate interferences of other drugs with the chromatographic analysis of FK506, we dissolved these drugs in acetonitrile/water (50/50 by vol) at a concentration of 1 g/L and injected 25 μL into the HPLC system for analysis as described above. We tested the following drugs: ace
tylsalicylic acid, acyclovir, amikacin, ampicillin, atenolol, azathioprine, azlocillin, cefotaxime sodium, cyclopentolate, cilastatin sodium, cimetidine, ciprofloxacin, clonidine hydrochloride, dexamethasone, diazepam, dil
tiazem, dopamine, epinephrine hydrochloride, erythromycin, fluocoxacin sodium, ganciclovir, gentamicin, imipenem, ketoconazole, lidocaine, mezlocillin, midazo
lam, nicardipine hydrochloride, nifedipine, omeprazole, pipercillin sodium, ranitidine, ramapen, rolitetracy
cline, spironolactone, sulfamethoxazole, trimcicinolone, trimethoprim, and vancomycin hydrochloride.

For HPLC/MS analysis, we searched the Wiley/NBS (John Wiley and Sons, Chichester, UK) and Pfieger/Maurer (VCH, Weinheim, FRG) MS spectra libraries for potential interferences.

Results

The limit of quantification of the HPLC/UV assay was 15 ng of FK506, the limit of detection was 5 ng [determined by injection of 10 μL of a 500 μg/L solution of FK506 in acetonitrile/water, pH 3.0 (75/25 by vol)], and the calibration curve in blood was linear to ≈225 ng (r = 0.998). The interassay coefficient of variance (CV) at 100 μg/L was 9.5% (SD 1.3%) (n = 8); close to the detection limit, it was >50%. Several drugs commonly used after organ transplantation were found to interfere with the HPLC/UV assay (Table 1). We tested various columns, filled with C18, C8, end capped C8, C4, and cyano-propyl materials. FK506, the internal standard, and the me-

---

**Table 1. Interference of Other Drugs with HPLC/UV Assay of FK506 and its Metabolites**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Retention time, min</th>
<th>Assay interfered with</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>18.2</td>
<td>Metabolites</td>
</tr>
<tr>
<td>Cyclosporine metabolites (AM1A, AM1)</td>
<td>33.5, 35.2</td>
<td>FK506</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>29.3, 35.5</td>
<td>FK506</td>
</tr>
<tr>
<td>Lidoacine</td>
<td>17.9</td>
<td>Metabolites</td>
</tr>
<tr>
<td>Midazolam</td>
<td>33.4</td>
<td>FK506</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>32.8</td>
<td>FK506</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>15.9, 17.3</td>
<td>Metabolites</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>18.7</td>
<td>Metabolites</td>
</tr>
</tbody>
</table>

Acetonitrile/water (pH 3.0) gradient: time 0 min, 43% acetonitrile; 20 min, 50% acetonitrile; 35 min, 59% acetonitrile; 50 min, 25% acetonitrile; detection wavelength, 205 nm; other conditions as in Fig. 3.
tabolites were eluted from the alkyl-modified materials in a double-peak elution pattern, caused by cis–trans isomerism of the C–N amide bond. Only the cyanopropyl modified silica gel resulted in a single but broad peak. There was no difference between endcapped and nonendcapped material. The use of narrow-bore HPLC had no significantly positive effect on the detection limit.

After incubation of FK506 in acetic anhydride, DCI-MS detected single-, double-, and triple-acetylated derivatives. The conditions described gave the highest yield of 32-O-acetyl FK506. The double- and triple-acetylated derivatives were less stable during ionization than was 32-O-acetyl-FK506.

The temperature of the MS source and the use of negative chemical ionization led to almost no fragmentation of FK506 and its metabolites during mass spectral analysis. The use of a higher source temperature enhanced the fragmentation, especially of the metabolites, whereas a lower source temperature led to faster contamination of the MS source, with a resulting loss of sensitivity. The limit of detection of the HPLC/MS assay was 25 pg after flow injection of 10 μL of FK506 standard solution (FK506 at 2.5 μg/L in acetonitrile/water, pH 3.0 (70/30 by vol)), resulting in a signal-to-noise ratio of 8:1. The mobile phase was acetonitrile/water (90/10 by vol). The calibration curve was linear from 25 pg to 50 ng (r = 0.999). Flow injection of the extracted blood samples showed peaks that interfered with FK506. This interfering material could be separated from FK506 and its metabolites by using a 100 × 4 mm C8 column and gradient elution (Figure 4). In a first step, the gradient eluted the interfering material and then eluted the FK506 metabolites, FK506, and the internal standard. A complete chromatographic separation was not necessary, because the metabolites could be differentiated by their molecular ions. It was much more important to elute the compounds of interest at almost the same time because the sensitivity of the MS detection was dependent on the acetonitrile/water or methanol/water ratio of the mobile phase. The use of a gradient allowed us to elute FK506 and its metabolites from the columns at a composition of the mobile phase that yielded the greatest sensitivity during particle beam separation and MS analysis (Figure 5). The sensitivity of the assay was best at a methanol/water volume ratio of 90/10.

The limit of quantification in blood was 250 pg, determined by extraction of 1 mL of blood and injection of 150 μL of the extract. The intra-assay CV was 10.5% at 5 ng (n = 10). The interassay CV was 12.3% at 1 μg/L, 11.3% at 5 μg/L, and 13.2% at 50 μg/L (all with n = 10).

The calibration curve after extraction of FK506 from blood had a correlation coefficient (r) of 0.998.

Analysis of extracts of human liver microsomal preparations after metabolism of 10 nmol of FK506 showed that all metabolites previously identified could be detected by HPLC/MS (Figure 6). No other clinically relevant drugs were found to interfere with the assay.

Stability of the HPLC/MS system was tested by 50

![Fig. 4. HPLC/UV and HPLC/MS analysis for FK506 in blood samples A, UV chromatogram of an extracted FK506-free blood sample; B, the same sample after injection into the HPLC/MS system; C, HPLC/MS analysis of a blood sample with 2 ng of FK506 and 5 ng of the internal standard 32-O-acetyl-FK506 added. HPLC/UV conditions: 100 × 4 mm (C8, 3-μm particle) analytical column. Acetonitrile/water gradient as follows: 0 min, 50% acetonitrile; 1 min, 60% acetonitrile; 8.1 min, 95% acetonitrile; 10 min, 50% acetonitrile; flow rate, 0.3 mL/min. Column temperature: 40 °C, detection wavelength: 205 nm. HPLC/MS: chromatographic conditions as described for HPLC/UV; reagent gas, methane; source temperature, 250 °C; quadrupole temperature, 120 °C. The detection mass was changed from 604 to 844 amu at 10.5 min](image)

![Fig. 5. Association of detection signal of the HPLC/MS analysis and composition of the mobile phase](image)

Repeated flow injections of 10 ng of FK506: mobile phase, methanol/water; flow rate, 0.3 mL/min; column temperature, 40 °C; reagent gas, methane; source temperature, 250 °C; quadrupole temperature, 120 °C. Shown are mean ± SD (n = 3)

repeated injections of 10 ng of FK506, with one injection every 1.5 min, beginning right after starting the HPLC pump. It took 20 min until a stable signal could be detected. The signal was stable for the other injections,
with a slight increase in sensitivity of ~20%, which could be attributed to a time-dependent change of the shape of repeller in the MS source. Long-term stability could be evaluated by analysis of quality-control samples during a sequence of 75 extracted blood samples; the change of signal was 9.3% (n = 8), which is almost equal to the intra-assay CV described above.

Cross-validation of the HPLC/MS blood assay of FK506 and its metabolites with an enzyme immunoassay of FK506 in plasma in 25 patients showed no significant correlation between both assays.

With the system described, as many as 75 samples could be run before the skimmers of the particle beam interface had to be cleaned or exchanged. The source of the MS had to be cleaned every 200–250 samples.

In blood samples, FK506 and a demethylated metabolite at 790 amu (demethyl-FK506), were found (Figure 7); in urine, FK506 was found, as were metabolites at 776 (di-demethyl FK506), 790 (demethyl FK506), and 792 amu (di-demethyl hydroxyl FK506) (Figure 8).

Discussion

The HPLC/MS assay had several advantages over the other assays tested. The HPLC/UV assay was not sensitive enough. Most patients measured in this study had trough blood concentrations of FK506 in the range of 5–10 µg/L, which was below the detection limit of the HPLC/UV assay. The major problem of HPLC of FK506, its metabolites, and the internal standard is the double elution pattern caused by the cis–trans isomerism of the C–N amide bond. Especially in low concentrations, FK506 and its derivatives were eluted as broad peaks, with incomplete separation of the rotamers, which caused high intra-assay variability. Furthermore, several clinically important drugs interfere with the HPLC/UV assay.

Detection of FK506 by MS had the advantage that, besides the different HPLC retention times and the different m/z values, a second criterion was available to identify FK506 and its metabolites. With HPLC alone, only an incomplete separation of the metabolites was possible (8, 10). The specific detection by MS allowed us to choose HPLC conditions that reduced peak broadening and the double-peak elution of FK506. The best
results were obtained with a short (100 × 4 mm) C₈ column. Sensitivity of the MS detection depended on the flow rate of the mobile phase: 0.3 mL/min gave the best results. Using an increased column temperature of 75 °C in the HPLC/UV assay (8, 10) increased the isomerization rate of the drug, which resulted in narrower peaks. However, because the step gradient and the analytical column used for HPLC/MS are not able to separate the FK506 rotamers, use of higher temperatures gave no advantage in the latter assay. The columns were maintained at 40 °C, which was enough above room temperature to be maintained by the column oven. Using either acetonitrile or methanol in the mobile phase made no significant difference in the detection limit, but the method involving methanol resulted in sharper peaks.

Liquid–liquid and solid–liquid extraction of plasma samples gave different results in the enzyme immunoassay—presumably because of different recoveries of the FK506 metabolites and cross-reaction of the antibody used for enzyme immunoassay with the metabolites. Thus, we checked the recoveries of isolated metabolites in the solid–liquid extraction methods used and found these not to be significantly different from that of FK506 or the internal standard. Solid–liquid extraction procedures were easier to handle for a large number of samples and could also be automated (23).

The use of an internal standard was required in the HPLC/MS assay for two reasons: to compensate for losses during extraction, and to compensate for the variable detection sensitivity of the MS. The more samples we ran on the MS, the more the skimmers and the MS source were contaminated, and the more the sensitivity decreased.

The question of whether to use blood or plasma for therapeutic drug monitoring of FK506 is still under discussion (24, 25). One major disadvantage of plasma as matrix is that the distribution of FK506 between blood cells and plasma depends on the temperature and hematocrit of the sample (26). Furthermore, it is not known whether the distribution coefficients between blood and plasma at a given temperature are the same for FK506 and its metabolites. For practical reasons, blood is much easier to handle, is reproducible, and is not subject to the erroneously high concentrations of FK506 seen in hemolyzed plasma. The problem of choice of matrix was exhaustively considered for the immuno-suppressant cyclosporine as well; today, blood is the generally accepted matrix of choice (27, 28) because the cyclosporine concentrations in blood show better correlation than plasma values with such clinical events as rejection and (or) cyclosporine toxicity, and methods based on blood can easier be standardized. Furthermore, the FK506 concentrations determined in plasma by enzyme immunoassay are in the ng/L range, which is at the detection limit of the analytical methods available. Concentrations of FK506 in blood are 20- to 50-fold higher than those in plasma (24, 29), and thus are in a concentration range that allows for more reliable and reproducible quantification. For these reasons we selected blood as matrix for this study.

The lack of a significant correlation between enzyme immunoassay in plasma and HPLC/MS in blood is mainly due to the different matrices used. As discussed above, the FK506 concentration in plasma is dependent on concentration, hematocrit, and temperature (24–26).

Thus far, only two specific assays for FK506 have been available with a sensitivity suitable for measurement in plasma or blood of patients. One was based on derivatization of FK506 with dansyl hydrazine, column-switching HPLC, and chemiluminescence detection (20). Derivatization is a time-consuming procedure and is usually accompanied by a high CV, because of the derivatization reaction. Only one assay (19) differentiates between FK506 and its metabolites: in this assay, FK506 and its metabolites are separated by HPLC, and the respective fractions collected are quantified by enzyme immunoassay. This is possible because the antibody used for the enzyme immunoassay obviously is not specific for FK506 but cross-reacts with the metabolites. However, quantification of the metabolites is not possible with that method because the extent to which the antibodies cross-react with the FK506 metabolites is not yet known.

In conclusion, HPLC/MS with a particle-beam interface allows the direct specific and sensitive quantification of FK506 and all its known metabolites within a 15-min HPLC run, after simple solid–liquid extraction with a commercially available extraction kit.

We thank F. Mandel (Hewlett-Packard, Waldbronn, FRG) for his support and fruitful discussions. This study was supported by DFG grant Pi 48/11-4, project D5, and SFB 265, project A7.

References
10. Christians U, Radeke HH, Kownatzki R, Schiebel HM, Schott-