FK506 (Tacrolimus) and Its Immunoreactive Metabolites in Whole Blood of Liver Transplant Patients and Subjects with Mild Hepatic Dysfunction

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Purpose. To determine the concentrations of FK506 and its metabolites in blood from liver transplant patients and subjects with hepatic dysfunction.

Methods. HPLC was combined with an enzyme-linked immunosorbent assay (ELISA) to determine the concentrations of FK506 and its immunoreactive metabolites in human whole blood.

Results. In four liver transplant patients, most of the immunoreactivity was seen in the HPLC fractions where unchanged FK506 eluted. FK506 accounted for about 95% or more of the total immunoreactivity in the first days posttransplant. Immunoreactivity observed in the nonFK506 fractions ranged from 1.6% to 10.7% of the total immunoreactivity; about 30% of the nonFK506 immunoreactivity was due to M-III (15-O-demethyl FK506). Blood from subjects with mild hepatic dysfunction was examined at 1.5 and 6 hours after an oral and intravenous dose, respectively, by HPLC-ELISA. Regardless of the route of administration, more than 96% of the total immunoreactivity was recovered in the FK506 fraction. M-III was detected in the blood of 3 of 6 subjects after an oral dose, but in none of these after an intravenous dose.

Conclusions. ELISA is an appropriate method for therapeutic drug monitoring of FK506.

KEY WORDS: FK506; tacrolimus; HPLC-ELISA; immunoreactive metabolites; liver transplantation; hepatic dysfunction.

INTRODUCTION

FK506 (tacrolimus, Prograf®) is a 23-membered ring, 822 dalton macrolide of fungal origin discovered in 1984 in Tsukuba, Japan.[1,2] This novel immunosuppressant agent is approved in the US, Europe, and Japan for prevention of allograft rejection in liver transplant patients.

Two immunoassays, based on the same murine anti-FK506 monoclonal antibody, were developed to monitor FK506 concentrations in blood or plasma: an enzyme-linked immunosorbent assay (ELISA) [3] and a microparticle enzyme immunoassay (IMx®; Abbott Laboratories).[4]

FK506 is metabolized by cytochrome P450 systems, specifically cytochrome P450 3A, to at least four major metabolites (first generation) and at least nine total metabolites.[5–9] *In vitro*, the predominant metabolite was 13-O-demethyl FK506 (M-I) and less common metabolites were O-demethylated at the 31- and 15-positions (M-II and M-III, respectively) and hydroxylated at the 12 position (M-IV).[9] Secondary metabolism appeared to involve activity by the same mechanisms at other sites on the molecule.[5,6]

The primary site of FK506 metabolism is the liver. The hepatic metabolism of FK506 might vary after liver transplantation or in subjects with reduced liver function. A potential problem with immunoassays was cross-immunoreactivity of metabolites with the antibody, making the assay results nonpredictive of immunosuppressant activity. In this study, blood obtained from such subjects was analyzed by HPLC-ELISA to determine the concentrations of FK506 and its immunoreactive metabolites.

MATERIALS AND METHODS

Patients

Blood samples were obtained from patients enrolled in clinical studies conducted by Fujisawa USA, Inc. All patients were required to give Informed Consent and the studies were reviewed by IRBs according to US FDA Regulations. Blood samples were collected into EDTA-tubes, shipped on dry ice, and stored at −20 °C until analyses. Samples were obtained from four liver transplant patients (three pediatric and one adult) and six subjects with mild liver dysfunction.

Chemicals and Reagents

FK506 analytical standard, FK506 metabolites (M-II and M-III), anti-FK506 monoclonal antibody, and FK-POD (an FK506-horseradish peroxidase conjugate) were supplied by Fujisawa Pharmaceutical Company, Ltd., Osaka, Japan. Goat anti-mouse polyclonal antibody was obtained from Incstar (Stillwater, Minnesota). Bovine serum albumin and o-phenylenediamine dihydrochloride tablets were obtained from Sigma Chemicals (St. Louis, MO). All other chemicals and reagents were obtained from suppliers offering the highest purity available.

HPLC Procedure

The reversed-phase HPLC system consisted of an L-6200A pump and AS-2000 autosampler (Hitachi Instruments), a T-6300 column heater (E. Merck), Intersil ODS-2 (4.6 × 10 mm) guard column, Altima C_{18} 5 μm (4.6 × 250 mm) column, and a 785A absorbance detector (Applied Biosystems). Detection was at 210 nm and the column was heated to 50 °C.

A volume of patient whole blood that contained the equivalent of 5 or 10 ng of FK506, as determined by ELISA or Abbot IMx, was added to 1 ml of 0.2M phosphate buffer (pH 7.0). Following addition of 100 μl of methanol, each tube was vortexed for approximately 30 sec and 8 ml of ethylacetate was added. The samples were shaken for 30 min at room temperature at low speed on a reciprocal shaker, then centrifuged at 3000 rpm for 10 min at 4 °C. Seven ml of the organic phase was removed and dried under nitrogen at 45 °C. Dried samples were resuspended in 175 μl of acetonitrile

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and vortexed for 3 min. Then, 100 μl of the sample was injected into the HPLC system.

Three mobile phase gradient systems were used to separate FK506 and its metabolites. Phase A consisted of 100% acetonitrile, Phase B of 20% acetonitrile, and Phase C of 100% methanol. A linear gradient elution was performed during the first 35 min and from 40 to 45 min. The mobile phase consisted of 100% Phase B at 0 min, 63% Phase A and 37% Phase B between 35 and 40 min, and 100% Phase A from 45 to 50 min. The column was then flushed with Phase C for 6 min.

Eluent was collected every minute for the period of 31 to 51 min after injection. Previous experience showed no significant immunoreactivity in the fractions which eluted before 31 min. Each fraction was dried under nitrogen at 45 °C, then reconstituted with 200 μl of FK506-POD solution and vortexed for 3 min.

ELISA Assay

The ELISA assay was previously reported [3] and has been used in patient monitoring.[10] Briefly, a microtiter plate was prepared by adding 200 μl of goat anti-mouse antibody solution (2.5 μl/ml). The plate was incubated overnight at 4 °C on a plate shaker, then the antibody solution was discarded. Each well was washed three times with 300 μl of phosphate-buffered saline (PBS), then 300 μl of bovine serum albumin (1%) in PBS-TWEEN 20 was added to each well to block nonspecific binding. The plate was incubated at room temperature for at least one hour. This solution was aspirated off and 160 μl of the reconstituted samples (FK506-POD plus FK506 samples) was applied sequentially to each well using a micropipettor. Anti-FK506 antibody solution (50 μl per well) was added to each sample well, followed by overnight incubation at 4 °C on a plate shaker.

The wells were aspirated and washed twice with 200 μl of PBS-TWEEN 20 followed by two washes with 200 μl PBS. Immediately thereafter, 200 μl of o-phenylenediamine solution was added to each well at 20-sec intervals. The plate was incubated for approximately 12 min at room temperature in the dark. The reaction was stopped by adding 50 μl of 4N H_2SO_4 to each well at 20-sec intervals. Absorbance values were read on a Bio-Tek Bio-Kinetics microplate reader at 490 nm.

RESULTS

A typical HPLC chromatogram of FK506 and metabolites in spiked blood and patient samples is shown in Figure 1.

The retention times for FK506, M-II, and M-III were 44.8, 39.2, and 36.6 min, respectively. The immuno-cross-reactivity of FK506, M-II, and M-III was then determined in spiked whole blood samples processed by the HPLC procedure. Most FK506 was recovered in fractions 45 and 46, collected from 44 to 46 min after injection (38% and 56%, respectively). M-II was recovered primarily in fractions 39 and 40, collected from 38 to 40 min (9% and 86%, respectively). M-III was recovered primarily in fractions 36, 37, and 38, collected from 35 to 38 min (4%, 89%, and 3%, respectively).

Blood from an adult liver transplant patient was analyzed by HPLC-ELISA on Days 1–9 posttransplant. This patient received 7.5 mg of FK506 as a continuous intravenous infusion over 36 hours, followed by five 8-mg doses orally every 12 hours and eight 10-mg oral doses every 12 hours. The majority of immunoreactivity was found in the FK506 fractions (45 and 46). FK506 accounted for 89–98% of the total immunoreactivity on Days 1–9. The amount of nonFK506 immunoreactivity increased slightly with time posttransplant (Figure 2). Immunoreactivity ascribable to M-III (fraction 37) was first noted on Day 5 and increased to almost 3% of the total immunoreactivity recovered on Day 9. Some of the nonFK506 immunoreactivity may have been due to M-II, as the amount of immunoreactivity in fractions 39 and 40 showed some increase with time.

The three pediatric patients received FK506 as a continuous intravenous infusion of 0.05 mg/kg/day or as 0.3 mg/kg/day orally. Blood from these patients was analyzed by HPLC-ELISA on Days 1, 8, and 40; Days 1, 12, and 29; and Days 3 and 6, respectively. In all three subjects, most immunoreactivity (>93%) was due to unchanged FK506 and nonFK506 immunoreactivity was relatively constant posttransplant (Figure 3). Immunoreactivity ascribable to M-III was detected on Day 1 in the first subject (1.3% of total immunoreactivity), Day 29 in the second (2.2% of total), and Day 6 in the third (1.0% of total). In the first subject, immunoreactivity related to M-III remained relatively constant (1.3–1.9% of total) from Day 1 to Day 40.

Blood samples were collected from six patients with mild liver dysfunction at 1.5 hours after a single oral FK506 dose of 0.12 mg/kg and 6 hours after a 4-hour iv infusion of 0.02 mg/kg. Following oral administration, most immunoreactivity (>96%) was observed in the unchanged FK506 fractions and nonFK506 immunoreactivity was 1.2–3.5% of the total immunoreactivity. Three patients showed some immunoreactivity in the M-III fraction (fraction 37), but this accounted for 0.6–1% of the total immunoreactivity. Following intravenous infusion, total nonFK506 immunoreactivity was 1.3–2.5% and there was no immunoreactivity in fraction 37.

DISCUSSION

An HPLC-ELISA procedure was used to investigate the concentrations of FK506 and its immunoreactive metabolites in whole blood of liver transplant patients and subjects with mild liver dysfunction. FK506, M-II, and M-III were directly measured by the current assay. M-I, the major metabolite of FK506,[6] and M-IV were not directly measured by the current assay because of little cross-immunoreactivity with the monoclonal antibody.[9]

Following liver transplantation, most of the immunoreactivity (≥89%) was recovered in the FK506 fractions in whole blood from both an adult and three pediatric patients. NonFK506 immunoreactivity accounted for only a small and relatively constant percentage of total immunoreactivity over time posttransplant. Immunoreactivity corresponding to M-III was seen in all patients and there was some immunoreactivity seen in the fractions corresponding to M-II in the adult patient.

Similarly, most immunoreactivity in the blood was ascribed to unchanged FK506 in subjects with mild hepatic dysfunction. The minor difference in metabolite patterns in

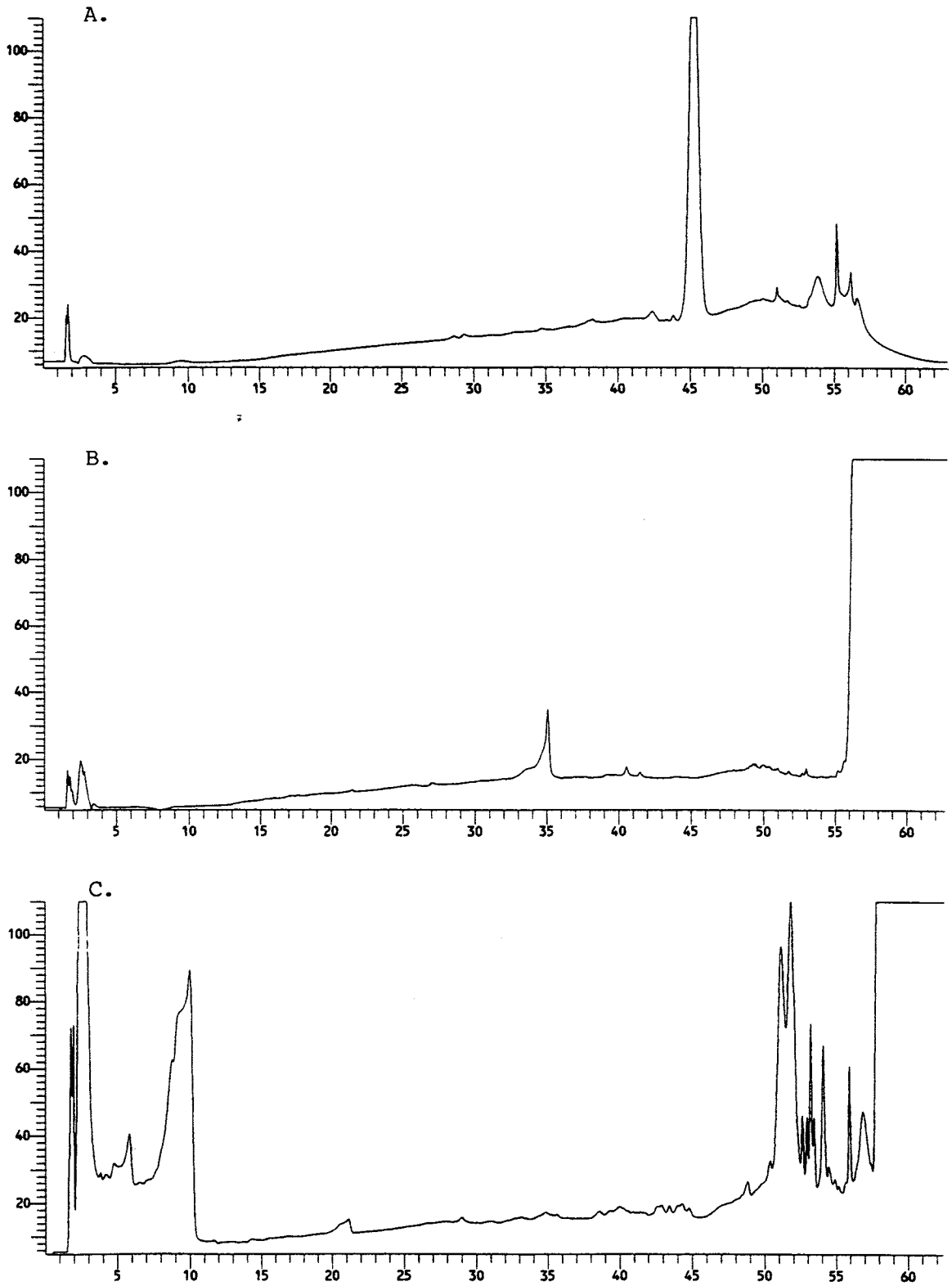

Fig. 1. Typical HPLC chromatograms of FK506 (A) and metabolites (B) in spiked blood and patient blood sample (C).

subjects administered FK506 orally or intravenously may reflect either differences in sampling times or in intestinal and hepatic metabolism of FK506.

Recently, the structures of three didemethyl metabolites of FK506, M-V (15,31-O-didemethyl FK506), M-VI (13,31-O-didemethyl FK506), and M-VII (13,15-O-didemethyl FK506, were proposed and their cross-immunoreactivity and pharmacological activities were characterized.[11] M-V

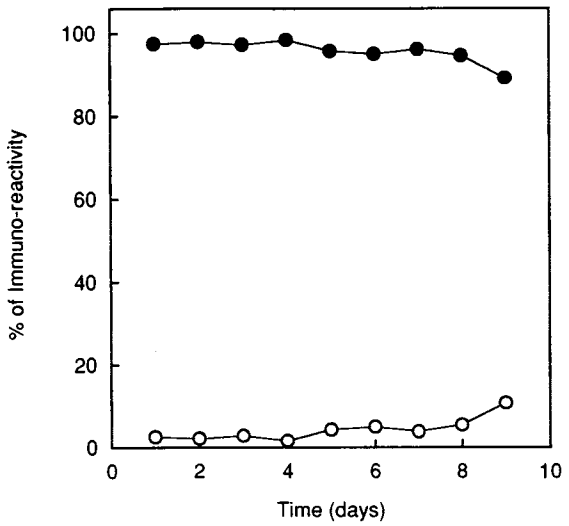

Fig. 2. Time-courses of immunoreactivity recovered in FK506 (●) and nonFK506 (○) fractions after liver transplantation in an adult.

exhibited 92% of the cross-immunoreactivity to the antibody of FK506, but M-VI and M-VII showed no reactivity. These three metabolites exhibited very little immunosuppressant activity in a mouse mixed lymphocyte reaction assay. Based on its cross-immunoreactivity, M-V should be directly detected by the current assay. The didemethyl and didemethylhydroxy metabolites have been reported to accumulate in patients with liver dysfunction.[12] In the present study, however, only minor nonFK506 immunoreactivity (less than 3.5%) was observed in the blood of the subjects with mild liver dysfunction. This suggests that there was little M-V in the blood of these subjects, at least at the times sampled.

The majority of immunoreactivity detected in whole blood of liver transplant patients or subjects with mild hepatic dysfunction administered FK506 was due to unchanged FK506. Therefore, ELISA is an appropriate assay method for therapeutic drug monitoring of FK506.

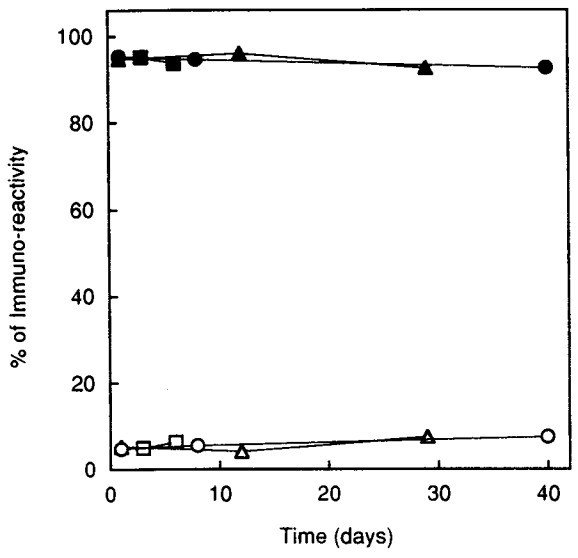

Fig. 3. Time-courses of immunoreactivity recovered in FK506 (closed symbols) and nonFK506 (open symbols) fractions after liver transplantation in pediatric patients (circles = patient 11901; squares = patient 11001; triangles = patient 11902).

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