

Tacrolimus Analysis: A Comparison of Different Methods and Matrices

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Summary: We determined the trough blood and plasma concentrations of tacrolimus from the day of transplantation through 30 days posttransplantation in four liver and four kidney transplant patients by three different methods. The first method involved a solid phase extraction of the blood or plasma using Sep-Pak columns (SPs) followed by quantitation of tacrolimus using an enzyme-linked immunosorbent assay (ELISA); the second method involved a liquid-liquid extraction using methylene chloride (MC) followed by quantitation of tacrolimus using the ELISA, and the third method involved a high-performance liquid chromatography (HPLC) fractionation of the extract obtained from the solid-phase extraction and quantitation of tacrolimus in the fractions by ELISA. The trough plasma tacrolimus concentrations ranged from 0.1 to 5.2 ng/ml. While the trough plasma concentrations of tacrolimus were similar and independent of the method of analysis in kidney transplant patients and in liver transplant patients with normal biochemical profile, in patients with liver dysfunction, tacrolimus plasma concentrations were higher when measured by SP-ELISA and MC-ELISA methods as compared to the HPLC-ELISA method. In plasma samples obtained from liver transplant patients with liver dysfunction, the presence of some metabolites that cross-reacted with the antibody used in the ELISA could be documented in the HPLC fraction corresponding to the metabolites. This indicates that while tacrolimus metabolites that cross-react significantly with the antibody used in the ELISA do not accumulate in kidney transplant patients, they can appear in the plasma of patients with liver dysfunction. The trough blood tacrolimus concentrations in patients were significantly higher than the corresponding plasma concentrations and ranged from 1.4 to 107 ng/ml. The trough blood tacrolimus concentrations were similar and independent of the method of analysis in kidney and liver transplant patients, suggesting unchanged tacrolimus to be the major component in the blood. The HPLC fractions corresponding to the metabolites of tacrolimus did not contain any components that cross-reacted with the antibody used. This study documents that the methods used in this study for the analysis of blood concentrations of tacrolimus appear to be specific for the parent tacrolimus and can be used in future pharmacokinetic and clinical studies. **Key Words:** Analysis—Immunosuppression—Tacrolimus—Therapeutic monitoring.

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Therapeutic monitoring of immunosuppressive drugs is an integral part of optimizing drug therapy in transplant patients because of the narrow therapeutic index of these agents. Since immunosuppressive drugs are used on a long-term basis, trough

blood or plasma concentration measurement of immunosuppressive drugs in organ transplant patients will also help in assuring compliance with the drug treatment protocol. Tacrolimus, a recently approved immunosuppressive drug, is effective in preventing graft rejection in patients after liver, kidney, and heart transplantation (1). Large interindividual variability in the pharmacokinetics (2) and the nephrotoxicity of tacrolimus (3) necessitates routine monitoring of this agent in transplant patients. Tacrolimus has been measured in plasma by an enzyme-linked immunosorbent assay (ELISA) that was originally developed by Tamura et al. (4), or by a modification of this ELISA (5–8), or by high-pressure liquid chromatography (HPLC)-ELISA (9,10), or by HPLC after derivatizing with dansyl hydrazine (11), or by a bioassay (12). Blood concentration of tacrolimus has been measured by ELISA (6,7,8,13), by a microparticle enzyme immunoassay developed by Abbott Laboratories (14–16), by a radioreceptor assay (17), or by HPLC-mass spectrometry (18,19).

Recently, we compared the plasma tacrolimus concentrations in kidney and liver transplant patients as measured by ELISA after a solid-phase extraction or a liquid-liquid extraction using methylene chloride or after a solid-phase extraction and HPLC fractionation to separate unchanged tacrolimus from metabolites that might be coextracted (10). We observed significant differences in the plasma concentration of tacrolimus between the different methods used only in samples obtained from patients with impaired liver function as determined by elevated bilirubin concentrations. This study was designed to extend our observations to whole blood tacrolimus concentration measurements in liver and kidney transplant patients during the immediate postoperative period.

MATERIALS AND METHODS

Materials

Tacrolimus pure drug, the monoclonal antibody for tacrolimus, and tacrolimus peroxidase enzyme conjugate were supplied by Fujisawa Pharmaceuticals, Osaka, Japan. Anti-mouse IgG was purchased from Atlantic Antibodies (Stillwater, MN, U.S.A.). Sep-Pak columns (SPs) (C-18) were obtained from Waters (Milford, MA, U.S.A.). O-Phenylenediamine was purchased from Sigma (St. Louis, MO, U.S.A.). Methylene chloride and methanol were

purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). The HPLC column used, a u-Bondapak C-18 column that is 15 cm long with an internal diameter of 3.9 mm was maintained at 60°C and the column eluents were monitored at 214 nm. Using a mobile phase of 80% methanol and 20% water (acidified to pH 6 with HCl) at a flow rate of 1.5 ml/min, the retention time of tacrolimus was 4.8 min.

Methods

Clinical Protocol

Patients received i.v. tacrolimus (0.1 mg/kg/day) as a continuous i.v. infusion during the immediate postoperative period and for up to 3–7 days after surgery. Oral therapy was initiated as soon as the patients were able to tolerate oral intake. Tacrolimus (0.1–0.24 mg/kg/day) was administered orally twice daily. Multiple serial blood samples were collected in heparinized tubes prior to the morning dose of tacrolimus from four liver and four kidney transplant patients from day 1 after surgery until approximately 30 days posttransplantation.

Analysis

Aliquots of the whole blood samples were separated and maintained at 4°C until analysis. The rest of the blood samples were incubated at 37°C for 45 min and centrifuged at 37°C for 10 min and the plasma obtained was analyzed on the same day or frozen at –20°C until analysis. The calibration curve for whole blood consisted of blank blood samples spiked with tacrolimus concentrations of 0, 0.8, 4, 8, 16, 40, and 64 ng/ml. Blood and plasma samples were extracted by the solid-phase extraction method, or by the liquid-liquid extraction method using methylene chloride, as described previously (10), except for the use of 1.0 N of HCl to assure complete lysis of whole blood samples prior to extraction. Additional samples were also extracted by the solid-phase extraction method and subjected to HPLC separation (as described in reference 10), and the fractions corresponding to the intact tacrolimus (fraction 2, 3.6–6 min) and tacrolimus-related materials (fraction 1, 0–3.6 min) were separately collected and evaporated to dryness under nitrogen. All the extracts were analyzed by ELISA, with overnight incubation.

A minimum of nine blood and plasma samples was analyzed from each of the patients observed.

Biochemical parameters indicative of the kidney function (serum creatinine level) and liver function (bilirubin and aspartate and alanine aminotransferase levels) were also measured in these patients over the entire study period.

RESULTS

The biochemical profiles of the patients observed are listed in Table 1. While all but one kidney transplant patient had normal liver function test results, not all the liver transplant patients had normal kidney function throughout the entire course of study. The interday coefficients of variation of the three methods used varied from 4.2 to 19.7% (Table 2), and were similar between the three methods used.

Figures 1 and 2 illustrate tacrolimus blood concentrations as measured by the three methods in liver and kidney transplant patients, respectively. In all the patients, the whole blood concentration of tacrolimus was very similar as measured by ELISA after the three different extraction/separation procedures. The regression equations of the tacrolimus concentrations measured by HPLC-ELISA versus SP-ELISA or MC-ELISA in whole blood are listed in Table 3. There was a significant correlation (0.821–0.949) between the two variables evaluated, and the slope of the lines was near unity (0.92–1.1).

Figures 3 and 4 illustrate the plasma concentrations of tacrolimus in liver and kidney transplant patients as measured by the three methods. Tacrolimus plasma concentrations were similar as measured by the three methods in the kidney transplant patients at all times. However, in the liver transplant patients, when the serum bilirubin concentrations were elevated, the tacrolimus concentrations

TABLE 2. Between-run precision of different assay methods

	Whole blood			Plasma		
	Mean, ng/ml	% CV	No.	Mean, ng/ml	% CV	No.
Solid-phase extraction						
LC	13.2	18.2	7	0.7	14.2	18
MedC	23.4	19.7	7	2.2	13.6	18
HC	36.3	15.4	7	5.8	12.0	18
Methylene chloride extraction						
LC	11.1	5.4	7	0.6	16.6	8
MedC	18.5	8.0	7	1.8	11.1	6
HC	30.6	13.0	7	5.7	15.7	5
HPLC						
LC	15.5	13.5	3	0.7	14.3	7
MedC	28.9	4.2	3	2.3	17.4	7
HC	35.1	5.4	4	5.6	10.7	7

CV, coefficient of variation; LC, low control; MedC, medium control; HC, high control; HPLC, high-performance liquid chromatography.

measured by SP-ELISA and MC-ELISA were higher than the values obtained by the HPLC-ELISA method. As the bilirubin concentrations recovered toward normal values, the concentration of tacrolimus measured by all three methods was almost identical. Correspondingly, Table 3 shows a slope of greater than unity (1.87 and 1.75, respectively) when plasma tacrolimus concentrations measured by SP-ELISA or MC-ELISA were compared with HPLC-ELISA measurements.

Figure 5 illustrates the correlation between plasma concentrations and whole blood concentrations in liver and kidney transplant patients as measured by all three methods. Blood and plasma con-

TABLE 1. Biochemical profile in four liver and four kidney transplant patients

Patients	Creatinine, mg/dl		Bilirubin, mg/dl		AST, IU/L		ALT, IU/L	
	Immediate postoperative period	3 wk after transplant	Immediate postoperative period	3 wk after transplant	Immediate postoperative period	3 wk after transplant	Immediate postoperative period	3 wk after transplant
Kidney								
CK	3.6	1.7	0.4	0.3	46	20	38	66
KW	1.2	1.2	0.9	0.7	712	33	1,476	89
JG	0.8	2.9	0.5	0.8	46	18	34	42
PC	2.3	2.2	0.7	0.6	20	44	27	140
Liver								
RC	4.1	3.3	8.0	2.9	101	90	93	96
GB	2.7	4.4	8.5	1.6	6,605	24	996	31
IZ	2.4	1.4	13.4	14.3	1,247	91	644	127
LT	1.6	1.3	14.5	26.3	863	136	725	495

AST, aspartate aminotransferase; ALT, alanine aminotransferase.

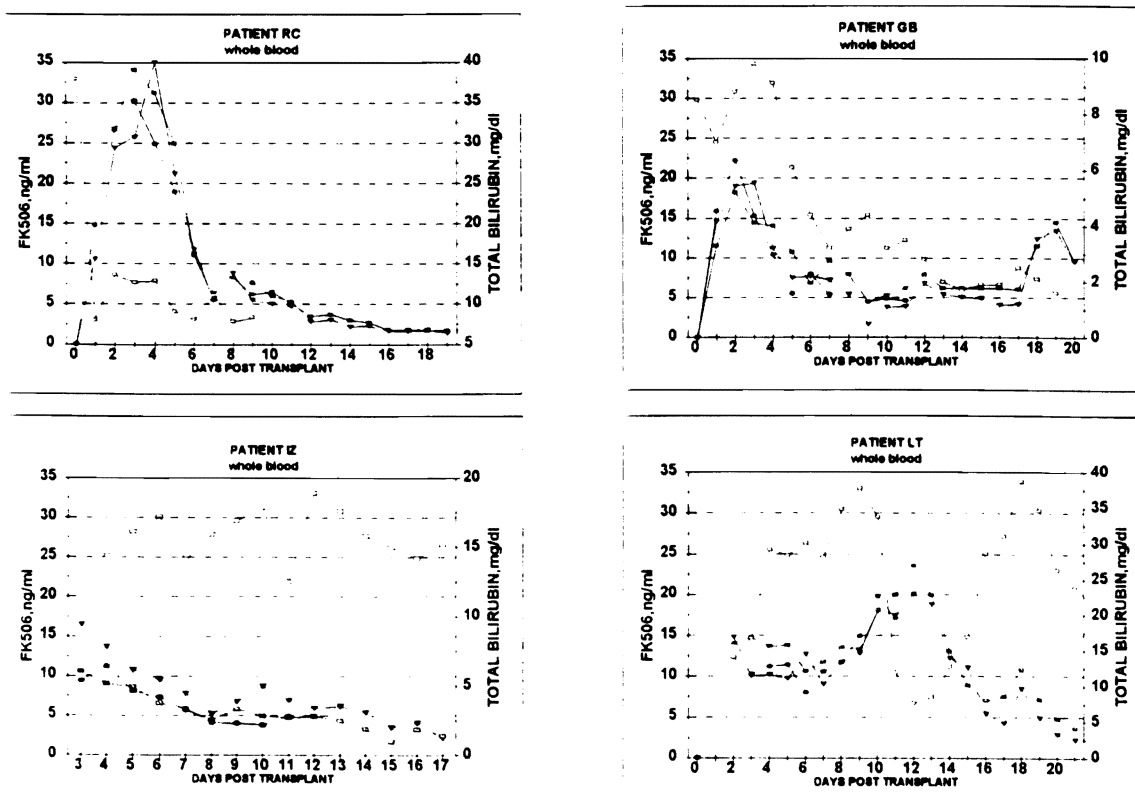


FIG. 1. Whole-blood FK 506 concentrations as measured by Sep-Pak enzyme-linked immunosorbent assay (ELISA) (triangles), methylene chloride ELISA (black boxes), high-performance liquid chromatography ELISA (circles), and bilirubin (white boxes) in four liver transplant patients.

centrations were poorly correlated ($r \leq 0.59$) with each other. The overall slope of blood versus plasma concentrations (4.1) in liver transplant patients was lower than the corresponding overall slope (11.7) in the kidney transplant patients (Table 4). The slope of the line comparing the plasma HPLC-ELISA concentrations versus whole blood HPLC-ELISA concentrations in liver transplant patients was also lower than the corresponding slope in the kidney transplant patients. The tacrolimus equivalent in fraction 1 (corresponding to HPLC fraction collected from 0 to 3.6 min) was <0.8 ng/ml in all cases.

DISCUSSION

In this study we compared three methods of measuring tacrolimus concentrations in plasma and whole blood. The sensitivity and the interday variations of the three methods were comparable and were acceptable for routine clinical use.

Over the past few years we have been using the SP extraction procedure for measuring plasma con-

centrations of tacrolimus in transplant patients. In a recent publication (10) we reported our observation of identical tacrolimus concentrations in plasma as measured by SP-ELISA, MC-ELISA, and HPLC-ELISA in kidney transplant patients and in liver transplant patients with biochemical profiles indicative of normal liver function. In patients with abnormal liver function, as determined by serum bilirubin concentrations >2 mg/dl, the SP-ELISA method tends to give higher estimates of tacrolimus concentration in comparison to the MC-ELISA method. However, both of these methods give values that are higher [slope of SP-ELISA vs. HPLC-ELISA was 1.53 ($n = 40$), vs. slope of MC-ELISA vs. HPLC-ELISA, 1.13 ($n = 40$)] than the HPLC-ELISA method, which measures only the parent tacrolimus in plasma. This is believed to be the result of coelution of some tacrolimus metabolites in the SP and MC extraction that cross-react with the antibody used in the ELISA procedure. When data from patients with a serum bilirubin concentration of <2 mg/dl were analyzed, the slope of the SP-ELISA versus HPLC-ELISA was 1.19 ($n = 27$),

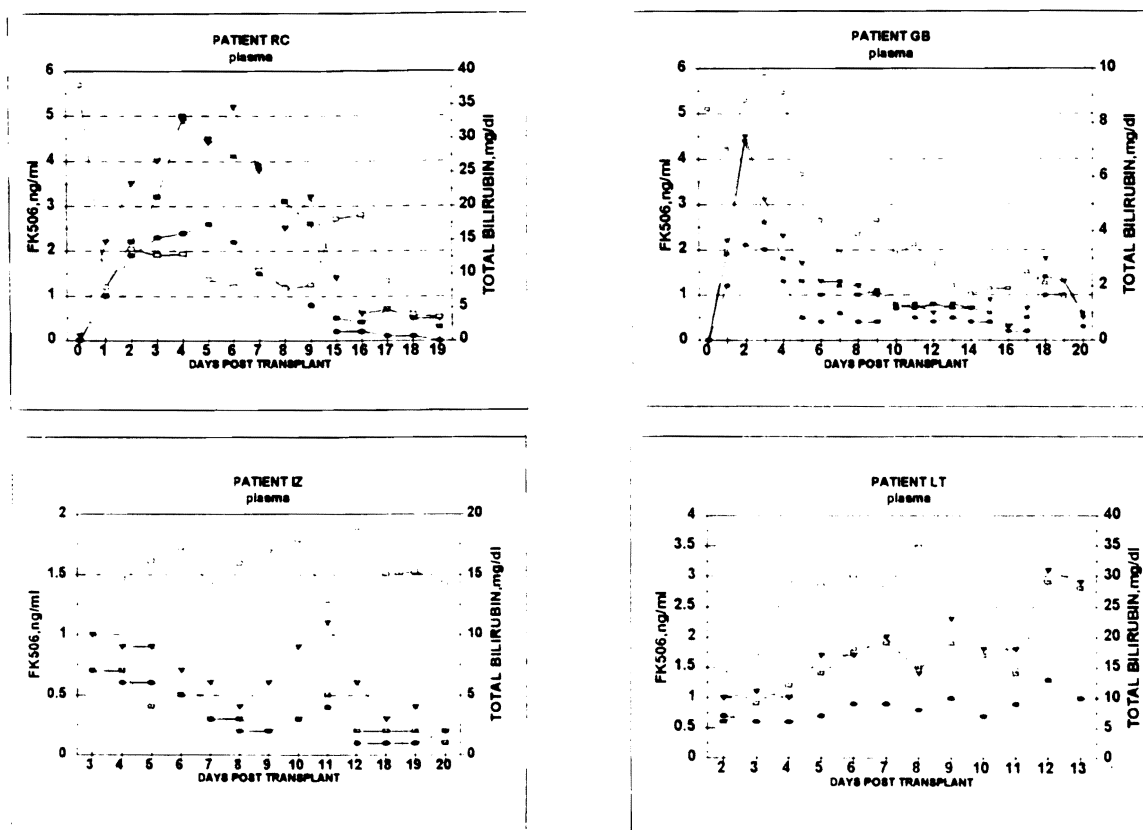


FIG. 3. Plasma FK 506 concentrations as measured by Sep-Pak enzyme-linked immunosorbent assay (ELISA) (triangles), methylene chloride ELISA (black boxes), high-performance liquid chromatography ELISA (circles) and bilirubin (white boxes) in four liver transplant patients.

is the predominant species in the whole blood and that relatively few metabolites accumulate in the blood. While similar correlation (0.786) has been reported for blood tacrolimus concentrations measured by SP-ELISA and MC-ELISA, lower slopes indicative of actual differences between these measurements have been reported (5,15). Our observations with regard to tacrolimus are consistent with the fact that little or no tacrolimus equivalent could be measured in fraction 1, collected on the HPLC. Whether the lack of tacrolimus equivalents in fraction 1 is due to the absence of cross-reacting tacrolimus metabolites in this fraction or to the potential instability of any tacrolimus metabolites in this fraction during the experimental procedure cannot now be clarified.

We have also noted the blood concentration of tacrolimus to be consistently higher [overall mean \pm SD blood-to-plasma ratio (B/P) was 12.8 ± 8.4 ; for kidney transplant patients, B/P was 16.8 ± 9.9 ; for liver transplant patients, B/P was 9.8 ± 5.5] than the corresponding plasma concentrations in all the patients observed at all times, as has been reported

earlier (2,6-8,10,13,15). These observations along with our earlier report would suggest that the parent tacrolimus is the primary species in the red blood cell and that tacrolimus metabolites do not appreciably partition into the red blood cells. The actual concentration of the metabolites that tend to accumulate in the plasma of patients with liver dysfunction appears to be very small compared to the total concentration of tacrolimus in the whole blood. Our conclusions differ from those of the recent publication (15), which suggests that the whole blood SP-ELISA and MC-ELISA are nonspecific and simultaneously measure tacrolimus metabolites as well. While these investigators showed some (10-15%) cross-reactivity of one of the metabolites (dimethyl hydroxy tacrolimus), three other metabolites tested did not show any appreciable cross-reactivity with the monoclonal antibody used in the ELISA procedure. These investigators also interpreted increased tacrolimus concentrations during episodes of liver dysfunction to be due to accumulation of tacrolimus metabolites. It should be realized that increased tacrolimus concentrations dur-

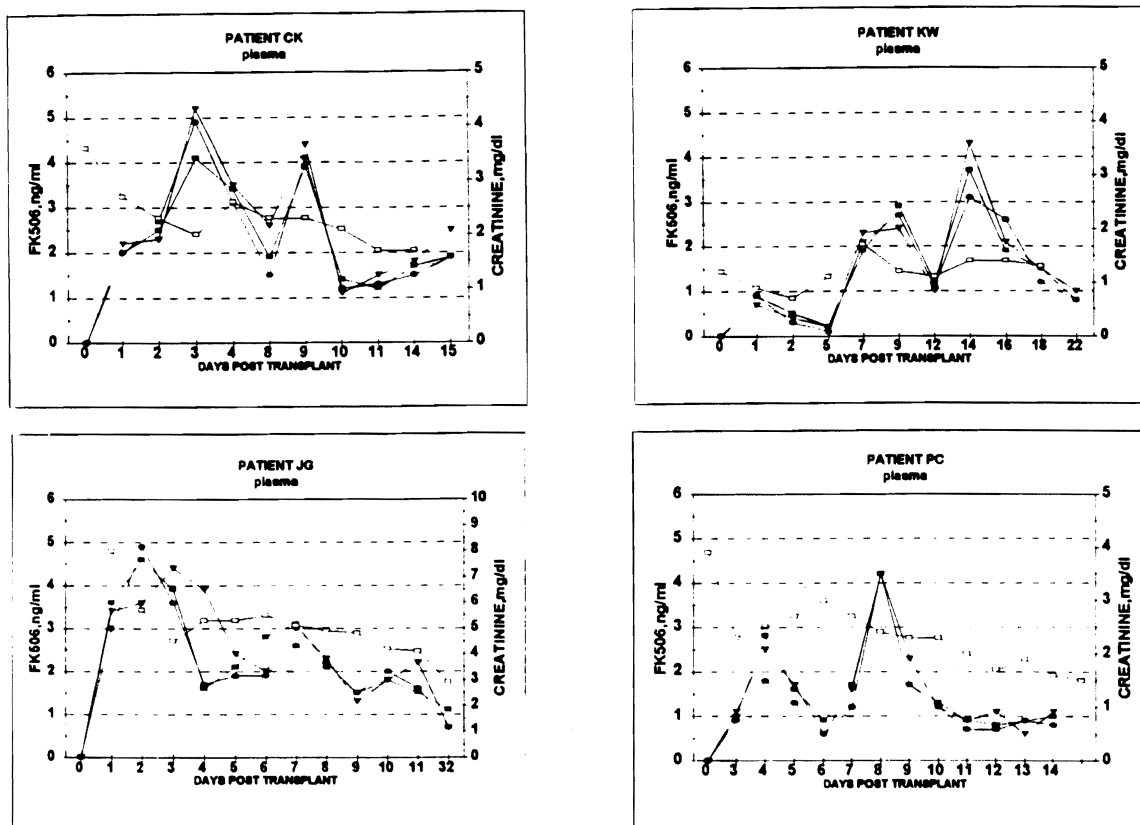


FIG. 4. Plasma FK 506 concentrations as measured by Sep-Pak enzyme-linked immunosorbent assay (ELISA) (triangles), methylene chloride ELISA (black boxes), high-performance liquid chromatography ELISA (circles), and creatinine (white boxes) in four kidney transplant patients.

ing episodes of rejection could very well be the result of impaired tacrolimus metabolism and accumulation of unchanged tacrolimus.

We have observed an overall poor correlation ($r = 0.44$ in liver and 0.4 in kidney transplant patients, respectively) between the trough blood and trough

plasma concentrations. This is consistent with published data (9,15). It is also of interest to note that the slopes of the lines describing the whole blood-to-plasma concentration of tacrolimus were 4.1 and 11.7 , respectively, in liver and kidney transplant patients (Fig. 5). When only HPLC-ELISA data were

FIG. 5. Correlation between whole blood and plasma as measured by solid phase extraction, liquid extraction, and high-performance liquid chromatography (HPLC) (circles), in four kidney transplant patients. Correlations between whole blood and plasma as measured by solid phase extraction and liquid extraction (boxes) and by HPLC (triangles) in four liver transplant patients.

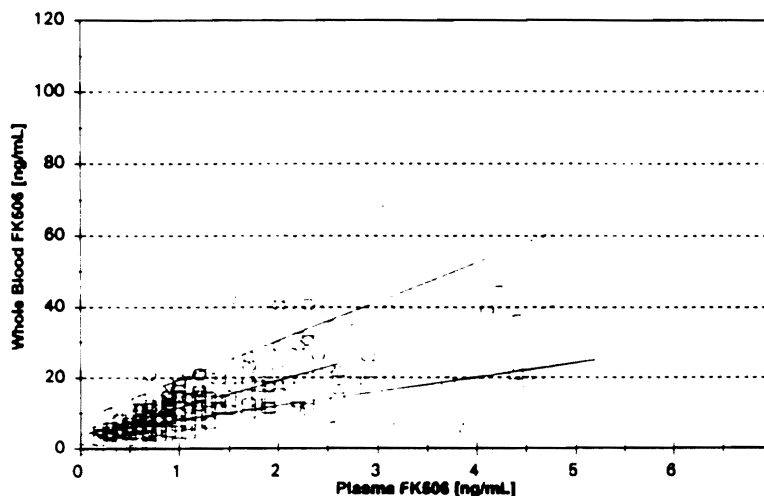


TABLE 4. FK506—whole blood vs. plasma correlation analysis as measured by different methods

	Slope	Intercept	r^2	No.
Liver transplant				
Sep-pak ELISA	4.0	3.5	0.52	58
methylene chloride ELISA	4.1	4.7	0.47	58
HPLC ELISA	7.7	3.9	0.59	36
overall (all three methods)	4.1	4.9	0.44	152
Kidney transplant				
Sep-pak ELISA	11.3	7.0	0.50	43
methylene chloride ELISA	12.2	7.5	0.31	37
HPLC ELISA	11.9	7.4	0.44	38
overall (all three methods)	11.7	7.4	0.4	118

ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography.

compared, the slopes were 7.7 and 11.9, respectively, in liver and kidney transplant patients. Our observations in kidney transplant patients indicate that the metabolites of tacrolimus do not accumulate in plasma and/or that they are removed by the extraction process used.

Our observations in the liver transplant patients can be partly explained by the accumulation and simultaneous quantitation of some ELISA cross-reactive metabolites of tacrolimus by SP-ELISA and MC-ELISA in plasma obtained from liver transplant patients with poor liver function. The higher slope of the blood-to-plasma concentration comparison, 11.7 and 7.7, respectively, in kidney and liver transplant patients suggests higher uptake/binding of tacrolimus to the red blood cells or decreased plasma protein binding of tacrolimus in kidney transplant patients as compared to liver transplant patients.

The significant positive intercept (3.5 to 7.5) observed is similar to what has been observed earlier (6.8,15) and is consistent with the reported nonlinear uptake of tacrolimus by the red blood cells (6.8). These observations indicate that it is inappropriate to extrapolate blood tacrolimus concentrations based on plasma tacrolimus concentration measurements made in transplant patients.

Our current observations are also consistent with our previous report that the concentrations of tacrolimus as measured by the three methods are also independent of the route of administration of tacrolimus (10). Tacrolimus is known to be metabolized by the cytochrome P-450 enzyme system (20–22), and this enzyme system is known to be present in the gut. In vitro studies with gut cytochrome P-450 (unpublished observations) provide indirect evidence of the involvement of gut metabolism in tac-

rolimus disposition. It is possible that in this patient population there was no route-dependent metabolism of tacrolimus, or that the metabolites produced in the gut do not cross-react with the monoclonal antibody used in this assay, or metabolites formed in the gut are removed during the extraction process.

Our study indicates that tacrolimus can be measured in whole blood by ELISA after SP or methylene chloride extraction. The trough blood concentrations of tacrolimus are seven to 12 times as high as the corresponding plasma concentrations. The blood-to-plasma ratio of tacrolimus is dependent on the assay method used, the nature of the organ transplanted, and the functional status of the liver.

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