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FK506 Measurement: Comparison of Different Analytical Methods

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Summary: In this study, we used solid-phase extraction with Sep-Pak and a liquid-liquid extraction with methylene chloride as two primary methods of extracting FK506 from plasma. The extracts were either analyzed directly by enzyme-linked immunosorbent assay (ELISA) or subjected to high-performance liquid chromatography (HPLC) separation and different fractions were analyzed by ELISA. Serial blood samples were obtained from four kidney transplant patients and four patients who underwent liver transplantation, from day 1 until day 30-35 posttransplantation. There was no significant difference in the FK506 plasma concentration as measured by all four methods in normal transplant patients. However, in liver transplant patients, the solid-phase extraction method gave higher FK506 concentrations than the methylene chloride extraction during the early postoperative period. The concentrations measured after methylene chloride extraction were higher than that after HPLC-ELISA. This higher FK506 concentration measured by direct ELISA could be attributed to possible cross-reacting metabolites that were present in the plasma of patients with abnormal liver functions. Once liver function returns to normal, all four methods give identical plasma concentrations for FK506. **Key Words:** FK506—Immunosuppression.

FK506 is currently under clinical investigation as an immunosuppressive agent in various organ transplant patients (1). It is a very potent compound that appears to have significant nephrotoxicity as a major side effect (2). Pharmacokinetic studies of FK506 have shown that there is a large inter- and intraindividual variation in its kinetics in organ transplant patients (3,4). These factors necessitate routine monitoring of plasma FK506 concentration in transplant patients in an effort to optimize FK506 therapy. Plasma FK506 concentrations are currently measured at the University of Pittsburgh

Medical Center by an enzyme-linked immunosorbent assay (ELISA) method after a solid phase extraction (5,6). In addition, FK506 can also be measured in whole blood by a radioreceptor assay (7), high-performance liquid chromatography-mass spectrometry (HPLC-MS) assay (8,9), Abbott IMx method (10), and in serum by a combined HPLC-ELISA method (11). A recent study has shown that FK506 concentrations, as measured by ELISA after a solid phase extraction, are higher than those measured after a liquid-liquid extraction, using methylene chloride (12,13). The primary objective of this study was to evaluate the effect of two different extraction procedures for measurement of trough FK506 concentrations in plasma samples obtained from liver and kidney transplant patients in comparison to a specific analytical procedure that

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uses a HPLC separation step, to separate potential FK506 metabolites from FK506 before use of ELISA.

MATERIALS AND METHODS

Materials

FK506 drug, monoclonal antibody for FK506, and FK506 peroxidase enzyme conjugate were supplied by Fujisawa Pharmaceuticals (Osaka, Japan). Anti-mouse IgG was purchased from Atlantic Antibodies (Stillwater, MN, U.S.A.). C-18 Sep-Pak columns were obtained from Waters (Milford, MA, U.S.A.). O-Phenylenediamine (OPD) was purchased from Sigma (St. Louis, MO, U.S.A.). Other reagents were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Methods

Clinical Protocol

Daily trough blood samples were collected from four liver and four kidney transplant patients from day 1 till 30–35 days posttransplantation. Biochemical parameters characterizing kidney function (serum creatinine) and liver function (bilirubin, aspartate aminotransferase, alanine aminotransferase) were measured in these patients over the entire study period. Blood samples obtained for FK506 measurement were incubated at 37° for 45 min. The plasma was separated at 37°C and extracted by the methods described below and subjected to ELISA analysis.

Solid-Phase Extraction

Plasma (100 μ l) in duplicate was acidified with 1 ml of 0.1 N HCl and passed through a C-18 Sep-Pak cartridge which was prewashed with 4% acetic acid. After an additional wash with 4% acetic acid, FK506 was eluted with 2 ml methanol (6). The methanol was evaporated under nitrogen and the FK506 content was analyzed by ELISA.

Liquid-Liquid Extraction with Methylene Chloride

Plasma (100 μ l) in duplicate was acidified with 1 ml of 0.1 N HCl and extracted with 5 ml of methylene chloride. This procedure is a minor modification of the procedure described by Kobayashi et al. (12). The organic layer was collected and evaporated

under nitrogen at room temperature and FK506 content was analyzed by ELISA.

High-Performance Liquid Chromatography

Plasma extracts prepared by solid phase extraction and methylene chloride extraction were dissolved in methanol and subjected to HPLC separation. HPLC was performed on a Waters 600E multisolvent delivery system equipped with a system controller and a 994 photodiode array detector. For the separation of FK506, its metabolites, and endogenous compounds, we used 3.9-mm ID \times 15.0-cm long analytical column filled with μ BondaPak C-18 (Catalog no. 86684, Waters Associates). The column temperature was set to 60°C for the analysis. A mobile phase consisting of 80% methanol and 20% H₂O (acidified to pH 6.0 with HCl) was used to elute different components from the column at a flow rate of 0.8 ml/min. The eluents were monitored at 214 nm. The retention time of FK506 was 4.8 min under these conditions. Two fractions were collected from 0–3.6 and 3.6–6 min with greater than 99% of the parent FK506 being collected in the second fraction. Both fractions were evaporated under nitrogen and the residue analyzed for FK506 using ELISA.

RESULTS

The biochemical profiles of the patients studied are listed in Table 1. Patients received intravenous FK506 at a dose of 0.05 mg/kg/day as a continuous

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

Patient name	Creatinine (mg/dl)		Bilirubin (mg/dl)		AST ^a (IU/L)		ALT ^b (IU/L)	
	c	d	c	d	c	d	c	d
Kidney								
HB	11.8	1.9	0.4	0.3	31	12	21	51
HG	10.0	2.3	0.4	1.2	51	46	80	86
GW	8.8	3.0	1.3	0.8	20	36	10	56
FB	7.5	1.9	0.5	0.4	15	9	32	20
Liver								
MK	1.8	1.9	2.8	0.9	952	21	1,188	29
JR	1.9	1.4	7.1	0.9	194	11	303	30
BC	0.3	1.3	1.8	0.7	69	49	1,423	61
AA	0.4	0.8	3.1	1.0	59	30	159	31

^a Aspartate aminotransferase.

^b Alanine aminotransferase.

^c During the immediate postoperative period.

^d Four weeks after transplantation.

infusion during the immediate postoperative time and for up to 3–7 days. They were converted to oral therapy at a dose of 0.15 mg/kg day as soon as they were ready for oral intake. All kidney transplant patients had normal liver function tests and all liver transplant patients had normal kidney function throughout the entire course of study. The interday coefficient of variation of the HPLC-ELISA method is 16.5% ($n = 20$). Because HPLC-ELISA after Sep-Pak and methylene chloride were similar ($r^2 = 0.95$), these values were averaged and compared with direct ELISA. The regression equation of the FK506 concentrations measured by methylene chloride-ELISA versus Sep-Pak-ELISA is given as follows: methylene chloride method (ng/ml) = $0.066 + 0.91$ [Sep-Pak method (ng/ml)], $r^2 = 0.94$; $p < 0.01$.

In all the kidney patients, the concentration of FK506 in plasma was very similar when measured by ELISA following Sep-Pak or methylene chloride extraction as indicated in Fig. 1. In addition, separation of potential FK metabolites from parent FK506 by HPLC and subsequent ELISA assay also gave similar FK506 concentrations. In this group of patients, there was no difference in FK506 concentration as measured by the three methods, irrespective of whether the patient was on intravenous or oral FK506 therapy, or irrespective of the functional status of the kidney.

In liver transplant patients, during the immediate postoperative period, the bilirubin concentration

was high and decreased to normal values over a time period, as indicated in Fig. 2. In three of the patients (JR, MK, AA), during the immediate postoperative period, serum bilirubin was elevated, and FK506 concentrations measured by Sep-Pak-ELISA was higher in comparison with methylene chloride-ELISA. However, both of these estimates were also higher than the values obtained by HPLC-ELISA. As the bilirubin concentrations returned toward normal values, the concentration of FK506 measured by all three methods was almost identical. In one patient (BC), however, from day 1 posttransplant all the assay methods provided the same values. The highest total bilirubin in this patient was only 1.8 mg/dl which is indicative of near normal function of the liver. In this patient, on days 10 and 32, FK506 concentrations were significantly elevated as measured by all three methods.

DISCUSSION

Over the past several years, we have been using a Sep-Pak extraction procedure for measuring plasma concentrations of FK506. Recently, a methylene chloride extraction procedure has been reported for measurement of FK506 in plasma (12). This procedure tends to give lower values in comparison to the Sep-Pak extraction procedure. It is believed that this is due to accumulated FK506 metabolites that co-elute in the solid-phase extraction and that are not extracted by the methylene chlo-

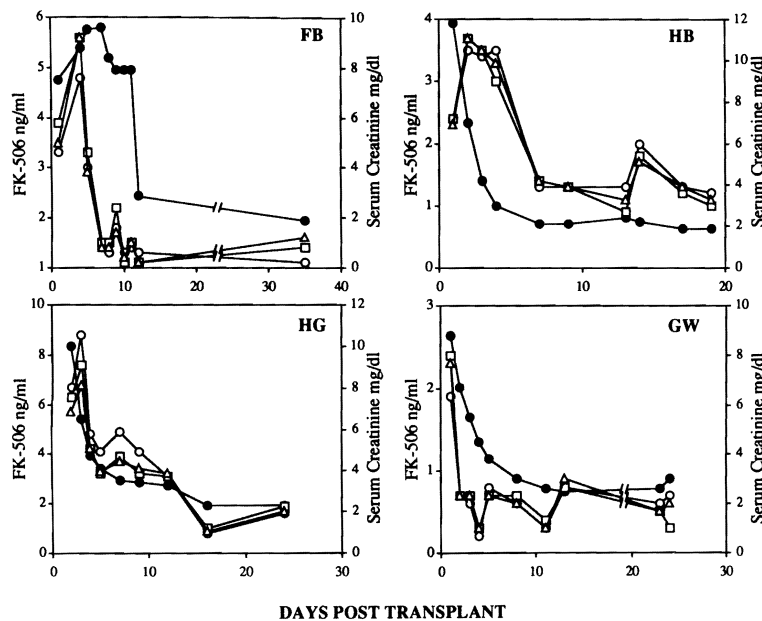


FIG. 1. Plasma FK506 concentrations as measured by Sep-Pak ELISA (○), methylene chloride ELISA (□), HPLC ELISA (△), and creatinine (●) in four kidney transplant patients.

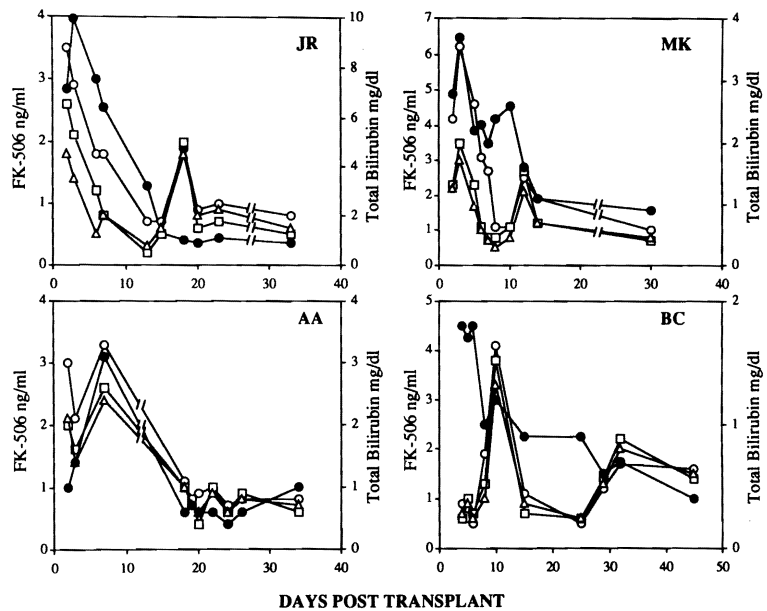


FIG. 2. Plasma FK506 concentrations as measured by Sep-Pak ELISA (○), methylene chloride ELISA (□), HPLC ELISA (△), and bilirubin (●) in four liver transplant patients.

ride. In order to evaluate the specificity of the Sep-Pak and methylene chloride methods, we developed an HPLC-ELISA method for measuring parent FK506 in plasma. Our results indicate that in patients with normal liver function, concentrations of FK506, as measured by a specific HPLC-ELISA method, is similar to the concentrations measured by Sep-Pak-ELISA or methylene chloride-ELISA. This is true independently of whether the organ transplanted is kidney or liver. This is perhaps due to the fact that in patients with normal hepatic function, very little, if any, of the metabolites of FK506 accumulate in the plasma and are, therefore, extracted from plasma. In liver transplant patients, during the immediate postoperative period when the liver function is not normal, as indicated by elevated bilirubin concentrations, the Sep-Pak procedure gives the highest values and HPLC-ELISA gives the lowest, indicating accumulation of the metabolites in these patients. These metabolites cross-react with the monoclonal antibody used in the ELISA procedure. Once hepatic function improves, the liver is able to clear most of the metabolites of FK506 with the result that there is no significant difference between concentrations measured by the different methods.

Our results also indicate that changes in kidney function, as measured by serum creatinine, did not have any influence on the FK506 concentration as measured by all three methods. This suggests that only a very small amount of FK506 metabolites are, in fact, excreted through the kidney. This fact is

supported by our previous observation of renal clearance of FK506 to be <1% of total body clearance (4).

The observation that FK506 levels, measured by the three different methods, are also independent of the route of administration, suggest the following: (a) There is no route-dependent metabolism of FK506, indicating minimal gut metabolism; and/or (b) any metabolite produced in the small intestine minimally cross-reacts with the monoclonal antibody used in the ELISA assay. Recent evidence points to the potential for gut metabolism of FK in humans (9). However, the extent of cross reactivity of the metabolites produced in the small intestine with the monoclonal antibody used in ELISA is not known at this time.

CONCLUSIONS

A solid phase extraction method and a liquid-liquid extraction method using methylene chloride were studied, in comparison to an HPLC method for measuring FK506 plasma concentrations. In kidney transplant patients, there was no difference in FK506 concentrations obtained by the different procedures used. In liver transplant patients, the solid-phase extraction method gave higher FK506 concentrations than the methylene chloride extraction only in patients with abnormal liver function. HPLC separation procedure before ELISA indicated that the elevated FK506 levels from solid-phase-ELISA and methylene chloride-ELISA were

due to cross-reacting metabolites that were present in these patients. Presently, at our medical center patients are maintained at trough plasma concentrations between 0.5 and 2.0 ng/ml. Therapeutic range for FK506 concentrations in plasma is independent of the assay method used in patients with normal liver function. However, in the presence of liver dysfunction, both methylene chloride-ELISA and Sep-Pak-ELISA will measure some of the metabolites and this should be kept in mind in interpreting the data.

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