HPLC Assay for FK 506 and Two Metabolites in Isolated Rat Hepatocytes and Rat Liver Microsomes

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Despite the current use of a standard two-step enzyme immunoassay in the clinical monitoring of the immunosuppressant FK 506, the lack of specificity for the parent drug in this assay renders it unsuitable for drug metabolism studies. An HPLC assay has been developed for studying the metabolism of FK 506 in isolated hepatocytes and microsomal mixtures. This assay allows simultaneous measurement of the parent drug and two of its time dependent metabolites. Metabolism of this drug was studied in intact rat liver cells and rat liver microsomes. We have shown that the metabolites observed are products of phase 1 oxidation reactions. Correlation of the 6β-testosterone hydroxylase activity with the FK 506 metabolite (M1) initial formation rate is consistent with the belief that CYP 3A isozymes are involved in FK 506 metabolism in male rats.

KEY WORDS: FK 506; *in vitro* metabolism; rat hepatocytes; rat liver microsomes; HPLC.

INTRODUCTION

FK 506 [1], a macrolide lactone, is an immunosuppressant that is at least 100 times more potent than cyclosporine (1), the current drug of choice in organ transplantation. Several analytical methods for the measurement of FK 506, and in some cases, the measurement of FK 506 metabolites, have been reported. The enzyme linked immunosorbent assay (2) and IMx (3), have high sensitivity but low specificity. The enzyme linked immunosorbent assay has been reported to cross react with at least 3 of FK 506 metabolites (4). A radioreceptor assay (5) also gives a combined measurement of FK 506 and its metabolites. High performance liquid chromatographic separation in conjunction with chemiluminescence detection (6), mass spectrometric detection (7) or UV detection (8) have been developed. Although the HPLC separation used with the chemiluminescence detection yields high sensitivity for FK 506, it does not measure FK 506 metabolites. The method also requires complicated column switching and derivatization of FK 506. The mass spectrometric and the UV detection methods allow specific measurements of both the parent drug and the metabolites, but require gradient solvent delivery. We report here a sample preparation method for rat hepatocytes and microsomes, together with HPLC separation using an isocratic solvent delivery system and UV detection that allows simultaneous monitoring of FK 506 and two time dependent metabolites.

Utilizing this assay which measures FK 506 and two metabolites, the metabolism time course of FK 506 was monitored in isolated rat hepatocytes from untreated rats and rat liver microsomes from untreated and dexamethasone-treated rats. The FK 506 metabolic activity and the 6β-testosterone hydroxylase activity were also compared between liver microsomes from untreated and dexamethasone-treated rats.

MATERIALS AND METHODS

Materials. FK 506 was kindly supplied by Fujisawa Pharmaceutical Company (Deerfield, IL). Dexamethasone was a gift from The Upjohn Company (Kalamazoo, MI). All solvents (HPLC grade) were obatined from Fisher Chemical (Fair Lawn, NJ). All chemicals for hepatocytes isolation and metabolic incubations were obtained from Sigma Chemical Company (St. Louis, MO). Biorad Protein Assay Kit with albumin protein standard was obtained from Biorad (Richmond, CA). FK 506 metabolite (M1) was produced as described in our previous work (9) by incubation of FK 506 (10–20 μM) with rat liver microsomes.

Animals. Male Sprague-Dawley rats, weighing 250–300 g, were obtained from Bantin and Kingman (San Leandro, CA). Dexamethasone pre-treatment involved intraperitoneal injection of dexamethasone suspended in corn oil at 100 mg/kg/day for 4 days (10).

Isolated Rat Hepatocytes. Liver perfusion with bacterial collagenase was carried out as described previously (11). Hepatocytes harvested were counted in a Coulter counter. Viability of cells was checked by the Trypan Blue Exclusion Test (12, 13). Only hepatocyte preparations with a viability index of higher than 85% were used in the experiments. The hepatocytes suspension was centrifuged at 1000 rpm for 5 min. The supernatant was carefully removed, and the hepatocytes were resuspended in a buffer containing 0.8% NaCl, 0.035% KCl, 0.016% $MgSO_4 \cdot 7H_2O$, 0.018% $CaCl_2 \cdot 2H_2O$, 0.18% glucose, 1% fatty-acid free BSA, and 0.24% HEPES, pH 7.45 (14).

Rat Liver Subcellular Fractions Preparation. Subcellular fractions were obtained by ultracentrifugation following standard methods (15-17). Protein concentration and P450 content were determined respectively using Biorad Protein Assay Kit and the method of Omura and Sato (18), as described in detail by Schoene et al. (19).

Metabolism Time Profile. Freshly harvested hepatocytes (5 million cells/ml) (10) were pre-incubated for 5 min at 37°C. The reaction was started by the addition of FK 506 (5 μM). Hepatic microsomes (2 mg protein/ml) and FK 506 (6 μM) were preincubated in the same manner as the hepatocytes. The reaction was started by the addition of NADPH (1 mM). Duplicate or triplicate samples (0.6 ml) were taken at 0.25, 3, 5, 10, 20, 30, 60 and 120 minutes. The reaction was stopped by quick freezing the sample in an acetone-dry ice mixture. All samples were stored at −40°C until analysis.

FK 506 Metabolite (M1) Initial Rate of Formation. Hepatic microsomes from dexamethasone-treated or untreated rats (0.25 and 2 mg protein/ml, respectively) were pre-

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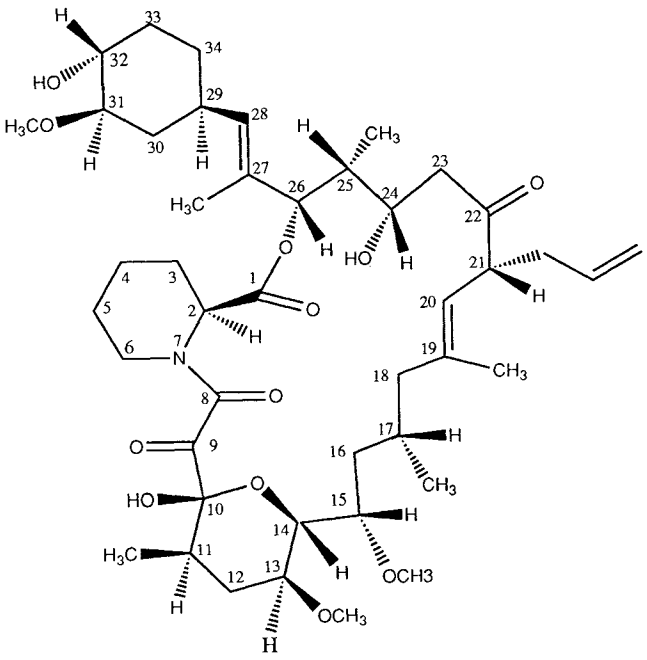
[1] FK 506



Scheme I

incubated for 3 min at 37°C with FK 506 (20, 30 and 40 μM). The reactions were initiated by the addition of NADPH (1 mM) and terminated after 5 and 3 min, respectively, in the untreated and the dexamethasone-treated microsomal mixtures.

Testosterone Metabolism. Testosterone hydroxylation activity was measured using a similar method described by Bornheim et al. (20).

Analysis. To all samples, 3 ml of protein precipitating agent (20% v/v acetonitrile, 30% v/v methanol and 5% w/v $ZnSO_4$ in water) was added. The mixture was vortexed and centrifuged for 10 minutes at 3500 rpm. The supernatant was passed through a BondElut C18 sample preparation column (Varian Sample Preparation Products, Harbor City, CA) that was pre-equilibrated with 1 ml 90.5% ethyl alcohol and 1 ml nanopure water. The sample preparation column was then washed consecutively with 1 ml of 40% methanol in water and 1 ml hexane. Compounds of interest were eluted from the sample preparation column with 1 ml methylene chloride. The methylene chloride was evaporated and the sample was reconstituted with the mobile phase prior to injection onto the HPLC column. The mobile phase was a solvent mixture of acetonitrile/methanol/diluted *O*-phosphoric acid pH 3, 49/3/48 (v/v/v). The column, a Beckman Ultrasphere ODS (5 μ, 250 × 4.6 mm), was warmed to 60°C in a Waters temperature control module. The mobile phase was delivered by a Beckman 110A solvent delivery module connected to a Beckman 420 controller. The initial flow rate was 1 ml/min for the first 20 minutes, stepped up to 1.5 ml/min within 0.5 min and maintained for the subsequent 30 minutes. The compounds of interest were detected by a Beckman 160 absorbance detector at 214 nm.

RESULTS

An HPLC assay with UV detection was developed to simultaneously monitor FK 506 and its two time dependent metabolites in rat hepatocytes and liver microsomes. The major metabolite-M1 seen is an O-desmethyl FK 506 (1). A representative chromatogram for a rat hepatocyte sample of FK 506 following a 30 min incubation is shown in figure 1. The retention times for the two observed metabolites, M1 and M2, and the parent FK 506 are 10.1, 14.2 and 44.1 min, respectively.

The concentrations of FK 506 and the metabolites were quantified by the construction of external standard curves using FK 506. For assay purposes, it is assumed that the extinction coefficient is the same for FK 506 and the two metabolites. The detection limit is 0.017 nmol. The inter-day and intra-day variabilties of FK 506 are 7.3 and 7.9%, respectively. Inter-day and intra-day coefficients of variation were estimated using FK 506 spiked microsomal samples. To estimate the absolute recovery, three different concentrations of FK 506 (1, 10, and 80 μM) and FK 506 major metabolite (M1) (0.15, 0.5, and 1.25 mM) were added to rat liver microsomal samples and extracted as described earlier. The average peak area obtained from the extracted samples was compared to the average peak area obtained from the corresponding unextracted samples. The results are summarized in tables 1a and b for the parent drug and metabolite respectively.

Figure 2 shows a representative time course of FK 506 metabolism in untreated rat liver microsomes. The decline of the parent FK 506 is accompanied by the formation of two time-dependent metabolites, M1 and M2. Incubation with different subcellular fractions of the liver cells showed that metabolism of FK 506 occurred mainly in the microsomes. The number of metabolites and the metabolic pattern observed in the microsomes were similar to that seen in hepa-

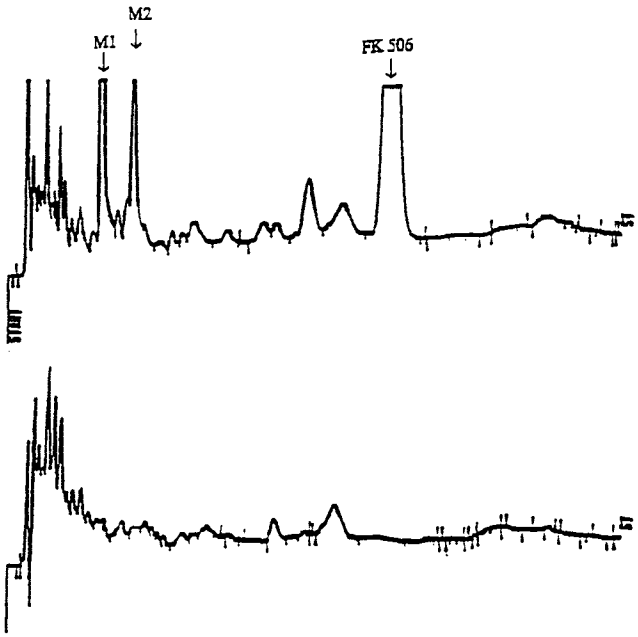


Fig. 1. Top: A representative chromatogram of FK 506 and its two time dependent metabolites in isolated rat hepatocytes samples. Retention time: FK 506 = 44.1 min, FK 506 metabolite M1 = 10.1 min, and FK 506 metabolite M2 = 14.2 min. Bottom: A representative chromatogram of a blank sample.

Table 1a
Percentages of FK 506 recovered following extraction from hepatocytes and liver microsomal samples at three different concentrations. (N = 5)

	% ± S.D. Recovered in:	
Concentration (μM)	Hepatocytes	Microsomes
1	78 ± 7	76 ± 11
10	65 ± 7	65 ± 12
80	82 ± 1	85 ± 4

tocytes (data not shown). The metabolism of FK 506 in liver microsomes was NADPH and oxygen dependent. The formation of the FK 506 metabolites was abrogated when carbon monoxide was bubbled into the incubation mixture 15 min before the addition of NADPH. Such evidence suggests that the enzyme involved in FK 506 metabolism is a microsomal P450 enzyme.

Testosterone 6β-hydroxylation activity, as well as the FK 506 metabolite (M1) initial formation rate, were higher in liver microsomes from dexamethasone pre-treated than the untreated rats by 5 and 5.15 fold, respectively.

DISCUSSION

This paper describes an HPLC assay that can simultaneously measure FK 506 and two time-dependent metabolites. This assay requires an isocratic pump and a UV detector which are standard equipment in most laboratories. While other assays may have higher sensitivity than the present method, the simple equipment requirements, the reasonable recoveries of 75 and 69% for FK 506 and FK 506 major metabolite, respectively, and <10% intra-day and inter-day variabilties make this assay well suited for experiments using rat liver samples. We have and will continue to utilize this assay in kinetic studies of FK 506 metabolite (M1) formation in both male and female rats.

Isolated rat liver hepatocytes provide an attractive model to study the metabolic events of FK 506 in the liver *in vitro*. Maintaining the precise inner architecture and the permeability of the plasma membrane, the hepatocytes allow the metabolic reactions to occur in an orderly sequence inside the cell. Because intact cells retain all the membrane bound and soluble enzymes, both phase 1 and 2 metabolic reactions can occur in the hepatocytes (13). In microsomes, the disruption of the plasma membrane makes the supplement of cofactors necessary for both phase 1 and 2 reactions. Since NADPH was the only cofactor added to the microsomal mixture in our experiments, the similar metabolic pattern in the hepatocytes and the microsomes suggests that the two observed metabolites are formed via phase 1 oxidation reactions instead of phase 2 conjugation reactions. The plateau or even gradual decline in the metabolite concentrations after 30 minutes, with continuing decline of FK 506, suggests further metabolism of the metabolites, presumably a phase 1 instead of a phase 2 metabolic reaction, based on the earlier discussion.

Table 1b
Percentages of FK 506 major metabolite M1 recovered following extraction from liver microsomal samples at three different concentrations. (N = 5)

Concentration (μM)	% ± S.D. Recovered
0.15	68 ± 5
0.45	69 ± 5
1.25	70 ± 6

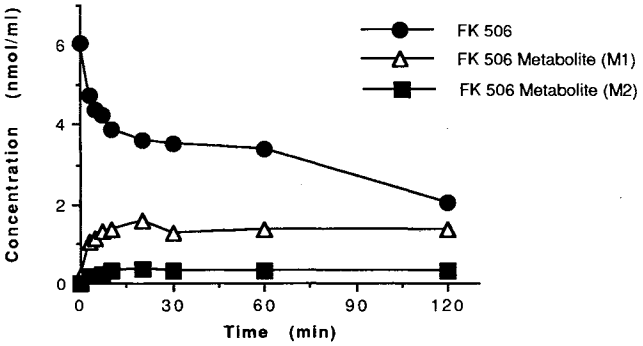


Fig. 2. A representative time course of FK 506 metabolism in rat liver microsomes. [FK 506] = 6 μM

Testosterone 6β-hydroxylation is a marker for CYP3A activity (21). In male rats, CYP3A2 is constitutive, while CYP3A1 is glucocortocoid inducible (22). Since microsomes from dexamethasone-treated rats exhibit higher testosterone 6β-hydroxylation activity as well as higher initial FK 506 metabolite M1 formation rates than those found in untreated rats, this data is consistent with the belief that CYP3A isozymes are responsible for the formation of the metabolite 13-desmethyl FK 506 in dexamethasone-treated male rats, as demonstrated by Vincent et al. (8) in an immunoinhibition study using anti-rat CYP3A IgG. In human liver microsomes, it was also shown by Sattler et al. that the formation of this metabolite is inhibited by 92% by rabbit anti-CYP3A4 (23).

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