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 immunosuppres-
sant 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 de-

livery 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 obtained 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 (Rich-

mond, 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 bacte-
rial 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 hepa-
tocytes were resuspended in a buffer containing 0.8% NaCl, 0.035% KCl, 0.016% MgSO4·7H2O, 0.018% CaCl2·2H2O, 0.18% glucose, 1% fatty-acid free BSA, and 0.24% HEPES, pH 7.45 (14).

Rat Liver Subcellular Fractions Preparation. Subcellu-
lar 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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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 variabilities 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 hepato-

RESULTS

An HPLC assay with UV detection was developed to