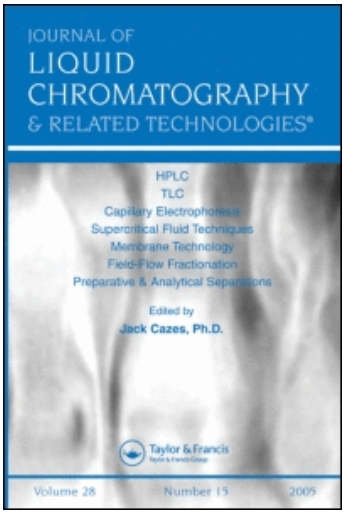

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ISOLATION AND SPECTROSCOPIC CHARACTERIZATION OF THREE MAJOR METABOLITES FROM THE PLASMA OF RATS RECEIVING RAPAMYCIN (SIROLIMUS) ORALLY*

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ABSTRACT

Three major metabolites of rapamycin (M2, M3, and M5) were isolated from pooled plasma of orally dosed rats. Metabolites were extracted from the plasma with ethyl acetate/methanol prior to isolation by HPLC using a Supelcosil SPLC-18, 5μm, 10 x 250 mm column. The mobile phase was a methanol/ammonium acetate linear gradient system. The isolated metabolites were characterized by negative ion FAB MS, ion-spray MS and ion-spray MS/MS. Metabolite M2 is oxygenated in the southern portion of rapamycin and the macrolide ring is opened. M3 is a structural isomer of rapamycin where the lactone ring is opened. M5 is O-demethylated on the C41 methoxy moiety and the macrolide ring is intact.

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INTRODUCTION

Rapamycin, [3S-[3R*[S*(1R*,3S*,4S*)],6S*,7E,9S*,10S*,12S*,14R*,15E,17E,19E, 21R*,23R*,26S*,27S*,34aR*]]-9,10,12,13,14,21,22,23,24,25,26,27,32,33,34a-Hexadecahydro-9,27-dihydroxy-3-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c] [1,4]oxaazacyclohentriacontine-1,5,11,28,29(4H,6H,31H)-pentone, (Figure 1), an antitumor and antifungal agent isolated from the fungus *Streptomyces hygroscopicus* (1,2,3), has been found to have potent immunosuppressive activity while exhibiting little toxicity in primates (4,5). Rapamycin is currently under clinical trials as an immunosuppressive agent. The drug demonstrates a synergistic effect when co-administered with cyclosporine (6,7).

We have reported the isolation and characterization of two major *in vitro* degradation products following incubation of rapamycin at 37°C in rat bile or ammonium acetate (pH 8.0) (8). Degradation product A was a macrolide ring-opened hydrolysis product of rapamycin where the C25 ester bond has been hydrolyzed. Degradation product B was a ring-opened isomer of rapamycin. Christians et. al. have isolated two metabolites after *in vitro* metabolism of rapamycin in human liver microsomes and rat small intestinal microsomes (9). One of the metabolites was 41-O-demethyl-rapamycin. The second was a hydroxylated metabolite. However, the identification of *in vivo* plasma metabolites of rapamycin has not yet been reported. The present study was therefore conducted for the isolation and structural characterization of rapamycin metabolites in the plasma of rats after receiving rapamycin orally. A semi-preparative HPLC system was developed for the separation of metabolites.

EXPERIMENTAL

Materials

Rapamycin was obtained from Wyeth-Ayerst Research, Rouses Point, NY. It was formulated in a vehicle consisting of 2% (v/v) ethanol, 0.03% (w/v) Polysorbate 80, 0.37% (v/v) Phosal 50 PG and 96.7% (v/v) water at a concentration of 1.05 mg/ml for the doses used in the study. Ammonium acetate (HPLC grade) was obtained from J.T. Baker (Phillipsburg, NJ). All solvents used in the study were HPLC grade.

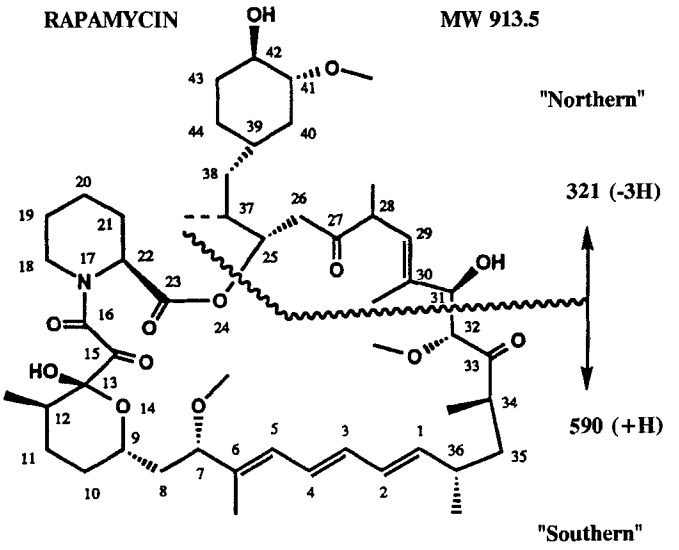

FIGURE 1 Chemical Structure of Rapamycin and its Major Fragmentation Pathway

Animals and Treatment

The study consisted of sixty-four Sprague-Dawley rats (Charles River Laboratory), weighing between 257 and 324 g. Two animals received only vehicle and were used as control. The remaining animals were dosed by gastric intubation with rapamycin at 10.5 mg/kg in a volume of 10 ml/kg. Blood was collected via cardiac puncture into tubes containing heparin at 1 hr post-dosing and immediately placed on ice. Plasma was separated by centrifugation at 2300 rpm for 10 min prior to freezing at -80°C until analysis.

Instrumentation

The HPLC system consists of a Waters 600E system controller and pump (Waters Associates, Milford, MA), a Waters 490E programmable multi-wavelength detector, a Waters U-6K manual injector and a Hewlett-Packard 3390A integrator. Isolation of metabolites was

TABLE 1

Stepwise gradient system used for the isolation of rapamycin metabolites[a]

Time (min)	Flow rate (ml/min)	Percent methanol	Percent 0.05M ammonium acetate
0.0	2.0	62	38
80	2.0	84	16
95	2.0	84	16
100	2.0	62	38

[a]Linear gradient was used between each time point; column was Supelco SPLC-18, 250 x 10.0 mm, 5 μm.

achieved using Supelcosil SPLC-18, 5 μm, 10 x 250 mm column (Supelco, Bellefonte, PA). A Sciex TAGA 6000A upgraded to API III ion spray-mass spectrometer operated under negative ion mode was used for the structural elucidation of metabolites.

Isolation of Rapamycin Metabolites in Rat Plasma

A total of 242 ml of plasma was used for metabolite isolation by HPLC. Five ml aliquots of plasma were mixed with 300 μl of methanol to which 10 ml of ethyl acetate was added and the samples were shaken for 20 min. Following centrifugation at 2500 rpm for 20 min, the ethyl acetate extract was removed and dried under nitrogen. The remaining aqueous layer was re-extracted as above and the combined organic layers were dried under nitrogen prior to reconstitution in 1 ml of methanol/water (65/35). Isolation of metabolites was by HPLC using a Supelco semipreparative SPLC-18 column and a mobile phase gradient listed in Table 1. Detection was by UV absorbance at 276 nm. Eluates containing each of the three individual peaks (M2, M3, and M5) were collected and pooled following repeated injections of the sample. After removal of methanol using a Savant speed-vac concentrator, the remaining aqueous layer was lyophilized to dryness. The dried samples were stored at -80°C prior to being characterized by ion spray mass-spectrometry.

Structural Elucidation of Metabolites by Mass Spectrometry

Metabolites were analyzed using ion spray-mass spectrometry (MS and MS/MS modes). The sample was initially dissolved in either dichloromethane or absolute ethanol and an aliquot was removed for analysis. Samples were then evaporated to dryness under nitrogen at room temperature. Prior to analysis, the residue was dissolved in 80 µl of 20% 4 mM ammonium acetate in methanol. The solution was infused at 4 µL/min into the mass spectrometer using a Harvard Syringe Pump. Mass spectra were acquired at Cornell University using a Sciex TAGA 6000E upgraded to an API III and a home made ion sprayer (10). The mass spectrometer was calibrated in the negative ion mode up to 2000 daltons with polypropylene glycol prior to analysis; final optimization of sprayer position and mass spectrometer conditions for maximum sensitivity was carried out with rapamycin standard (1 mg/mL). The reconstituted residue was analyzed by infusion into the mass spectrometer at
-3.2 Kv and a declustering potential of -68 V. The product ion mass spectra were acquired at collision energies of 50-53 eV and at collision gas pressure of approximately 5 x 10^{12} atoms/cm^2 of Argon.

RESULTS

Identification of Rapamycin and its Metabolites in Rat Plasma

Metabolites M2, M3, and M5 were isolated from a semipreparative column using a gradient mobile phase system. A typical HPLC chromatogram showing separation of rapamycin and its metabolites is shown in Figure 2. The molecular weights (MW) and possible metabolic transformation of these metabolites are summarized in Table 2. Further insight into the possible sites of metabolic transformation was provided by tandem mass spectrometry (MS/MS) as described below. The product ion mass spectrum of rapamycin shows two complimentary diagnostic product ions at m/z 321 and 590 which correspond to the "northern" and "southern" portions of the molecule (Figure 1), respectively. The latter two product ions are useful for monitoring biotransformation of the rapamycin molecule by the mass shift technique (11).

M2: The full scan mass spectrum of M2 shows the presence of a low abundance deprotonated molecular anion at m/z 928.6 which is 16 daltons more than rapamycin. This suggests a

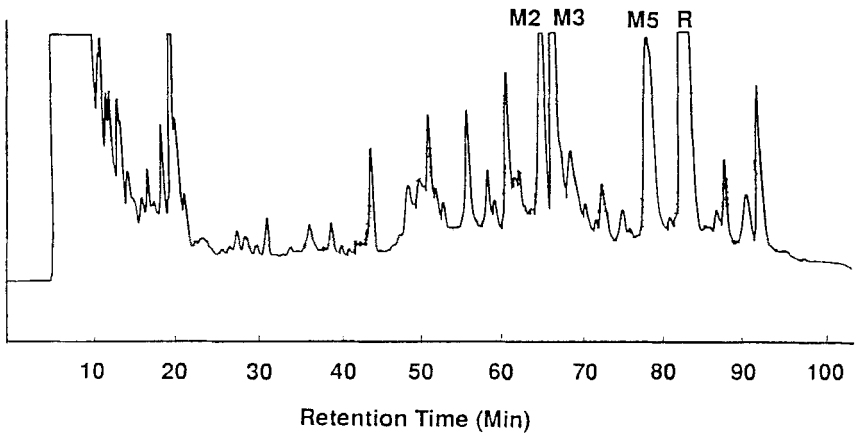

FIGURE 2 HPLC (Supelco LC-18, 25 cm x 10 mm, 5 μm) Chromatogram of Pooled 1 Hour Post-dosing Plasma from Rats Dosed Orally with Rapamycin Showing M2, M3, M5, and Rapamycin (R). The major metabolite peaks were over scale because data was acquired with low attenuation for the collection of metabolite eluates.

TABLE 2

Summary of MS analysis of major plasma metabolites of rapamycin in rats

Metabolite Number	MW[a]	Possible Metabolic Transformation
M2	929	monohydroxylation or epoxidation
M3	913	ring opened isomer
M5	899	monodemethylation

[a]MW based on nominal monoisotopic mass.

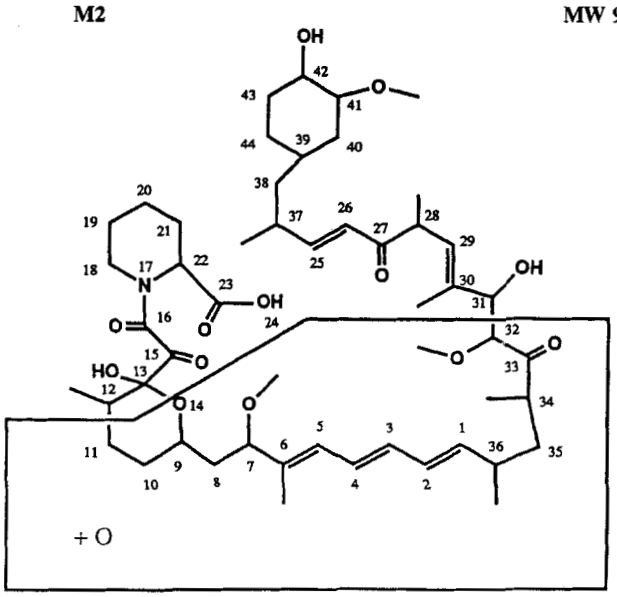

FIGURE 3 Proposed Site of Metabolic Transformation of Rapamycin to Metabolite M2

biotransformation by either aliphatic hydroxylation or epoxidation. The site of biotransformation can be located from the product ion mass spectrum which shows the complimentary fragment ions at m/z 321 and 606. The fragment ion at m/z 321 suggests that the "northern" portion of rapamycin is unchanged. That biotransformation occurred at the "southern" portion of rapamycin is inferred from the ion at m/z 606 which is 16 daltons higher than the corresponding fragment ion from the parent compound. Furthermore, the base peak at m/z 240 indicates that the change is unlikely on carbons 12 to 23. The proposed site of metabolic transformation of M2 is depicted in Figure 3.

M3: This metabolite has the same deprotonated molecular anion (m/z 912.6) as rapamycin in the full scan ion spray mass spectrum. That this metabolite is isomeric with rapamycin is indicated by the similarity of its product ion mass spectrum to that of rapamycin. M3 has been identified and reported as a degradation product of rapamycin (8). Figure 4 shows the structure of M3.

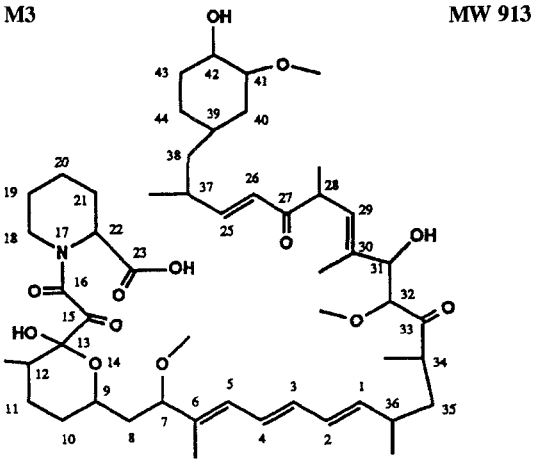

FIGURE 4 Proposed Structure of M3

M5: The full scan ion spray mass spectrum of M5 shows the presence of a low abundance deprotonated molecular anion at m/z 898.6 which is 14 daltons less than rapamycin. This would correspond to the loss of a methyl group. The product ion mass spectrum shows the presence of two complimentary fragment ions at m/z 307 and 590. The weak fragment ion at m/z 590 suggests that the "southern" portion of the rapamycin molecule is intact. Therefore, biotransformation must have occurred on the "northern" portion of rapamycin and is corroborated by the fragment ion at m/z 307 which is 14 daltons less than the corresponding "northern" fragment of rapamycin. Examination of the "northern" portion of rapamycin indicates a metabolic soft spot at carbon 41 which would likely be O-demethylated. The fragment ion at m/z 321 in the product ion mass spectrum may suggest the presence of another minor isomeric demethylated metabolite in this sample, that is, either one of the metabolic soft spots on carbon 7 or 32 in the "southern" portion of rapamycin is likely to be O-demethylated. The structure of M5 is proposed in Figure 5.

DISCUSSION

A semipreparative HPLC procedure using a C-18 column for the isolation of three major plasma metabolites of rapamycin (M2, M3, and M5) has been developed successfully. The

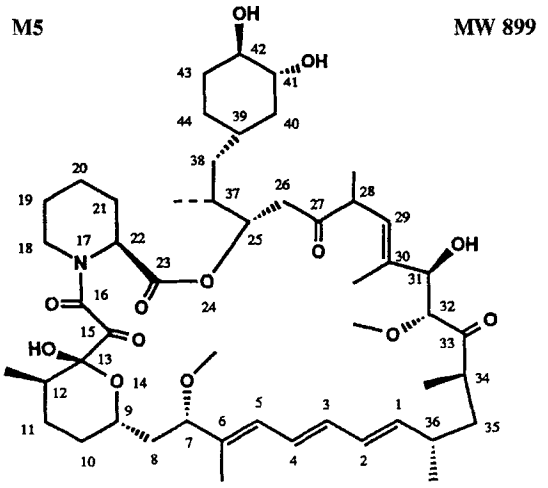

FIGURE 5 Proposed Structure of M5

baseline separation of these metabolites was achieved using a reverse phase gradient system. Metabolites M2, M3, and M5 were isolated and identified. All other minor metabolites were not investigated due to their lower quantities. In our experience, these metabolites are not stable; thus, sample handling is critical for the successful isolation of the metabolites. Rats were given a high dose to produce large quantities of metabolites and therefore to facilitate metabolite isolation and identification. Structural elucidation by mass spectrometry indicated that metabolites M2, M3, and M5 are the oxygenated, ring opened and O-demethylated metabolites of rapamycin, respectively. M3, a degradation product of rapamycin found *in vitro* (8), was also present *in vivo*. The M5 metabolite identified in this study may be the same as the previously reported *in vitro* O-demethylated metabolite of rapamycin at carbon 41 (9). The pattern of metabolism of rapamycin is similar to that of FK506, another immunosuppressive agent, in which major metabolites also produced via demethylation and oxygenation (12).

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