Pharmacokinetics and Metabolic Disposition of Sirolimus in Healthy Male Volunteers After a Single Oral Dose

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Abstract: The pharmacokinetics and metabolic disposition of sirolimus (rapamycin, Rapamune), a macrocyclic immunosuppressive agent for the prevention of allograft rejection in organ transplantation, were investigated in 6 healthy male volunteers after a single nominal 40-mg oral dose of the ^{14}C-radiolabeled drug, with the added aim of assessing the potential role of sirolimus metabolites in the clinical pharmacology of the parent drug. The absorption of parent drug and derived materials was rapid (t_{max} 1.3 ± 0.5 hours, mean ± SD), and the elimination of sirolimus was slow ($t_{½}$ 60 ± 10 hours, mean ± SD) in whole blood. The high whole blood to plasma (B/P) concentration ratio of sirolimus (142 ± 39) was consistent with its extensive partitioning into formed blood elements. The markedly lower B/P value based on radioactivity (2.7 ± 0.4) suggested that drug-derived products partitioned into formed blood elements to a much lesser extent. Based on AUC_{0-144h} values, unchanged sirolimus represented an average 35% of total radioactivity in whole blood. Drug-derived products in whole blood were characterized by HPLC, LC/MS, and LC/MS/MS as 41-O-demethyl, 7-O-demethyl, and several hydroxy, dihydroxy, hydroxy-demethyl and didemethyl sirolimus metabolites. The percentage distribution of sirolimus metabolites in whole blood ranged from 3%–10% at 1 hour to 6%–17% at 24 hours after drug administration. Based on their low immunosuppressive activities and relative abundance in whole blood of humans after sirolimus administration, metabolites of sirolimus do not appear to play a major role in the clinical pharmacology of the parent drug. A majority of the administered radioactivity (91.0 ± 8.0%) was recovered from feces, and only 2.2% ± 0.9% was renally excreted.

Key Words: sirolimus, pharmacokinetics, metabolic disposition, immunosuppressive activity, metabolites

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Sirolimus (rapamycin, Rapamune®), a macrolide fermentation product of *Streptomyces hygroscopicus*, is used as an immunosuppressive agent to prevent allograft rejection in kidney transplantation. The mechanism of immunosuppressive action and the side effect profile of sirolimus are distinctly different from those of tacrolimus and cyclosporine.[1,2] The differences in the mechanism of immunosuppressive action most likely explain the synergistic effects of sirolimus and cyclosporine to prevent rejection of renal allografts.[3]

Sirolimus was metabolized primarily by rat and human liver microsomal cytochrome P450 CYP3A enzymes to several demethyl, hydroxy, and hydroxydemethyl and didemethyl metabolites.[4–7] The macrolide ring-opened isomer (*seco*-sirolimus) was characterized as a degradation product of sirolimus in various matrices, including buffer, whole blood, plasma, bile, and in liver microsomal incubation of sirolimus.[6,8] In rats after a single oral dose of sirolimus, major drug-derived products in plasma were characterized as 41-O-demethyl sirolimus, hydroxylated sirolimus, and *seco*-sirolimus.[9] In trough whole blood of renal transplant patients receiving multiple oral doses of sirolimus, cyclosporine, and prednisolone, several oxidative metabolites were characterized by LC/MS/MS.[6,10]

Sirolimus exhibited extensive distribution into formed blood elements.[2,11,12] The mean (%CV) whole-blood to plasma concentration ratio (B/P) of sirolimus in renal graft recipients receiving single doses of sirolimus was 49 (31%),[2] presumably because of binding to intracellular FKBP12. The pharmacokinetics of sirolimus have been characterized after single-dose[2,13] and multiple-dose[14,15] sirolimus administration by oral solution in stable renal transplant recipients receiving concomitant steady-state regimens of cyclosporine, azathioprine, and corticosteroids. Absorption of an oral dose was rapid, with mean (%CV) peak whole-blood sirolimus concentrations occurring at 1.4 (79%) and 1.4 (86%) hours after single and multiple sirolimus doses, respectively, with an oral bioavailability of approximately 20%. The declining portion of whole-blood sirolimus concentration–time profiles was bi- or triexponential in individual patients. Mean (CV%) terminal half-lives of 57 (23%) and 62 (26%) hours were observed after single and multiple sirolimus doses, respectively. Detailed information on the absorption, metabolism, distribution, and excretion of sirolimus using a radiolabel assay is currently not available. The objectives of the present study were to characterize the pharmacokinetics and metabolic disposition of sirolimus in healthy male volunteers after a single oral dose of the ^{14}C-radiolabeled drug, with the added aim of assessing the potential role of sirolimus metabolites in the clinical pharmacology of the parent drug.

MATERIALS AND METHODS

The study was approved by an institutional review board (IRB) recognized by the US Food and Drug Administration

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(FDA). All subjects signed a witnessed and written informed consent. The clinical phase of the study was conducted at Innovex Inc (Lenexa, KS).

Study Design

Each of 6 fasted subjects received a single nominal 40-mg oral dose (actual dose was 42 mg) of the ^{14}C-radiolabeled drug in a drinking solution. The 40-mg dose was chosen because it was within the range of doses identified as safe and well tolerated in ascending single oral dose studies. Subjects were fasted for about 10 hours before drug administration and continued to abstain from food for at least 4 hours after dosing. Water was not allowed from 2 hours before to 2 hours after dosing. The observation period was 15 days. Whole blood was collected (see below) until 144 hours, and excreta were collected quantitatively until day 15. Citrus juices were not allowed during the in-house trial period.

Shortly before dosing, each dose of the drug product was mixed thoroughly with about 2 ounces of water. The volunteers drank the dosing solution, the bottles were rinsed twice with additional volumes of water, and the volunteers drank the washings followed by 120 mL of water. Venous whole-blood samples were collected by multiple punctures from a forearm vein before drug administration (1 × 10 mL) and at 0.5, 1, 2, 4, 12, 24, 48, 72, 96, and 144 hours (4 × 10 mL each) postdosing. Whole-blood samples were collected in evacuated whole-blood tubes containing solid heparin and gently inverted several times after collection. Aliquots of the whole-blood samples (5 mL predose and 10 mL postdose) were removed; the remaining whole-blood samples (5 mL at predose and 30 mL at other time points) were centrifuged to obtain plasma. Whole-blood and plasma samples were kept on ice after collection and promptly stored at −80°C to ensure sample integrity. Complete urine output was collected by spontaneous voiding into polypropylene bottles at predose and during 0–12 hours, 12–24 hours, and each subsequent 0- to 24-hour interval after dose administration until day 15. Urine samples were kept refrigerated during the collection period. Total fecal output was collected in plastic bags as voided until day 15. All whole blood, plasma, urine, and fecal samples were stored promptly at approximately −80°C after collection.

Volunteer Selection and Safety Assessment

Six healthy male subjects (4 white, 1 Hispanic, and 1 black) participated in the study, with mean (range) values for age, body weight, and hematocrit of 29 (19–42) y, 73 (66–92) kg, and 44 (41–48)%, respectively. It was not the objective of this study to evaluate ethnic or gender differences in the metabolic disposition of sirolimus. Subjects were randomly chosen provided that they met established inclusion criteria. Subjects were selected based on a prestudy screen with routine clinical laboratory tests, which included ECG, physical, and laboaratory evalutions. Subjects had no evidence of current clinically significant metabolic or other diseases or known history of significant allergic conditions or hypersensitivity to macrolide compounds. No psychoactive, recreational, or prescription drugs were allowed within 30 days, or episodic use of over-the-counter drugs (except occasional acetaminophen) within 14 days of study drug administration. Subjects had average alcohol consumption of less than 24 oz beer or the equivalent per day and were nicotine-free for the 3 months and abstained from alcohol from 48 hours before study drug administration until discharge from study. All subjects were negative for HIV and/or HbsAg serology.

^{14}C-Radiolabeled Drug Substance

Sirolimus (released batch, lot OM 4657) was obtained from Wyeth-Ayerst Research, Rouses Point, New York. [^{14}C]Sirolimus (Lot CFQ 8054, 64 mCi/mmol), radiolabeled at the 2 and 6 positions of the piperidine ring, was purchased from Amersham International (Buckinghamshire, England) and purified by HPLC to achieve a radiochemical purity of 95% before use. Each dose of the drug product contained nominally 40 mg (actual dose was 42 mg) and 100 μCi of sirolimus in a vehicle (8 mL) of ethanol (2% vol/vol), polysorbate 80 (1% vol/vol), in Phosal 50 PG. The drug product was stored in amber bottles at 2° to 8°C until use.

Chemicals

Sirolimus (released batch, lot OM 4657) was obtained from Wyeth-Ayerst Research, Rouses Point, New York. WAY-126792 (*seco*-sirolimus) and WAY-125286 (32-O-demethyl sirolimus) were prepared at Wyeth-Ayerst Research, Princeton, New Jersey. WAY-138769 and WAY-138772 (7-O-demethyl sirolimus epimers) were obtained from Wyeth-Ayerst, Pearl River, New York. Organic solvents and water were HPLC grade and were obtained from EM Science (Gibbstown, NJ). Polysorbate 80 was purchased from Baker Analytical. Solid-phase extraction cartridges (C_{18}, 500 mg) were purchased from Waters (MA). β-Glucuronidase and sulfatase (Type H-1 from *Helix pomatia*) were obtained from Sigma Chemical Co (St. Louis, MO).

Determination of Radioactivity Concentration in Whole-Blood, Plasma, and Excreta

Thawed whole-blood aliquots (0.5 mL) in triplicates were placed in tared Combusto-Cones (Canberra-Packard Co., Downers Grove, IL) and weighed. They were air dried and oxidized to $^{14}CO_2$ using a Model 307 Tri-Carb Sample Oxidizer (Canberra-Packard Co.). The efficiency of oxidation was determined by the oxidation of methylmethacrylate standards of known radioactivity (New England Nuclear, Boston, MA). Thawed plasma aliquots (0.5 mL) in triplicates were placed in tared scintillation vials and weighed. To each aliquot was added deionized water (0.5 mL), followed by liquid scintillation cocktail (10 mL, 3a70b, Research Products International, Mt. Prospect, IL) and directly counted. Urine samples were thawed and volume measured, and duplicate aliquots (1 mL) were directly counted in 10 mL of scintillation cocktail. Thawed fecal samples were weighed and homogenized in a known volume of water:ethanol (1:1 vol/vol) using a Polytron PT-6000 (Brinkman Instruments Inc, Westbury, NY). Duplicate weighed aliquots (1 mL) of the fecal homogenates were combusted according to procedures used for whole-blood samples. Radioactivity measurement was performed using a Packard 2500 TR liquid scintillation spectrophotometer (Canberra-Packard Co.). Samples were counted for 10 minutes or until % σ of 0.2 was achieved.

Counts per minute (cpm) were converted to disintegrations per minute (dpm) using a quench curve generated from external standards of known radioactivity.

Determination of Sirolimus Concentration in Whole-Blood and Plasma

Sirolimus concentrations were determined using validated high-performance liquid chromatographic/tandem mass spectrometry (LC/MS/MS) methods described previously, using 32-desmethoxyrapamycin as the internal standard.[16] Using a 1-mL sample, the methods had a limit of quantification of 0.1 ng/mL and were linear over the range of 0.10 to 50 ng/mL for both whole-blood and plasma. Between-day coefficients of variation were ≤20% in both matrices.

Metabolite Profile in Whole-Blood and Plasma

A HPLC method with radioactivity detection was developed to determine the metabolite profile of sirolimus in whole-blood and plasma. Whole-blood samples (5 mL) from each of subjects 1 to 6 at 1 hour and 4 hours postdosing were used for evaluation. For metabolite characterization, pooled whole-blood samples (approximately 10 mL pooled from 1.7 mL of each of 6 subjects) at 2, 12, and 24 hours postdosing were used for analyses by both HPLC with radioactivity detection and by LC/MS/MS. Similarly, pooled plasma samples at 1 and 2 hours postdosing were used for analyses by HPLC and LC/MS/MS. Whole-blood and plasma samples were extracted by liquid-liquid extraction as follows. Methanol (0.375 mL) was added to each 5 mL of whole-blood aliquot and thoroughly mixed. Following the addition of ethyl acetate (12.5 mL), the mixture was shaken on an automatic shaker for 20 minutes. The organic layer was removed following centrifugation at 2300 rpm at 25°C using an IEC CRU-5000 centrifuge. The extraction procedure was repeated once. The combined organic fractions were evaporated under a stream of nitrogen to dryness, reconstituted with methanol:-water 65:35 (300 μL), and filtered through a Nylon 66 membrane filter (0.22 μm, Rainin Instruments). Aliquots were analyzed by HPLC (100 μL) or LC/MS (20 μL). The recovery of radioactivity following sample extraction was monitored by measuring radioactivity in duplicate aliquots of the initial extracts and in the reconstituted solutions. The efficiency of the extraction method for sirolimus was assessed by determining the recovery of added ^{14}C-sirolimus (5 μg, 0.1 μCi in 20 μL ethanol) in triplicates from 5 mL of control whole-blood or plasma. The contribution of major sirolimus metabolites to total radioactivity in whole-blood and plasma extracts was estimated by integration of corresponding peaks in the HPLC radiochromatograms. For samples at 1 hour and 4 hours post–drug administration, percentage contribution to total radioactivity was expressed as mean ± SD of 6 subjects. For pooled samples at 2, 12, and 24 hours, percentage contribution to total radioactivity represented the mean values of 6 subjects.

Pooled whole-blood samples at 12 hours and pooled plasma samples at 1 hour after dose administration were subjected to hydrolysis with β-glucuronidase/sulfatase. The enzyme solution was prepared by dissolving 400 mg of β-glucuronidase (Sigma Chemical Co, Type H-1 *Helix pomatia*) in each 800 μL of 0.2% NaCl. An aliquot of the enzyme solution (200 μL, 100 mg, approximately 35,000 units β-glucuronidase activity, 1500–4000 units sulfatase activity) was added to aliquots of whole blood or plasma samples (10 mL) in sodium acetate buffer (10 mL, 0.1 M, pH 5). Control incubations contained an equal volume of 0.2% NaCl instead of the enzyme solution. The mixtures were incubated at 37°C in a shaking water bath for an hour to minimize the degradation of sirolimus or metabolites. Control incubations contained 5 mM of the β-glucuronidase inhibitor D-saccharic acid 1,4-lactone. Hydrolysis samples were extracted and prepared for LC/MS analyses according to procedures described above for untreated whole-blood samples.

Metabolite Profile in Urine and Feces

Urine samples from each of the subjects containing the highest amount of excreted radioactivity (0.3% to 1% of dose) were used. Dependent on the subject, the volume of the urine samples ranged from 10 to 50 mL, and the highest amount of excreted radioactivity occurred from 0 to 72 hours. Urine samples were lyophilized to dryness using a Labconco freezer dryer, and residues were dissolved in methanol (10 mL), filtered through a Nylon 66 membrane filter (0.22 μm, Rainin Instruments), and evaporated under a stream of nitrogen to remove all volatile components. The final residue was reconstituted with water and analyzed by HPLC with radioactivity and mass spectrometric detection.

Fecal homogenates from each of the subjects containing a majority of the excreted radioactivity (10% to 90% of dose) were used. Dependent on the subject, the volume of the fecal homogenates ranged from 5 to 10 mL, and the majority of excreted radioactivity occurred within 24 to 72 hours. Fecal homogenates were centrifuged at 50,000 rpm using a Sorvall RC M120 high-speed centrifuge, and resulting supernatants were filtered through Nylon 66 membrane filters (0.22 μm, Rainin Instruments). Aliquots (100–200 μL) of the filtrate were subjected to HPLC (100–200 μL) and/or LC/MS (20 μL) analyses using conditions described in preceding sections. Selected fecal and urine samples were subjected to hydrolysis by β-glucuronidase similar to procedures described above for blood and plasma. Details of the HPLC and LC/MS methods employed for the metabolite profiling of sirolimus in blood, plasma, urine, and feces are described below.

High-Performance Liquid Chromatography (HPLC)

HPLC analyses were performed on a Waters HPLC system consisting of a 715 Ultra WISP autosampler, a 600E system controller, a 486 Tunable Absorbance Detector, and a Radiomatic Flow-One A-500 radioactivity detector. Data acquisition and analysis were performed by Radiomatic and PE Nelson Turbochrom software. Separation of sirolimus and drug-derived products was achieved on a Keystone BDS Hypersil C18 column (150 × 2 mm, 5 μm) using a linear gradient of 65:35 to 86:14 methanol: 5 mM ammonium acetate over 80 minutes at a flow rate of 0.2 mL/min; or a Supelcosil LC-18 column (250 mm × 4.6 mm, 3 μm) using the same gradient solvent system and at a flow rate of 0.5 mL/min. The eluent was monitored at 276 nm. Because of the low levels of

radioactivity in whole-blood and plasma samples, the eluent was collected in scintillation vials (20 mL) on a Waters fraction collector. Fractions were collected at 1 fraction per minute, and their radioactivity contents determined by liquid scintillation counting. Radiochromatograms were reconstructed by plotting radioactivity content (dpm) of each fraction versus fraction number using QuattroPro 5.0 or Excel 6.0 spreadsheets.

Electrospray Ionization LC/MS and LC/MS/MS

All electrospray ionization (ESI) LC/MS and LC/MS/MS analyses were performed using an HP 1090M HPLC connected via 0.005-inch ID peek tubing to the Sciex API III$^+$ mass spectrometer. Chromatographic separation of sirolimus and its metabolites was achieved on a BDS Hypersil C18 column (150 × 2 mm, 5 μm) using a linear gradient of 58:42 to 85:15 methanol:10 mM ammonium acetate over 80 minutes at a flow rate of 0.2 mL/min. The column was kept at 40°C. The eluate was split postcolumn with 50 to 80 μL/min being pneumatically sprayed into the mass spectrometer. The spray needle was maintained at +5 kV for ESI LC/MS analysis in the positive ion mode. The interface heater was kept at 55°C during analysis. Desolvation of the solvent droplets was performed at a declustering potential of +30 V. The $(M + NH_4)^+$ ions of sirolimus and its metabolites were monitored at unit mass resolution in the ESI LC/MS-SIM experiment. Ions monitored were: *m/z* 903.7, didemethyl sirolimus; *m/z* 917.7, demethyl sirolimus; *m/z* 931.7, sirolimus and isomers; *m/z* 933.7, hydroxydemethyl sirolimus; *m/z* 947.7, hydroxysirolimus; *m/z* 949.7, sirolimus plus water; and *m/z* 963.7, dihydroxysirolimus.

Full-scan ESI LC/MS/MS analysis in the negative ion mode was conducted by spraying the LC eluate at −4 kV. Declustering potential was set at −70 V. The molecular anions $(M - H)^-$ corresponding to sirolimus and its metabolites were selected by Q1 at resolution 3 to 4 Da at half-height. Collision-activated dissociation of the molecular anion was induced by argon at a collison gas thickness of 240 to 250 × 10^{12} atoms of argon/cm^3 and at collision energy of 33 eV. The resultant product ions were mass analyzed at unit mass resolution by scanning Q3 from *m/z* 50 to 950 in 0.17 milliseconds and at step size of 0.1 Da. Ions were detected with electron multiplier set at saturation (4500 V). Structural elucidation of sirolimus-derived products was based on the mass shift of the *m/z* 321 and *m/z* 590.[17] Peak area integration was performed manually using MacSpec 3.22.

Pharmacokinetic Analysis

Pharmacokinetic parameters were determined was using noncompartmental analysis. The peak concentrations (C_{max}) and time to peak concentration (t_{max}) of sirolimus and total radioactivity in whole-blood and plasma were taken directly from the observed data. The area under the concentration–time curve (AUC_{0-144h}) was calculated by applying the linear/log-linear trapezoidal rule. The ratio of total radioactivity in whole-blood to plasma was estimated as the ratio of concentration in whole blood to that in plasma at the individual time points. Total radioactivity (dpm) was expressed in nanogram (ng) equivalents. For sirolimus in whole-blood, the time points that were judged to be in the terminal phase were used to obtain the terminal disposition slope (λ_z) by log-linear regression. The area under the concentration–time curve (AUC_t) and the area under the first moment curve ($AUMC_t$) truncated at the last observable concentration (C_t) at time t_{last} were calculated by applying the linear/log-linear trapezoidal rule. The total AUC and AUMC were estimated as follows: $AUC = AUC_t + C_t/\lambda_z$ and $AUMC = AUMC_t + t_{last} \cdot C_t/\lambda_z + C_t/\lambda_z^2$. The terminal disposition half-life ($t_{½}$) was calculated as $0.693/\lambda_z$. The mean residence time (MRT) was calculated from AUMC/AUC. Oral-dose clearance (CL/F) was calculated from the relationship CL/F = Dose/AUC, and the apparent oral-dose steady-state volume of distribution (V_{ss}/F) was calculated from $V_{ss}/F = CL/F \times MRT$. Weight-normalized estimates of CL/F and V_{ss}/F were obtained by dividing by body weight (kg).

Because of extensive partitioning into formed blood elements, plasma sirolimus concentrations were low and were not detectable over the entire 144-hour sampling period. Therefore, complete concentration–time profiles could not be obtained. The estimation of plasma sirolimus pharmacokinetic parameters was limited to C_{max}, t_{max}, AUC_t, and the ratio of rapamycin concentration in whole blood to plasma (B/P ratio). An overall B/P ratio value for each subject was obtained as the average of available B/P values at individual time points over the initial 24 hours.

Descriptive statistics (mean, standard deviation, coefficient of variation) were obtained for subject demographic characteristics, concentrations of sirolimus, total radioactivity, and sirolimus pharmacokinetic parameters. Statistical analysis was performed using the SAS System.[18]

RESULTS

Radioactivity and Sirolimus Concentration in Whole-Blood and Plasma

The concentration–time curves of radioactivity in whole-blood and plasma are shown in Figure 1. Corresponding pharmacokinetic parameters are shown in Table 1. Peak concentrations (C_{max}) of radioactivity were measured in whole-blood and plasma at 1.3 ± 0.5 and 2.0 ± 1.1 (mean ±SD) hours, respectively. Mean whole-blood exposure of total radioactivity based on C_{max} was approximately 4-fold greater than that in plasma (267 versus 68 ng/mL), whereas whole-blood exposure based on AUC over 144 hours was approximately 2.7-fold greater than in plasma (5371 versus 2004 ng·h/mL). The higher mean concentrations in whole-blood than in plasma suggested that total radioactivity partitioned into formed blood elements. The partitioning of radioactivity decreased over time as reflected by the mean whole-blood-to-plasma (B/P) ratios shown in Figure 1. The observed decline in the partitioning of total radioactivity may be caused by changes in the relative concentrations of sirolimus and its metabolites over time, together with qualitative differences in their partitioning into formed whole blood elements.

Figure 2 depicts the concentration–time curves of sirolimus in whole blood and plasma. Tables 2 and 3 provide sirolimus pharmacokinetic parameters for whole-blood and plasma, respectively. As for total radioactivity, the absorption of sirolimus was rapid, with whole-blood (144 ± 32 ng/mL)

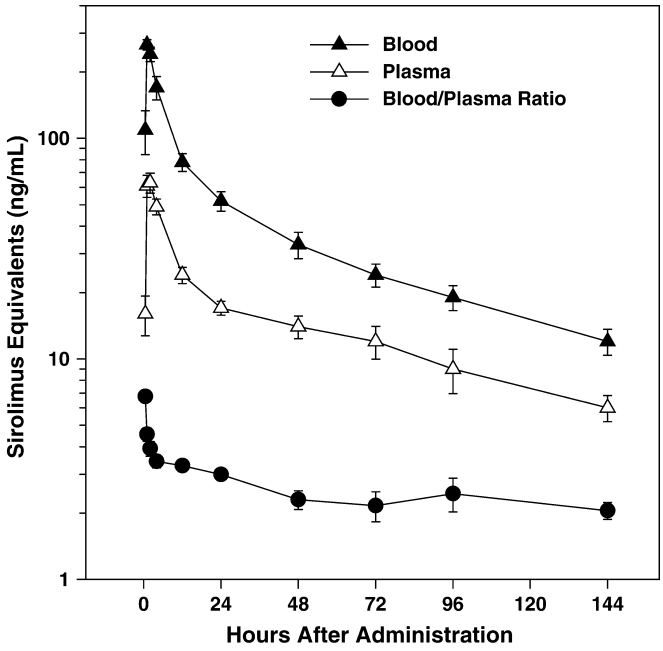

FIGURE 1. Radioactivity concentration and distribution within whole blood and plasma of healthy male subjects after a single nominal 40-mg oral dose of [^{14}C]sirolimus.

and plasma (2.13 ± 1.13 ng/mL) peak concentrations achieved at 1.3 ± 0.5 and 1.2 ± 0.4 hours, respectively. Elimination of the drug was slow, as reflected by elimination half-life ($t_{½}$) of 59.8 ± 9.7 hours in whole-blood. Sirolimus was not detectable in plasma at time-points greater than 24 hours, which precluded the estimation of elimination half-life in plasma. The area under the concentration–time curve over 0–144 hours ($AUC_{0–144h}$) in whole blood represented approximately 86% of the total area under the curve ($AUC_{0–\infty}$). The intersubject variability in parameter estimates was generally low in whole-blood, with values of oral-dose clearance (CL/F) and apparent oral-dose steady-state volume of distribution (V_{ss}/F) ranging only about 2-fold. The exposure of sirolimus in plasma was greatly reduced compared with exposure in whole-blood. In contrast to the sharp decline in partitioning observed over time for total radioactivity, a relatively constant partitioning of sirolimus between whole-blood and plasma was observed over the initial 24 hours, as shown in Figure 2. The elimination half-life of sirolimus in plasma could not be determined because drug concentrations were below detection at time points greater than 24 hours. The mean ± SD B/P ratio in individual patients over 24 hours was 142 ± 39, which was much greater that the partitioning of radioactivity between whole-blood and plasma (2.7 ± 0.4). Based on $AUC_{0\text{-}144h}$ values, sirolimus represented an average 35% of total radioactivity in whole-blood.

Metabolite Profile in Whole-Blood

The average recovery of added sirolimus from naive whole-blood by liquid-liquid extraction was 85%. The recovery of radioactivity from whole blood ranged from approximately 87% to 62% between 1 and 24 hours after drug administration. These recoveries translated to approximately 100% to 75% when normalized to that of the control sample. The decrease in the recovery of radioactivity over time suggested that concentrations of nonextractable drug-derived products present in the systemic circulation of man increased over time.

Figure 3 shows a representative HPLC radiochromatogram and the corresponding LC/MS total ion chromatogram of whole-blood extracts of subjects at 2 hours after dose administration. Several drug-derived peaks were detected, with unchanged drug representing the single most abundant drug-related product present. Representative reconstructed selected ion chromatograms (RIC) of whole-blood extracts are shown in Figure 4. Unchanged sirolimus (t_R = 51.6 minutes) was detected by monitoring the $(M + NH_4)^+$ ion in the RIC of *m/z* 931.7. Peak B′ (t_R = 28.6 minutes) was detected in the didemethyl sirolimus RIC of *m/z* 903.7. Peaks C and H were detected in the demethyl sirolimus RIC of *m/z* 917.7. Structural elucidation of metabolites of sirolimus was by LC/MS/MS analyses under negative ion mode. Collisonal-activated decomposition (CAD) of the molecular anion of sirolimus at *m/z* 912 under negative ion mode resulted in 2 complementary diagnostic molecules. The product anion at *m/z* 321 corresponded to the fragment C25 to C31 and C37 to C44 of the sirolimus molecule, whereas the anion at *m/z* 590 was the fragment containing C1 to C24 and C25 to C36 (Fig. 5).

TABLE 1. Pharmacokinetic Parameters for Total Radioactivity in Whole-Blood and Plasma of Healthy Male Subjects After a Single Nominal 40-mg Dose of [^{14}C]Sirolimus

	Whole Blood			Plasma		
Patient No.	C_{max} (ng·equiv/mL)	T_{max} (h)	$AUC_{0–144h}$ (ng equiv–h/mL)	C_{max} (ng·equiv/mL)	T_{max} (h)	$AUC_{0–144h}$ (ng equiv·h/mL)
1	266	1	6907	60	4	2007
2	299	1	4403	88	1	1659
3	217	1	3753	48	1	1342
4	265	2	6194	54	2	2408
5	311	1	5642	85	2	2100
6	244	2	5329	73	2	2508
Mean	267	1.3	5371	68	2	2004
SD	35	0.5	1155	17	1.1	444
CV%	13	39	21	24	55	22

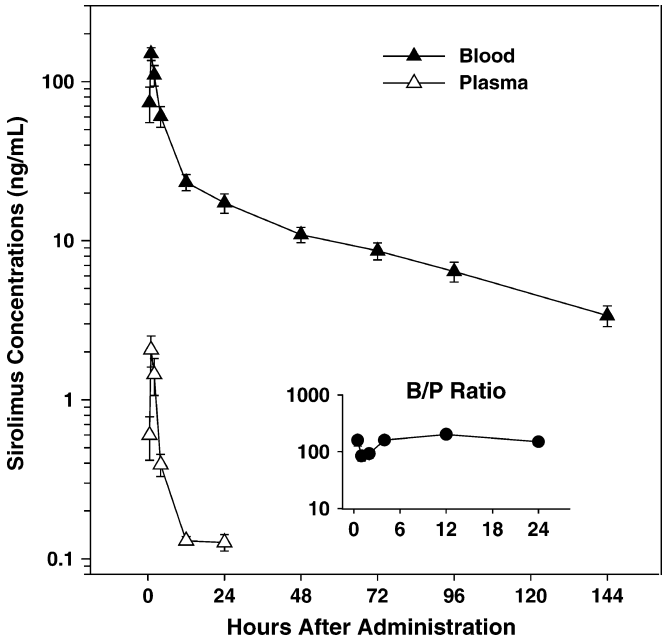

FIGURE 2. Sirolimus concentration and distribution within whole-blood and plasma of healthy male subjects after a single nominal 40-mg oral dose of [^{14}C]sirolimus.

The assignment of these anions was by negative ion high-resolution FAB/MS measurements of sirolimus at resolution of 10,000 with peak matching.[17] This cleavage of the sirolimus molecule has also been reported to occur following CAD of the positive lithium or sodium adduct ion of sirolimus.[7]

For demethyl metabolite peak C, the detection of anions at *m/z* 321 and 576 following CAD of the molecular anion of demethyl sirolimus at *m/z* 898 suggested that the fragment anion of sirolimus molecule at *m/z* 590 has shifted by 14 days, and therefore, demethylation has to occur on this portion of the molecule. There were two methoxy groups at C7 and C32 in this fragment that could be demethylated, and the precise location could not be established from their product ion mass spectra. The availability of authentic 32-O-demethyl sirolimus helped to establish that this metabolite was 7-O-demethyl sirolimus (Fig. 5).

Another demethylated metabolite corresponding to peak H (t_R = 47.7 minutes) produced fragment anions at *m/z* 307 and 590 from CAD of molecular anion at *m/z* 898. The fragment anion of sirolimus at *m/z* 321 showed a 14-day mass shift, and hence, demethylation occurred on this portion of the sirolimus molecule. The most likely position for metabolic demethylation was the 41-O-methyl group (Fig. 5).

Peak A′ (t_R = 25.6 minutes) was detected in the RIC of *m/z* 933.7 and corresponded to demethylhydroxy- or dihydrosirolimus. In the RIC of *m/z* 947.7, several peaks corresponding to hydroxy metabolites were detected at retention times of 23.0 (peak A), 29.8 (peak B), and 32.2 (peak C′). The product ion mass spectra of the hydroxyl metabolites contained two product anions at *m/z* 321 and 606, which suggested that the fragment anion of sirolimus at *m/z* 590 was shifted by 16 days, and therefore, hydroxylation occurred on this portion of the sirolimus molecule (Fig. 5). In the reconstructed ion chromatogram of *m/z* 963.7, several relatively minor peaks were detected as dihydroxylated sirolimus metabolites. Because of their low concentrations, no attempt was made to determine the locations of the sites of hydroxylations.

HPLC and LC/MS analyses showed that there was virtually no difference between extracts of whole-blood treated with and without β-glucuronidase/sulfatase (data not shown). These observations suggested that sirolimus or metabolites were not present in any significant extent as glucuronide or sulfate conjugates.

Quantitative Estimates of Sirolimus Metabolites in Whole Blood

The quantitative estimate of major sirolimus metabolites in whole blood of subjects at different time points until 24 hours after drug administration was accomplished by integration of drug-derived peak areas in the HPLC radiochromatograms (Table 4). Because not all drug-related peaks were completely resolved under the chromatography method used, they were grouped with respect to their percentage contributions. Sirolimus represented an average of 65% of total drug-derived products at 1 hour and decreased to approximately 30% at 12 and 24 hours after dose administration. Between 1 and 24 hours after sirolimus administration, the percentage distributions of hydroxy and hydroxydemethyl

TABLE 2. Pharmacokinetic Parameters of Sirolimus in Whole-Blood of Healthy Male Subjects After a Single Nominal 40-mg Dose of [^{14}C]Sirolimus

Subject No.	C_{max} (ng/mL)	T_{max} (h)	AUC_{0-144h} (ng·h/mL)	$AUC_{0-\infty}$ (ng·h/mL)	CL/F (mL/h/kg)	V_{ss}/F (L/kg)	MRT (h)	$t_{½}$ (h)
1	112	2	2581	3040	182	13.2	72.4	60.3
2	138	1	1369	1553	391	23.5	60.2	54.8
3	103	1	1329	1557	279	19.7	70.8	59.4
4	175	1	2019	2340	253	16.2	64.0	56.9
5	160	2	2129	2323	261	12.5	48.1	49.6
6	178	1	1879	2378	234	20.3	86.9	78.0
Mean	144	1.3	1884	2199	267	17.6	67.1	59.8
SD	32	0.5	477	566	69	4.3	13.1	9.7
%CV	22	39	25	26	26	25	19	16

TABLE 3. Pharmacokinetic Parameters of Sirolimus in Plasma of Healthy Male Subjects After a Single 42-mg Oral Dose of [^{14}C]Sirolimus

Subject No.	C_{max} (ng/mL)	T_{max} (h)	AUC_{0-t}[a] (ng·h/mL)	B/P Ratio
1	1.64	1	5.63	192
2	2.14	1	4.62	116
3	1.09	1	2.22	114
4	1.28	1	3.00	193
5	4.19	1	12.03	117
6	2.43	2	8.36	122
Mean	2.13	1.2	5.98	142
SD	1.13	0.4	3.67	39
%CV	53	35	61	27

[a]AUC_{0-24h} or less.

(or dihydro) metabolites increased from approximately 3% to 17%, hydroxy and didemethyl metabolites increased from approximately 5% to 13%, hydroxy and 7-O-demethyl metabolites increased from approximately 7% to 15%, and 41-O-demethyl sirolimus ranged from approximately 6% to 12%.

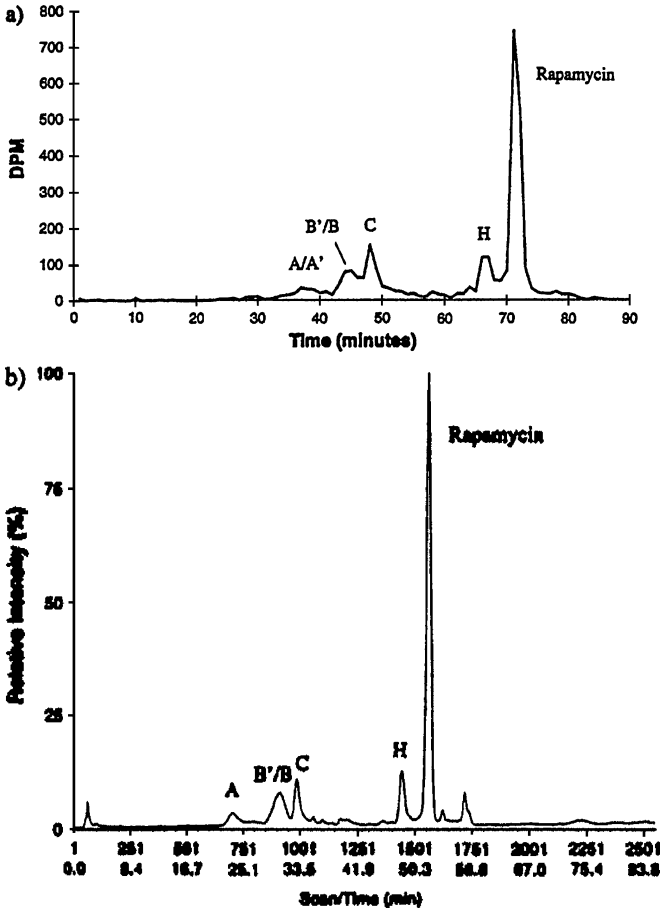

FIGURE 3. Representative HPLC radiochromatogram and LC/MS total ion chromatogram of whole-blood extracts of healthy male subjects (n = 6) at 2 hours after an oral dose of [^{14}C]sirolimus.

Metabolite Profile in Plasma

The average recovery of added sirolimus from naive plasma by liquid-liquid extraction was 92%. Using the same procedures, the recovery of radioactivity from the plasma of subjects treated with sirolimus ranged from 67% to 72% at 1 hour and 2 hours after drug administration, respectively. The lower recovery of radioactivity from plasma suggested the presence of nonextractable drug-derived products. In part because of low radioactivity levels, HPLC analyses of the 1- and 2-hour plasma extracts showed no distinguishable drug-derived peaks (data not shown). Analyses by LC/MS SIM revealed the presence of low levels of unchanged drug, *seco*-sirolimus, 41-O-demethylsirolimus, and several monohydroxylated metabolites. The sirolimus metabolite profile in plasma was not affected by treatment with β-glucuronidase/sulfatase (data not shown).

Recovery of Radioactivity in Urine and Feces

The total recovery from urine and feces was 93.2% ± 7.6% (mean ± SD) of the administered radioactivity over the 15-day study period, with most of the administered radioactivity (91.0% ± 8.0%) being recovered from feces. A majority (84.1% ± 9.6%) of the administered radioactivity was recovered within 4 days after drug administration.

Metabolite Profile in Urine and Feces

In both urine and fecal extracts, virtually all radioactivity was accounted for by polar and unidentified drug-related materials, with little unchanged drug detected. Treatment with β-glucuronidase or sulfatase had little effect on urinary or fecal metabolite profiles (data not shown).

DISCUSSION

The administration of [^{14}C]sirolimus to healthy male volunteers permitted a comparison of the distributions of total radioactivity and unchanged drug between whole-blood and plasma. The mean whole-blood-to-plasma (B/P) concentration ratio of sirolimus (142) over the initial 24 hours after drug administration was markedly higher than the B/P ratio for total radioactivity (2.7), indicating that sirolimus metabolites partitioned into formed whole-blood elements to a much

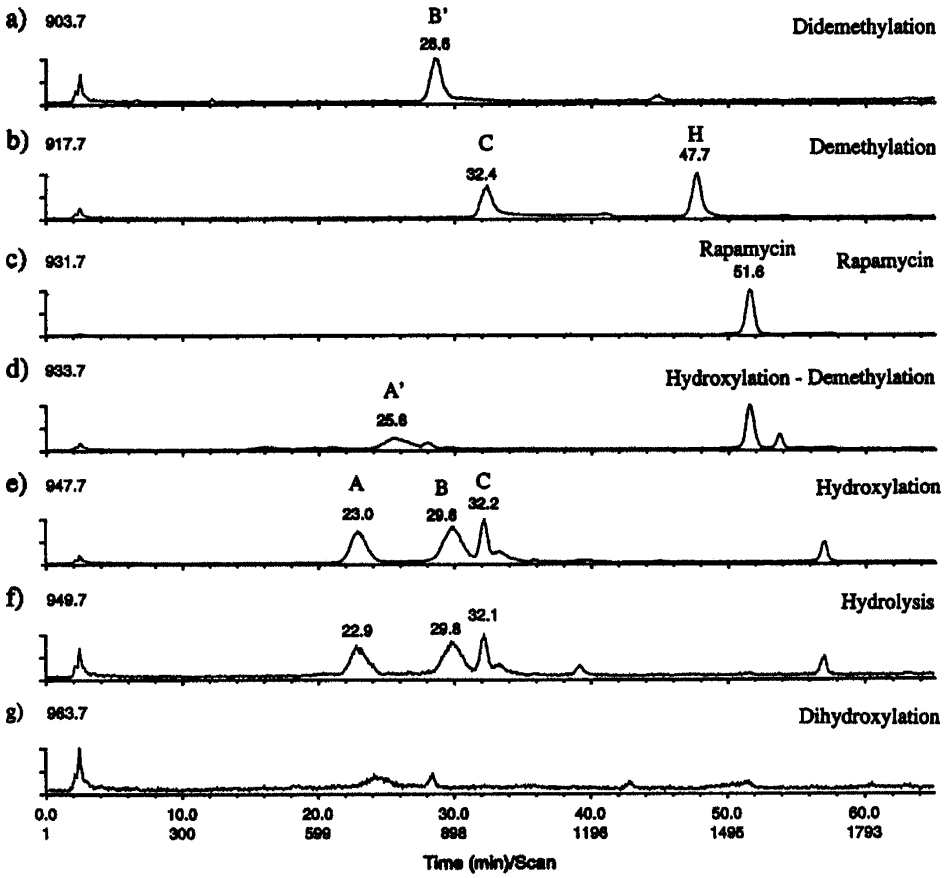

FIGURE 4. Representative reconstructed selected ion chromatogram of whole-blood extracts of healthy male subjects (n = 6) at 12 hours after an oral dose of [^{14}C]sirolimus.

lesser extent than the parent drug. The large differences between B/P ratios of total radioactivity and unchanged drug in humans are consistent with previous findings,[19] where the FKBP-binding affinity of several putative metabolites and degradation products of sirolimus was up to 5-fold lower than that of the parent drug. Though sirolimus concentrations in blood were many times higher than that in plasma as a result of the binding of sirolimus to FKBP in formed blood elements, it could not be concluded that sirolimus decreased faster in plasma than in blood because the elimination half-life of sirolimus in plasma could not be determined because drug concentrations were below detection at time points greater than 24 hours. In contrast, a relatively constant partitioning of sirolimus between blood and plasma over the initial 24 hours implied that the drug concentrations declined at a similar rate in both blood and plasma.

The pharmacokinetics of sirolimus (t_{max}, $t_{½}$, and CL/F) determined from whole-blood of normal subjects in the present study were very similar to parameters reported previously in normal fasted subjects after either a single 15-mg oral dose[16] or single oral 0.3, 1, 3, 5, and 5 mg/m^2 doses[20] and in renal transplant recipients receiving single oral 3, 5, 10, and 15 mg/m^2 doses of the drug.[2] The mean sirolimus B/P ratios for healthy subjects in this study (B/P = 142 ± 39, n = 6) were similar to those reported for the healthy fasting subjects receiving a single 15-mg dose of sirolimus (B/P = 84.4 ± 35.9, n = 22),[16] and were considerably greater than previously reported estimates of mean B/P ratios in stable renal transplant subjects receiving multiple-dose CsA and either concomitant single-dose[14] (B/P = 34.4 ± 17.6, n = 36) or concomitant multiple-dose[15] (B/P = 38.8 ± 12.8, n = 30) sirolimus. The lower estimates of B/P in stable renal transplant subjects receiving concomitant CsA administration may have been caused by the known hyperlipidemic effect of sirolimus.[21] Based on a 42-day study in renal allograft subjects (n = 6) receiving sirolimus at a dose of 10 mg/d,[22] significant increases were observed not only in total plasma cholesterol (+50%) and triglycerides (+95%) but also in the apolipoproteins ApoB-100 (+28%) and ApoC-III (+92%). Because sirolimus is approximately 40% bound to the lipoprotein fraction in blood over a sirolimus concentration range of 5 to 100 ng/mL,[11] increases in plasma lipoproteins may increase the sirolimus plasma concentrations, leading to a lower B/P ratio in renal allograft subjects. Despite large differences in sirolimus B/P ratios between normal subjects and renal allograft patients, sirolimus whole-blood pharmacokinetics between the groups remain similar. These observations suggest that factors that may affect sirolimus B/P distribution, such as hematocrits and hyperlipidemia, are expected to have little effect on the whole-blood pharmacokinetics or systemic exposure of sirolimus.

Whole-blood sirolimus concentrations, when determined by a validated LC/MS/MS method, represented an average 56% of total whole-blood radioactivity at 1 hour after drug administration, 46% at 2 hours, 36% at 4 hours, and

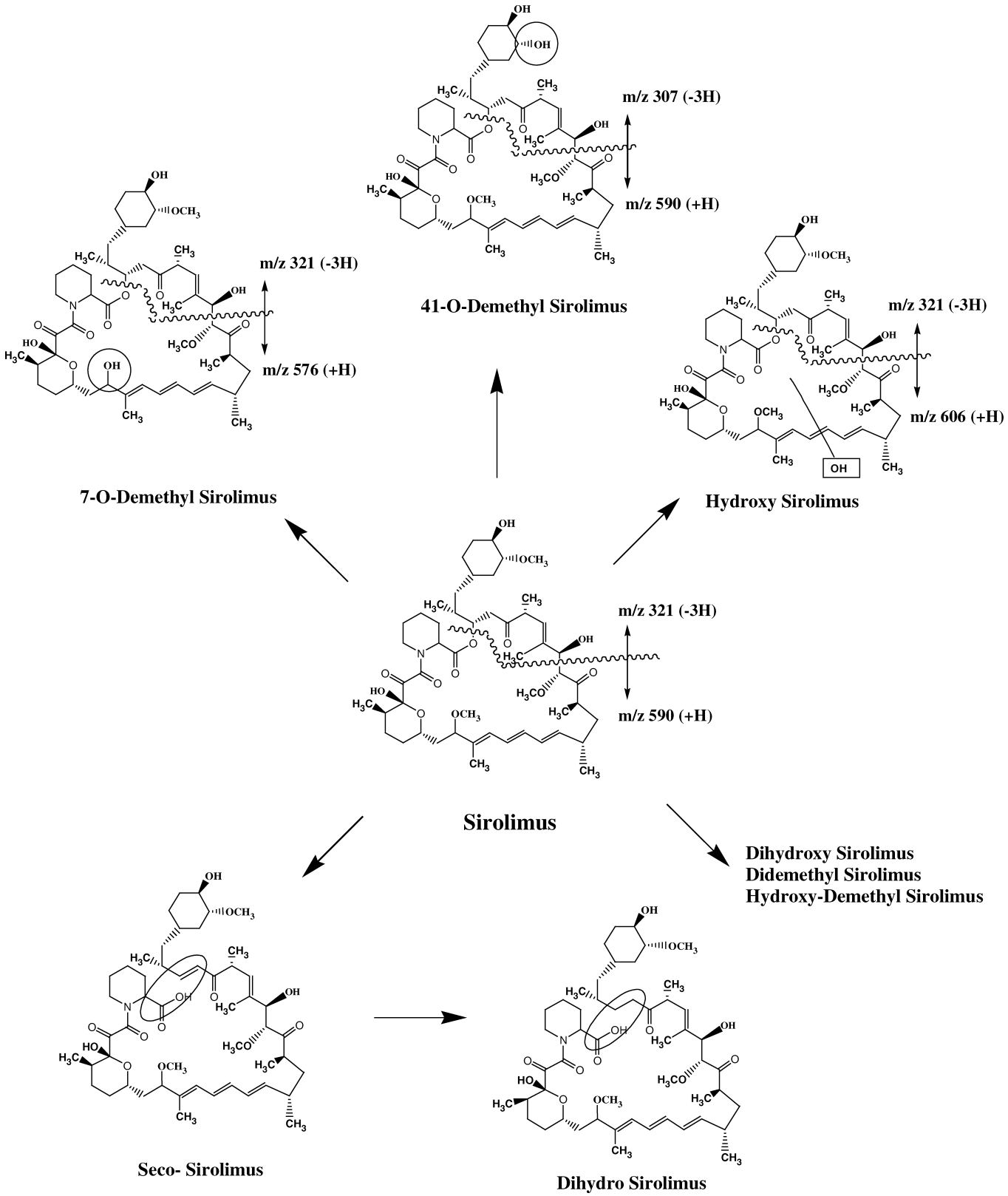

FIGURE 5. Proposed metabolite pathways of sirolimus in healthy male subjects.

TABLE 4. Percentage Distribution (%) of Sirolimus and Its Major Metabolites in Whole-Blood of Healthy Male Subjects After a Single Nominal 40-mg Oral Dose of [^{14}C]Sirolimus

Metabolite	Peak[†]	Hours After Drug Administration*				
		1[‡]	2[§]	4[‡]	12[§]	24[‖]
Hydroxy/Hydroxy-demethyl/Dihydro	A/A′	2.7 (1.6)	6.1	8.4 (1.4)	15.4	17.1
Hydroxy/Didemethyl	B/B′	4.9 (1.9)	8.9	13.4 (5.6)	12.5	13.1
Hydroxy/7-O-Demethyl	C	6.5 (3.2)	10.9	14.2 (2.9)	16.9	14.9
41-O-Demethyl	H	9.8 (2.7)	10.1	11.8 (1.7)	7.0	5.9
Sirolimus		65.4 (5.8)	48.7	39.9 (3.2)	30.3	34.2

*Percentage distributions are estimated by area integration of major drug-derived peaks in the HPLC radiochromatograms.
[†]Peaks A/A′, B/B′, and C each contains at least 2 closely eluting peaks based on LC/MS SIM analyses.
[‡]Results are mean (SD) of 6 subjects.
[§]Results represent mean values from samples pooled from 6 subjects.
[‖]Results represent mean values from samples pooled from 5 subjects (Nos. 2 to 6).

remained relatively constant (33%) thereafter up to 24 hours. These results were in good agreement with values obtained by HPLC with radioactivity detection, where sirolimus was estimated to represent approximately 65% of total drug-related peaks in whole-blood extracts at 1 hour after drug administration, 49% at 2 hours, 40% at 4 hours, and 34% at 24 hours. The similarities between the results obtained by the two different methods indicate that there was little nonextractable radioactivity in whole blood. These data indicate that sirolimus metabolites represented approximately 35% to 45% of total drug-related materials at 1 hour after drug administration and increased with time to approximately 65% at 24 hours.

The structural elucidation of metabolites of sirolimus was greatly aided by the assignment of the structures of the fragment ions of sirolimus by high-resolution measurements of anions using negative ion FAB/MS[17] and positive lithiated ions by liquid secondary ion mass spectrometry.[23] These fragment ions were also detected following analysis of sirolimus by ESI/MS/MS under negative[8,9,17] or positive[7] ion mode. The formation of same fragment ions by ESI/MS/MS has facilitated the structural elucidation of trace quantities of metabolites of sirolimus by on-line LC/MS/MS. It is well established that sirolimus has a tendency to form adduct ions with ammonia, sodium, or potassium instead of being protonated under positive ion mode.[7] The type of positive adduct ion formed is highly dependent on the mobile phase used and the type of API interface.[24] This was illustrated by the formation of the $(M + NH_4)^+$ instead of the $(M + Na)^+$ adduct ion as the most abundant ion using the acetonitrile/ammonium acetate mobile phase and curtain-gas API interface available on the API III$^+$ mass spectrometer. This, together with the uninformative product ions from CAD of $(M + NH_4)^+$ ions, were the rationale for the use of negative ion ESI/MS/MS for structural elucidation. In addition, high-resolution mass measurements of the fragment anions were available for assignment of structures.

The large fraction of the drug recovered in feces suggests that biliary excretion is likely the principal route of drug excretion. It is possible that hepatic impairment, but not renal impairment, may decrease the biliary clearance and therefore increase the systemic exposure of sirolimus. The presence of little unchanged drug in excreta indicated that sirolimus underwent extensive biotransformation and/or degradation during the absorption and excretion processes in humans. Biliary excretion was also the principal route of drug elimination in humans for the structurally similar immunosuppressant tacrolimus.[25] As for sirolimus, only trace levels of unchanged tacrolimus were detected in feces after an IV or oral dose in healthy human subjects.

Using ESI positive ion LC/MS SIM and negative ion LC/MS/MS analyses, several demethyl, hydroxy, didemethyl, dihydroxy, and demethylhydroxy sirolimus compounds were detected and characterized in the whole blood of healthy volunteers after single oral-dose administration. The contribution of sirolimus metabolites to total radioactivity in whole-blood was estimated by integration of their respective peaks in the HPLC radiochromatograms. Because not all sirolimus metabolites were separated under the chromatography conditions used, each radioactive peak on the HPLC may have consisted of more than one sirolimus-derived product. During the 1-hour to 24-hour interval after drug administration, the percentage distribution of hydroxy and hydroxydemethyl metabolites increased from approximately 3% to 17%, hydroxy and didemethyl metabolites increased from approximately 5% to 13%, hydroxy and 7-O-demethyl metabolites increased from approximately 7% to 15%, and 41-O-demethylsirolimus ranged from approximately 6% to 12%. Several hydroxy, dihydroxy, demethyl, and didemethyl metabolites of sirolimus were characterized in the whole-blood of stable renal allograft patients after administration of sirolimus, cyclosporine, and corticosteroids.[6,10] Coadministration with cyclosporine and corticosteriods therefore did not appear to qualitatively affect the metabolism of sirolimus.

Sirolimus was metabolized by human liver microsomal CYP3A enzymes,[4,5,7] pig small intestinal microsomes,[26] human intestinal microsomes, and Caco-2 cells expressing CYP3A4[27–29] to various demethyl and hydroxy metabolites. Therefore, CYP3A-mediated metabolism of sirolimus in the liver and small intestine can both contribute to the formation of sirolimus metabolites detected in humans. The macrocyclic ring-opened isomer (*seco*-sirolimus), a degradation product formed in liver microsomes,[6–8] was detected in plasma but not in the whole blood of humans after sirolimus administration. These observations are consistent with the increased stability

of sirolimus in whole blood compared with plasma.[30] A ring-opened dihydro rapamycin metabolite was characterized from incubation of sirolimus in human liver microsomes,[31] and in human liver and intestinal fractions and modified Caco-2 cell monolayers.[27–29] It is not clear whether a similar dihydro sirolimus metabolite is present in human subjects after sirolimus administration. Although a peak in the RIC of *m/z* 933.7 was detected in whole-blood of normal subjects in the present study, this ion can correspond to either a dihydro or a hydroxydemethyl rapamycin metabolite. Sirolimus, *seco*-sirolimus, and the dihydro metabolite were subjected to P-gp-mediated efflux using modified Caco-2 monolayers.[27,29] Extensive CYP3A-catalyzed first-pass metabolism and intestinal P-gp-mediated efflux of sirolimus and its metabolites, therefore, collectively may contribute to the low oral bioavailability of sirolimus (~20%).[14] Because there is no evidence that clinically relevant differences in either intestinal or hepatic CYP3A or P-gp occur in normal subjects versus stable renal graft patients or between the two genders, the pharmacokinetics and metabolic disposition of sirolimus may be expected to be similar between normal and renal transplant subjects and between male and female patients. Hepatic impairment, in addition to affecting biliary clearance, may also be expected to decrease the metabolic clearance of sirolimus, leading to increased systemic drug exposure. Several sirolimus metabolites, including 41-O-demethyl, 7-O-demethyl, and hydroxy sirolimus, were isolated from liver microsomal[4,6] or microbial[19] incubation of sirolimus. The immunosuppressive activities of sirolimus metabolites were several fold lower than that of sirolimus in the thymocyte proliferation assay or mixed lymphocyte reaction.[4,19] Metabolites of sirolimus, based on their relative abundance in whole-blood of humans after sirolimus administration, therefore do not appear to play a major role in the clinical pharmacology of the parent drug.

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