Rifapentine and 14C-rifapentine were synthesized by Hoechst Marion Roussel (Kansas City, MO). Acetonitrile and methanol, HPLC grade, were used as received from Burdick and Jackson (Muskegon, MI). Heptfluorobutyric acid (HFB), ascorbic acid, Trizma® buffer, sulfatase (type VI), β-glucuronidase (type VII-A), p-nitrocatechol sulfate, and phenolphthalein glucuronidic acid were purchased from Sigma Chemical Co. Potassium phosphate (dibasic) was received from Mallinckrodt (Paris, KY). FloScint® III, Ultima Gold®, and Permafluor® E® scintillation fluids were obtained from Packard (Meriden, CT). Human serum was obtained from Valley Biomedical, Inc. (Winchester, VA), human plasma from Rockland, Inc. (Gilbertsville, PA), and human serum albumin and α1-acid glycoprotein from Sigma Chemical Co. (St. Louis, MO).

Dose Formulation. The labeled drug was prepared with 14C in the 3-position (fig. 1). Radioactive doses were prepared by mixing 25 ml alcohol U.S.P. and 10 ml of potassium phosphate buffer (pH 7.4) in vials containing 600 mg (108 μCi; specific activity 0.18 μCi/mg) 14C-rifapentine. The radiochemical purity was 98.41%, and the chemical purity was 97.71%.

Study Design. Four healthy male volunteers between the ages of 18 and 45...
years and within ±10% of ideal body weight participated in this single-dose, open-label investigation. Prior to enrollment, all subjects underwent a drug abuse screen and tested negative for HIV antibodies and hepatitis B antigen. Subjects reported to the study site on the day before study drug administration and remained there until all sampling was complete. At 8:00 a.m. on study day 1, each subject received a single 600-mg dose of the prepared 14C-rifapentine solution orally. Each dosing container was rinsed five times with deionized water and ingested by each subject for a total volume of 243 ml. Subjects were fasted for 10 hr before 14C-rifapentine administration, and for 5 hr after the dose. For the remainder of the study, they received regularly scheduled high fiber–content meals. To ensure complete ingestion of the dose, dosing vials were saved for measurement of residual radioactivity. In all determinations, negligible levels of 14C remained in the vials.

Blood, urine, and feces were collected before and after study drug administration. Whole blood (10 ml) was collected immediately before 14C-rifapentine administration on day 1 and at 2, 4, 5, 6, 8, 10, 12, 18, 24, 36, 48, and 72 hr after dosing. Additional blood samples were collected at 24-hr intervals until two consecutive samples contained less than 3 times the background radioactivity. Plasma was separated from whole blood using centrifugation at 2500 rpm for 10 min. After the complete transfer of plasma, the buffy coat was removed from the collection tube so that red blood cells could be obtained.

Urine was collected before dosing and at intervals of 0–12, 12–24, 24–36, 36–48, 48–60, and 60–72 hr after dosing. Total urine was then collected in 24-hr intervals until two consecutive samples contained less than 3 times the background radioactivity. Plasma was separated from whole blood using centrifugation at 2500 rpm for 10 min. After the complete transfer of plasma, the buffy coat was removed from the collection tube so that red blood cells could be obtained.

Asterisk (*) indicates position of 14C label.

FIG. 1. Proposed metabolic scheme for rifapentine.

Assay of Total Radioactivity. Fecal samples were collected in 2-liter polypropylene jugs and homogenized with 4 ml diluent (75:25, MEOH:H2O, v/v, with 2.5 μg/ml ascorbic acid added as an antioxidant) per g of feces, using the Teckmar SD-45 (Tekmar Corp., Cincinnati, OH) homogenizer. Duplicate aliquots (0.5 ml or 0.5 g) from each matrix sample were prepared and counted by liquid scintillation analysis. Aliquots of urine were measured by volume, and aliquots of plasma, whole blood, red blood cells (RBCs), and feces homogenate were measured by weight. Urine and plasma aliquots were counted with 10 ml of liquid scintillation cocktail (Ultima Gold™). Whole blood, red blood cells, and feces homogenate aliquots were added to ashless Combustipads™ in Combusticones™, dried overnight, and burned with the addition of Combustaid™ using a Packard 307 oxidizer. The 14CO2 was absorbed into 10 ml Carbo-Sorb® E, and after the addition of 10 ml Permafluor® 1, each sample was counted by liquid scintillation analysis.

Radioactivity counting for each sample was performed on a Tri-Carb 1600TR Liquid Scintillation Analyzer (Packard). The observed counts per min were converted to dpm using a previously prepared quench curve. The dpm data were then used to calculate concentration (μg equivalents/g or ml) and percentage of dose recovered in urine and feces.

Metabolite Characterization: Quantitation of Radiolabeled Compounds in Urine and Feces. Feces homogenate samples were extracted with methanol and acetonitrile, each containing 10 μg/ml ascorbic acid. Aliquots (5 ml) of methanol and acetonitrile solutions were added to 3 ml homogenate in test tubes. Tubes were capped, vortexed, and centrifuged. The supernatant from each tube was transferred to a separate tube and dried under vacuum in a SpeedVac (Savant, Holbrook, NY). Aliquots (4 ml) of methanol and acetonitrile solutions were then added to the residual solid. Samples were again vortexed and centrifuged. The supernatant was added to the corresponding tube in the SpeedVac. Samples were evaporated to dryness and reconstituted in 250 μl water and 500 μl methanol. The reconstituted samples were filtered into HPLC vials and assayed by HPLC with visible and radioactivity
detection. Urine samples were centrifuged and directly injected onto the HPLC system.

The HPLC system used to quantitate the radiolabeled compounds included a 600E controller and pump (Waters, Milford, MA), a WISP 712 autoinjector (Waters), a 486 ultraviolet/visible detector (Waters), and a Radiomatic radioflow detector (Packard) with a liquid cell (0.5 ml). The wavelength of the UV/VIS detector was kept at 480 nm. A Zorbax RX-C8 column (250 x 4.6 mm, 5 μm; Mac Mod, Chadds Ford, PA) with a gradient mobile phase was used to separate 14C-rifapentine–related compounds in urine and feces extracts. Mobile phase A was 71/29/0.1 water/acetonitrile/HFB and mobile phase B was 100% A. The flow rates of the mobile phase and scintillation fluid (FloScint) were 1 ml/min and 3 ml/min, respectively. Feces extracts were quantitated by integration of 14C peak areas from the radioflow detector chromatograms. Because of the low concentration of radioactivity in the urine, the radioflow detector was not used for quantitation. Instead, 1-min fractions of column effluent were collected in scintillation vials using a Foxey fraction collector (Isco, Lincoln, NE). Ultima Gold scintillation fluid (10 ml) was added to each urine fraction and counted on a 1900TR scintillation counter (Packard).

Column recovery of feces extract was determined by fraction collection of two feces extracts using the HPLC conditions described above. The sum of the dpm in the fractions was compared with the dpm/ml in the extract injected onto the column. Recovery of urine samples was established by comparison of the sum of dpm in urine fractions with the dpm concentration of the urine injected onto the column. Because column recovery was established, compounds were quantitated by multiplying the percentage of dose in the original sample by the percentage contribution of the peak or fraction to the total chromatogram.

Test for Conjugated Metabolites. To evaluate the presence of conjugates in the feces, feces extract samples were treated with β-glucuronidase. Feces extract samples were pooled and aliquoted (0.3 ml) into test tubes, p-Nitrocatechol sulfate and phenolphthalein glucuronic acid were added to separate tubes as positive controls. Samples were dried under vacuum in a SpeedVac. β-Glucuronidase (5000 units) in phosphate buffer (0.075 M, pH 6.8) was added to dried extract samples including samples with phenolphthalein glucuronic acid. Sulfatase (5 units) in Tris buffer (0.05 M, pH 7.4) was added to another set of dried extracts, including samples with p-nitrocatechol sulfate. Phosphate and Tris buffer were added to selected samples as negative controls. Samples were incubated at 37°C for 18 hr. After incubation, samples for analysis were dried and reconstituted in 0.1 ml water and 0.2 ml methanol (with ascorbic acid). Samples were assayed as described above for feces extracts. Positive control samples were reconstituted in 0.5 ml water and treated with 0.1 M NaOH. The appearance of an orange-red color indicated that the positive control samples had undergone hydrolysis.

Metabolite Characterization. An HPLC/MS system was used to separate and provide molecular weight information on components in fecal and urine samples. The system consisted of a Gibson 231 autosampler, a HPLC pump (Michrom BioResources, Inc.), and a Waters 490-MS Programmable Detector set at 480 nm. The column separation procedure and mobile phase were identical to those previously described for the profiling analysis. A Finnigan MAT TSQ 710 mass spectrophotometer with a scan range of 400-1400 atomic mass units and a scan time of 2 sec per scan was used. Electrospray ionization mode was used to produce the mass spectra. The heated capillary temperature was 150°C, and spray voltage was set at 4500 volts. The flow from the LC column was split 10:1 before entering the mass spectrophotometer.

HPLC Assay for Rifapentine and 25-Desacytetyl-rifapentine in Plasma. An analytic method using HPLC with visible detection was developed by the Hoechst Marion Roussel Bioanalytics Department for the determination of rifapentine and 25-desacyetyl-rifapentine in heparinized human plasma. Using visible detection at a wavelength of 480 nm, the method was validated over a range of 0.5–60 μg/ml for both analytes. A structurally related compound, 25-desacytelrifampin, was used as an internal standard.

Sample preparation consisted of adding 100 μl of plasma sample, 100 μl of methanol (containing drug as appropriate standards), and 500 μl of internal standard in methanol to an autosampler vial. The contents of the vial were mixed to precipitate the plasma proteins, then centrifuged at approximately 3000 rpm for 5 min. The supernatant (20 μl) was directly injected into the HPLC system, without separation of the supernatant, by programming the depth of the autosampler injection needle to avoid contact with the protein pellet. The concentration of rifapentine and 25-desacytetyl-rifapentine in unknown sample was calculated by interpolation from the line of best-fit for calibration standards run simultaneously with unknown samples. The line of best-fit for calibration standards was calculated by weighted (1/x2) quadratic least squares regression based on peak-height ratios. Validation of this procedure demonstrated the method to be reliable, accurate, precise and specific for quantitation of rifapentine and 25-desacytetyl-rifapentine, without interference from the plasma matrix. Using 0.1 ml of plasma, the validated assay had nominal standard curve ranges of 0.5–60 μg/ml and 0.45–54 μg/ml for rifapentine and 25-desacytetyl-rifapentine, respectively.

Data Analysis. Plasma pharmacokinetic parameters for radioactivity, rifapentine, and 25-desacytetyl-rifapentine were calculated from plasma concentration-time data by model-independent methods. Pharmacokinetic parameters estimated from these data included Cmax, tmax, and t1/2. The linear trapezoidal rule was applied to calculate the cumulative area under the plasma concentration-time curve (AUC) from time zero to the last data point (AUC(t→)). AUC from the last data point to infinity (AUC(t→)) was estimated by dividing the last plasma concentration by the terminal elimination rate constant (k). AUC(t→) was the sum of AUC(0→t) and AUC(t→).

Protein Binding. The in vitro protein binding of 14C-rifapentine in human plasma and human serum was determined over the concentration range of 0.5–50 μg/ml. Human plasma and serum spiked with the appropriate concentrations of rifapentine were processed by ultrafiltration (1 ml filters, with molecular weight cut-off of 30,000; Amicon, Beverly, MA). Binding of rifapentine to human serum albumin (HSA) was also determined by ultrafiltration of 10 μg/ml 14C-rifapentine in a 45 mg/ml buffer solution of HSA. Rifapentine binding to α1-acid glycoprotein (AAG) was determined by equilibration dialysis of 10 μg/ml 14C-rifapentine in a buffer solution of 0.7 mg/ml AAG. Equilibrium dialysis was conducted using the Spectrum equilibrium dialyzer (20-cell model, Spectrum Medical Industries, Los Angeles, Califaria); the dialysis membrane had a 12,000–14,000 molecular weight cut-off. Drug concentrations were determined by liquid scintillation counting.

Results

Four healthy male subjects completed the study. All subjects met release criteria on or within 18 days after dosing. Concentrations of rifapentine and 25-desacytetyl-rifapentine in plasma were below the limits of quantitation by 72 hr after dosing. Radioactivity was detectable in whole blood and red blood cells for up to 72 hr, in plasma for up to 192 hr, in urine for up to 168 hr, and in feces for up to 18 days.

Total Radioactivity. Mass balance determination included 14C-rifapentine and 25-desacytetyl-rifapentine represented 99% of the 14C dose at 600 mg oral dose of 14C-rifapentine. Radioactivity was detectable in plasma for 192 hr in three subjects and for 240 hr in one. Rifapentine plus 25-desacytetyl-rifapentine represented 99% of the 14C AUC(t→) in plasma, indicating that 25-desacytetyl-rifapentine is the major metabolite.

Fig. 3 presents the plasma concentration-time profiles for rifapentine, 25-desacytetyl-rifapentine, and total radioactivity in plasma after the 600-mg oral dose of 14C-rifapentine. Radioactivity was detectable in plasma for 192 hr in three subjects and for 240 hr in one. Rifapentine plus 25-desacytetyl-rifapentine represented 99% of the 14C AUC(t→) in plasma, indicating that 25-desacytetyl-rifapentine is the major metabolite.

Fig. 4 presents the profile for mean radioactivity in blood components during the 72-hr period after dosing. Radioactivity appeared rapidly in whole blood and plasma. Maximum radioactivity detected was highest in plasma and lowest in RBCs. The RBC-plasma AUC ratio was 15%, and the whole blood/plasma AUC ratio was 56%. The average time to peak radioactivity for all blood matrices ranged from 4 to 5 hr.

Pharmacokinetics. Key pharmacokinetic parameters for rifapen-
tine and 25-desacetyl-rifapentine are summarized in table 1. Plasma concentrations of the metabolite peaked at a later time (13.25 hr) compared with parent drug (4.25 hr), but both compounds followed the same terminal profile (fig. 3). Disposition profiles of rifapentine and the 25-desacetyl derivative were similar, with mean $t_{1/2}$ of 14.0 and 12.1 hr, respectively. The AUC (0-$\infty$) and the $C_{\text{max}}$ ratios of 25-desacetyl-rifapentine:rifapentine were 64% and 36%, respectively, indicating that 25-desacetyl-rifapentine is a primary metabolite in plasma (table 1).

Metabolite Profiles in Urine and Feces. Recoveries of radioactivity from the HPLC column were 95% and 107% in two fecal extract samples. Urine samples also yielded acceptable column recoveries, averaging 109%.

Fig. 5 is a representative $^{14}$C chromatogram of a feces extract sample obtained from one subject (subject 3) 3 days after the oral rifapentine dose was given. Most radiochromatograms of feces extracts consisted of eight distinct peaks and one region of low-level radioactivity eluting between 10 and 22 min that may represent many different compounds. This area between 10 and 22 min in the LC/$^{14}$C chromatogram was integrated and labeled F3. Of the nine peaks, F1, F5, F6, F8, and F9 accounted for most of the radioactivity. Initial assignments of peaks were made by matching LC/$^{14}$C/UV retention times with standards. Separate LC/MS analyses were subsequently performed to verify some of the assignments. Results of the peak assignments based on LC/MS were consistent with results noted in the companion animal studies (Emary et al., 1998). LC/MS analysis provided molecular weight information for peaks F5, F6, F8, and F9, which were consistent with structures for 25-desacetyl-rifapentine, rifapentine, 3-formyl-25-desacetyl-rifapentine, and 3-formyl rifapentine, respectively. In addition, HPLC retention times for F5, F6, and F9 were congruent with standards for 25-desacetyl-rifapentine, rifapentine, and 3-formyl rifapentine, respectively. The proposed structures for these compounds are illustrated in fig. 1.

Attempts were made to identify unique masses for the less retained components of the feces extract sample, particularly the large peak at approximately 4-min retention (F1). None of the mass spectra at this retention time were distinguishable, except in overall intensity from the predose sample. Therefore, no molecular weight information was obtained for peaks F1 or F2. It is important to note that rifapentine and rifapentine analogs give a relatively poor response by LC/MS. The molecular ion intensity of rifapentine is approximately 100 times less than that of the typical pharmaceutical compounds we study. In an effort to improve MS response, different mobile phase buffers and pH conditions were tested but did not yield any improvements. Treatment of feces samples with $b$-glucuronidase and sulfatase did not result in the disappearance of either F1 or F2 peaks, which indicates that these polar peaks do not consist of glucuronide- or sulfate-conjugated metabolites.

Urine compounds were profiled by reconstruction of histograms from fraction collection data. Peak assignments were made based upon co-elution of reference standards and correlation with peaks in $^{14}$C chromatograms of feces samples. Retention times for U1 through U9 were consistent with F1 through F9 because the same chromatographic conditions were used for urine and feces samples.

Table 2 lists the quantitative results for radioactive components in urine and feces. An average of 92% of the excreted radioactivity was quantitated as $^{14}$C chromatographic peaks, and 50% of the excreted radioactivity was structurally characterized. These characterized compounds found in feces and urine were rifapentine, 25-desacetyl-rifapentine, 3-formyl-25-desacetyl-rifapentine, and 3-formyl rifapentine. In urine, the 25-desacetyl metabolite was the most abundant compound, contributing 54% to the profiled radioactivity in urine. In
feces, 25-desacetyl-rifapentine was also the most abundant compound, contributing 22% to the profiled radioactivity in feces.

**Protein Binding.** The in vitro protein binding characteristics of 14C-rifapentine in human serum and plasma were evaluated. The percentage of protein-bound 14C-rifapentine ranged from 97.0% to 99.0% in human serum and 96.5% to 98.8% in human plasma. The bound concentration increased slightly (<2%) as the concentration of 14C-rifapentine increased from 0.5 to 10 μg/ml. At concentrations above 10 μg/ml, protein binding did not appear to vary with increasing concentration. The binding of 14C-rifapentine (10 μg/ml) to HSA (45 mg/kg) and AAG (0.7 mg/ml) was 92.5 ± 0.12% and 15.1 ± 3.50%, respectively. In a subsequent in vivo study (unpublished data), the mean percentages of protein-bound rifapentine and 25-desacetyl-rifapentine in plasma obtained from healthy volunteers were 97.7% and 93.1%, respectively.

**Renal Clearance.** The average amount of rifapentine recovered unchanged in the urine was approximately 6 mg. Renal clearance of rifapentine was estimated to be approximately 0.285 ml/min, which was less than glomerular filtration rate of the free fraction (120 ml/min × 3.23% = 2.76 ml/min). These data suggest rifapentine was reabsorbed in the renal tubules. Similarly, approximately 38 mg of 25-desacetyl-rifapentine was recovered in the urine and CLR was estimated to be approximately 2.56 ml/min. Renal clearance of 25-desacetyl-rifapentine was also less than glomerular filtration rate of the free fraction (120 ml/min × 6.8% = 8.18 ml/min), suggesting that the 25-desacetyl metabolite was also reabsorbed in the renal tubules.

**Discussion**

Fecal excretion is the primary route of rifapentine elimination in humans. A total of 86.6% of the oral 14C-rifapentine dose was recovered within 18 days after ingestion in healthy volunteers, with most (70.2% of dose) found in the feces. In comparison, 76%, 100%,
and 85% of dose was recovered as excreted radioactivity in feces of mice, rats, and monkeys, respectively, after single oral doses of 14C-rifapentine (Emary et al., 1998). In bile-cannulated rats, biliary excretion was the major excretory pathway (approximately 51% of total radioactivity excretion) after iv 14C-rifapentine doses (Emary et al., 1998). In humans, rifapentin is also eliminated primarily through excretion into bile, with only about 8 to 24% of an oral rifapin dose excreted by urine (Acocella, 1978; Loos et al., 1985). In contrast, about half of the radioactivity recovered after a single oral 270 mg dose of 14C-rifabutin in healthy volunteers was found in urine, and only about 30% of the dose was recovered in feces (Battaglia et al., 1990).

Metabolic profiling of feces, urine, and plasma after the oral doses of radioactive rifapentine in this study identified four primary components: parent drug, the active 25-desacetyl metabolite, and the 3-formyl derivatives of the parent drug and 25-desacetyl metabolite. These same four components were identified in the mouse, rat, and monkey studies (Emary et al., 1998). Although the biotransformation pathways for rifapentine appear to be similar in animals and humans, the ratio of components in the various excreta differ. In man, 25-desacetyl-rifapentine was the most abundant compound in plasma and bile is 25-desacetyl-rifapentine, M. tuberculosis

Fig. 6 outlines a proposed scheme for absorption and disposition of rifapentine in humans that we believe best represents the data currently available. We postulate that after rifapentine is absorbed from the gastrointestinal tract, it can be metabolized in the liver to 25-desacetyl-rifapentine in humans. Rifapentine desacetylation may also occur in other tissues, including the blood. Rifapentine and 25-desacetyl-rifapentine account for virtually all (99%) of the circulating radioactivity in humans. Biliary excretion, gastrointestinal secretion, metabolism, and renal clearance all contribute to the elimination of rifapentine from the systemic circulation, based upon the human and animal data (Emary et al., 1998). Most of the oral dose is eliminated in feces (70.2%) and a minor percentage (16.6%) is eliminated by the kidney. In the gastrointestinal and urinary tracts, rifapentine and 25-desacetyl-rifapentine undergo nonenzymatic degradation to formyl derivatives and rifapentine may undergo desacetylation.

The disposal scheme outlined for rifapentine parallels that previously described for its homologue, rifampin (Acocella, 1978; Loos et al., 1985). Similar to rifapentine and rifampin, a primary metabolite of rifabutin is the 25-desacetyl derivative. However, in addition to desacetylation, rifabutin undergoes further metabolism through a number of different pathways to more than 20 different compounds (Cocchiara et al., 1989). Two of these metabolic pathways in man include oxidation on a methyl group of the ansa chain to produce 31-OH-rifabutin, 32-OH-rifabutin, and 32-OH-25-O-desacetyl-rifabutin, and oxidation at the piperidine nitrogen to produce 25-O-desacetyl-rifabutin-N-oxide (Cocchiara et al., 1989).

Although a total of 36% of the dose after rifapentine administration represented unidentified chromatographic peaks, it is unlikely that these byproducts were formed by oxidation. In an unpublished in vitro study, rifapentine and 14C-rifapentine were incubated in human S9 fraction with and without the presence of cofactors. A small peak of 25-desacetyl-rifapentine was found (3%–4% area by LC/UV or LC/14C) demonstrating that rifapentine was metabolized in S9 to a small degree. However, neither the percentage of 25-desacetyl-rifapentine formed nor the mean peak area ratio of rifapentine to 25-desacetyl-rifapentine changed in the presence of cofactors, which indicates the formation of the decacylated metabolite is not cytochrome P450-dependent. The metabolism of 25-desacetyl-rifapentine was also evaluated in this study. After incubation of the 25-desacetyl derivative in human S9 microsomes with and without the addition of cofactors, 25-desacetyl-rifapin was added as an internal standard. The mean peak area ratios (by LC/UV) changed little with the addition of cofactors, offering no evidence of metabolism of 25-desacetyl-rifapentine by S9.

In summary, rifapentine, a rifamycin antibiotic with activity against M. tuberculosis, is excreted primarily in feces in man. The primary metabolite found in plasma and bile is 25-desacetyl-rifapentine, formed by an esterase found in many tissues. The other degradation products found in feces and urine are the 3-formyl derivatives, formed by nonenzymatic hydrolysis. In contrast to rifabutin, no oxidative metabolic pathways have been identified for rifapentine.

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References


