Absorption and Disposition of the Sphingosine 1-Phosphate Receptor Modulator Fingolimod (FTY720) in Healthy Volunteers: A Case of Xenobiotic Biotransformation Following Endogenous Metabolic Pathways

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Running title:
Absorption and disposition of fingolimod in man

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Number of text pages: 31
Number of tables: 4
Number of figures: 6
Number of references: 26
Number of words in abstract: 206
Number of words in introduction: 313
Number of words in discussion: 1374

Abbreviations:
AUC, area under the concentration-time curve; C_{max}, maximum concentration; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry.
Abstract

Fingolimod, a new drug for the treatment of relapsing multiple sclerosis, acts through its phosphate metabolite which modulates sphingosine 1-phosphate receptors. This represents a novel mechanism of action. In the present work, the absorption and disposition of \(^{14}\)C-labeled fingolimod was investigated in healthy male volunteers after a single oral dose of 4.5 mg. Total radioactivity was determined in blood, urine, and feces. Fingolimod was quantified in blood. Metabolite profiles were determined in blood and excreta, and metabolite structures were elucidated by mass spectrometry, wet-chemical methods, and comparison with reference compounds. Fingolimod was absorbed slowly but almost completely. The biotransformation of fingolimod involved three main pathways: (i) reversible phosphorylation to fingolimod phosphate ((S)-enantiomer, active principle), (ii) \(\omega\)-hydroxylation at the octyl chain, catalyzed predominantly by CYP4F enzymes, followed by further oxidation to a carboxylic acid and subsequent \(\beta\)-oxidation, and (iii) formation of ceramide analogs by conjugation with endogenous fatty acids. This metabolism is quite unusual as it follows metabolic pathways of structurally related endogenous compounds rather than biotransformations typical for xenobiotics. The elimination of fingolimod was slow and occurred predominantly by oxidative metabolism while fingolimod phosphate was eliminated mainly by dephosphorylation back to fingolimod. Drug-related material was excreted mostly in the urine in the form of oxidation products.
Introduction

Fingolimod (FTY720, Gilenya™) is a new chemical entity recently approved in the US as a therapy for relapsing multiple sclerosis (Kappos et al., 2006; Brown et al., 2007). The compound is metabolically phosphorylated to form (S)-configured fingolimod phosphate. The latter is a potent agonist at different sphingosine 1-phosphate receptors, including those of type 1, while the (R)-enantiomer, which was not observed as a metabolite, and fingolimod itself show low or negligible activity at these receptors (Hale et al., 2004; Albert et al., 2005). Activation of type 1 sphingosine 1-phosphate receptors on lymphocytes by (S)-fingolimod phosphate results in a suppression of the egress of lymphocytes from lymph nodes and a prevention of their re-circulation to other organs, including the central nervous system (Brinkmann, 2007; Chiba, 2005). Hence, fingolimod exhibits a novel mechanism of action. The compound does not impair T- or B-lymphocyte activation, proliferation, or effector functions at clinically relevant concentrations (Pinschewer et al., 2000). Fingolimod may also have direct effects in the central nervous system because sphingosine 1-phosphate receptors are present in this tissue (Bryan et al., 2008) and (S)-fingolimod phosphate can be assumed to be present as well, based on results in the rat (Foster et al., 2007; Novartis, unpublished data).

The pharmacokinetics of fingolimod and fingolimod phosphate in man have been described previously (Kovarik et al., 2007a,b). Two metabolites of fingolimod other than its phosphate have been quantified in clinical studies (e.g. Kovarik et al., 2005; Kovarik et al., 2006; Kovarik et al., 2007b). However, a full description of the biotransformation of the compound has not been published. In this communication we present the results of an absorption, distribution, metabolism, and excretion study in healthy male volunteers following a single oral dose of 4.5 mg ¹⁴C-labeled fingolimod. Complementary data on the oxidative metabolism of fingolimod
in vitro and the involved enzymes are presented in an accompanying publication (Jin et al., 2010).
Methods

Study Drug. [14C]Fingolimod hydrochloride ([2-14C]-2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride) with a specific radioactivity of 432 kBq/mg (referring to the hydrochloride salt; 149 MBq/mmol) and a radiochemical purity of >98% was synthesized by the Isotope Laboratory of Novartis, Basel, Switzerland. The structure of the compound and the position of the label are shown in Fig. 1.

Chemicals and Standards. Authentic standards of the metabolites M1 to M4 were obtained from Yoshitomi Pharmaceutical Industries Ltd. (Tokyo, Japan). Authentic standards of the metabolites M29 and M30, unlabeled and 14C-labeled (S)-fingolimod phosphate as well as (1,1,3,3-2H4)fingolimod, used as internal standard for the quantitation of fingolimod in blood, were synthesized at Novartis, Basel, Switzerland. All other chemicals and solvents were of analytical grade and from commercial sources.

Subjects and Study Design. The clinical part of the study was performed at Swiss Pharma Contract Ltd. (Allschwil, Switzerland) in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki (1964 and subsequent revisions). All subjects gave written informed consent before entering the study. The protocol and the radiosafety assessment were approved by the Ethics Committee of the Two Basel, Switzerland, and the Swiss Federal Office of Public Health (Radioprotection Division, Bern, Switzerland), respectively.

Four healthy male subjects participated in this open-label single oral dose absorption, distribution, metabolism, and excretion study. They were of good health as determined by past medical history, physical examination, vital signs, electrocardiogram, and laboratory tests. The subjects were non-smokers, had no history of alcoholism or drug abuse, and did not use any prescription drug, over-the-counter drug, or herbal medication between 2 weeks prior to dosing
and 5 weeks after dosing, except for medication required to treat adverse events. The subjects had to fast for at least 10 h prior to dosing and to continue fasting for at least 4 h thereafter.

Each subject received a single oral dose of 5 mg [\textsubscript{14}C]\textit{fingolimod hydrochloride} (4.5 mg referring to free base), formulated as a drinking solution. The radioactivity dose was 2.16 MBq. The whole body radiation dose was below 1.0 mSv.

Blood was collected at predose, 1, 2, 4, 6, 8, 12, 16, 24, 36, 48, 72, 96, 120, 144, 168, 192, 216, 240, 312, 480, and 816 h postdose into EDTA-containing vacuum tubes by direct venipuncture or by an indwelling cannula inserted in a forearm vein. Up to 72 h, blood volumes of 23 ml were taken. At later times, the sampling volume was reduced to 5 ml. After aliquoting for the different assays, the blood samples were frozen and stored at approximately -80°C until analysis.

All urine was collected in time-fractions of 0-6 h, 6-12 h, 12-24 h, and thereafter in 24-h fractions up to 240 h. In addition, all urine was collected during Day 13 (288-312 h), Day 20 (456-480 h), and Day 34 (792-816 h). During the collection periods, the urine was kept refrigerated at 4-8°C. All feces were collected after dose administration up to 240 h postdose. Urine and feces samples were stored at ≤ -20°C until analysis.

Aliquots of blood, urine, and feces were assayed for radioactivity by liquid scintillation counting. These analyses were performed by RCC Ltd. (Itingen, Switzerland). Blood concentrations of fingolimod were determined by a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay at Novartis. Metabolite profiles in blood, urine, and feces were obtained by high-performance liquid chromatography (HPLC) with radioactivity detection, and metabolite structures were characterized by mass spectrometry, wet-chemical methods, and comparison with reference compounds at Novartis. Details are given below.
Safety assessments included the monitoring and recording of all adverse events, regular checks of routine blood chemistry, hematology, and urine values, electrocardiogram recordings, measurements of vital signs, and physical examinations.

**Liquid Scintillation Counting.** Blood samples were analyzed in triplicates (300 µl each), feces (homogenates in two volumes of water) were analyzed in quadruplicates (200-1000 µl each), and urine samples were analyzed in duplicates (1000 µl each). Blood and feces samples were solubilized by standard procedures and mixed with Hionic Fluor liquid scintillation cocktail (Packard, Meriden, CT, USA). Urine samples were measured directly after addition of Insta Gel Plus (Packard). The measurements were performed on a Packard Tri-Carb 2500 TR liquid scintillation counter or a Packard 2550 TR/LL low-level counter with counting times of 10-30 min for blood, 10 min for urine, and 10 min for feces. The long counting times of 30 min were used to improve the counting statistics in case of blood samples containing low levels of radioactivity (20-100 dpm/ml). Quench correction was performed by an external standard method.

**Determination of Fingolimod Concentrations in Blood.** Fingolimod in blood was quantified by LC-MS/MS after liquid-liquid extraction. Aliquots of 0.5 ml were spiked with 100 µl of internal standard solution, containing 4 ng of (1,1,3,3-²H₄)fingolimod, alkalinized with 0.5 ml of 100 mM aqueous NaOH, and extracted with 5 ml of a 75:25 (v/v) mixture of tert-butyl methyl ether and dichloromethane. The samples were shaken for 30 min and centrifuged. The organic layer was removed, evaporated to dryness, and re-constituted in 200 µl of a 1:1 (v/v) mixture of 20 mM aqueous ammonium acetate and methanol. A 100 µl aliquot of the final extract was injected onto a 4.6 x 50 mm Symmetry Shield RP8 column from Waters (3.5 µm particles; Milford, MA, USA), protected by a 3.9 x 20 mm pre-column of the same stationary phase. The components were eluted with a gradient of 20 mM aqueous ammonium acetate versus methanol.
at 40°C using a total flow rate of 2 ml/min. The eluent was directed into the atmospheric pressure chemical ionization source of a Finnigan (San Jose, CA, USA) TSQ7000 tandem quadrupole mass spectrometer, operated in the multiple reaction monitoring mode. For fingolimod and the internal standard, the transitions from $m/z$ 308 ([M+H]$^+$) to $m/z$ 255 and from $m/z$ 312 ([M+H]$^+$) to $m/z$ 259 were monitored, respectively. The lower limit of quantitation was 0.26 pmol/ml (0.080 ng/ml).

**Determination of Metabolite Profiles in Blood, Urine, and Feces.** For metabolite profiling in blood, either individual samples or pools across the subjects of samples taken at the same time postdose were analyzed, depending on the available sample volumes and the concentrations of radioactivity. Each blood sample was extracted three or four times with methanol. The methanolic extracts were combined and evaporated to dryness. The residue was re-constituted sequentially in four different solvents of decreasing polarity. The first re-constitution was performed with methanol/acetonitrile/water 7.5:7.5:85 (v/v) containing 0.5% (v/v) of formic acid. After sonication and centrifugation, the supernatant (extract A) was removed. The pellet was re-suspended by sonication in methanol/acetonitrile/water 25:25:50 (v/v) containing 0.5% (v/v) of formic acid. After centrifugation, the supernatant (extract B) was removed. The remaining pellet was re-constituted two more times in the same way, using methanol/water 70:30 (v/v) (extract C) or methanol/water 90:10 (v/v) (extract D), both acidified with 0.5% (v/v) of formic acid. The extracts A to D were analyzed all together in one HPLC run after injecting them sequentially onto the column in the order D-C-B-A. By this procedure, both polar and moderately non-polar components were dissolved and concentrated on the head of the HPLC column before the start of the elution. As the pellet remaining after the forth re-constitution (pellet D) still contained a significant amount of radioactivity, especially at later times after dosing, this pellet was re-constituted again by sonication in a purely organic solvent (dimethyl sulfoxide/methanol 1:4,
v/v). After centrifugation, the supernatant was removed and subjected to a separate HPLC analysis. This fraction of fingolimod-related material is called "nonpolar fraction" while the fingolimod-related material in extracts A to D is called "polar fraction".

Urine of each subject was pooled across the collection period of 0 to 240 h. To an aliquot of each pool, 5% (v/v) of methanol/acetonitrile 1:1 (v/v) was added, and the pH was adjusted to 2.6 with formic acid. Following centrifugation, the supernatant was analyzed by HPLC.

Feces homogenates of each subject were pooled across the collection period of 0 to 240 h. An aliquot of each pool was extracted twice with acidified methanol (0.017 mol/l hydrochloric acid) and once with pure methanol. Preliminary tests had shown that the addition of acid increased the recovery of radioactivity. The combined methanolic extracts were evaporated to dryness. The residue was re-constituted in a similar way as described above for the blood samples. Three different solvents were used for this purpose: methanol/acetonitrile/water 7.5:7.5:85 (v/v; extract A), methanol/acetonitrile/water 25:25:50 (v/v; extract B), and methanol/water 70:30 (v/v; extract C), all acidified with formic acid (0.15-0.5%, v/v). The extracts A to C were analyzed all together in one HPLC run after sequential injection onto the column in the order C-B-A.

The stability of fingolimod and (S)-fingolimod phosphate during the blood and feces preparation and HPLC analysis was investigated using blank blood and feces spiked with [14C]fingolimod or [14C](S)-fingolimod phosphate, respectively. No degradation of either compound was observed.

The chromatography was performed on an HP 1090 (Hewlett-Packard, Palo Alto, CA, USA) or an Agilent 1200 liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA). The components were separated at 40°C on a 150 x 4.6 mm Advantage Armor C18 column (5 µm particles; Analytical Sales and Services, Pompton Plains, NJ, USA), protected by a 40 x 4.6 mm pre-column of the same stationary phase. For the elution, a gradient of 0.5% (v/v) formic acid in
water (pH 2.5; mobile phase A) versus 0.5% (v/v) formic acid in methanol/acetonitrile 1:1 (v/v; mobile phase B) was used. The proportion of the mobile phase B was kept at 5% till 10 min after injection and was then increased linearly to 100% at 55 min where it was kept for another 10 min (polar blood fractions, urine, feces) or 45 min (nonpolar blood fractions). The total flow rate was 1.2 ml/min. Radioactivity was detected by off-line solid scintillation counting. For this purpose, the column effluent was collected in 0.15 min (180 µl) fractions on yttrium silicate scintillator-coated 96-well plates (Deepwell LumaPlates; Packard BioScience, Groningen, The Netherlands) using a 222 XL Liquid Handler from Gilson (Villiers-le-Bel, France). Strongly colored fractions, obtained after injection of blood extracts, were bleached by treatment with 20 µl of 30% (w/v) aqueous H₂O₂ for 30 min at room temperature. The solvents were removed in a vacuum centrifuge and the dry plates were measured in a TopCount NXT microplate scintillation counter (Packard).

Concentrations in blood and amounts in excreta of fingolimod and its metabolites were estimated from the radiochromatograms, based on the relative peak areas and the concentrations or amounts of radioactivity in the original biological samples, reduced by the losses during sample preparation and chromatography.

**Structural Characterization of Metabolites.** The structures of the polar fingolimod-related components were characterized by liquid chromatography-mass spectrometry (LC-MS), nano-LC-MS/MS, and comparison with the available authentic standards (M1, M2, M3, M4, and (S)-fingolimod phosphate). The nonpolar metabolites M27, M28, M29, and M30 were assigned by comparison of retention times with metabolites identified in animals. In addition, metabolite M22 and the nonpolar metabolites M27 to M30 were investigated by wet-chemical methods.

Briefly, the polar metabolites were isolated from blood, urine, and feces by HPLC using similar sample preparation procedures and the same HPLC method as described above for the
metabolite profiling. LC-MS analysis (single-stage mass separation) of the isolated metabolites (or parent compound) was performed on a Micromass (Wythenshawe, UK) Q-Tof 2 hybrid quadrupole time-of-flight mass spectrometer using electrospray ionization in the positive ion mode. For the chromatographic separation, a Waters 150 x 1.0 mm Symmetry C18 column (3.5 µm particle size) and a gradient of 0.5% (v/v) formic acid in water vs. acetonitrile was used. Product ion spectra of the [M+H] + ions of the isolated components were obtained by nano-LC-MS/MS analysis on the same instrument, using a Waters 150 mm x 75 µm Symmetry C18 column (3.5 µm particle size) and the same mobile phases as for LC-MS.

To check whether metabolite M22 might be an acyl glucuronide, its stability in alkaline solution was investigated. For this purpose, the metabolite was isolated from urine by HPLC and incubated for 2 h at room temperature in a 1:2 (v/v) mixture of methanol and 0.4 M aqueous ammonium acetate buffer of pH 10, followed by HPLC analysis.

For obtaining further structural information on the nonpolar metabolites M27 to M30 and additional nonpolar trace components, these metabolites were subjected to alkaline hydrolysis, followed by analysis of the products. To this end, the nonpolar metabolites were isolated from a representative blood sample (36 h blood pool across all four subjects) all together in one HPLC fraction. The pool of nonpolar metabolites was subjected to hydrolysis in a 4:1 (v/v) mixture of ethanol and 2.5 M aqueous KOH for 2 h at 90°C, followed by neutralization and HPLC analysis.

**Pharmacokinetic Evaluations.** Pharmacokinetic parameters were calculated from blood concentration-time data using non-compartmental methods. Areas under the concentration-time curve (AUC values) were determined by the linear trapezoidal method and extrapolated to infinity using the equation $\text{AUC}_{0-\infty} = C_t \cdot t_{1/2} / \ln 2$, where $C_t$ is the last observed concentration above the lower limit of quantitation (fingolimod) or limit of detection (metabolites). The $t_{1/2}$ values were calculated by log-linear regression of blood concentrations vs. time across the
terminal elimination phase. The amounts of radioactivity excreted in urine or feces between the end of consecutive sampling and infinity were estimated by dividing the excretion rate during the last of the consecutive sampling periods by the rate constant of the urinary or fecal excretion, respectively. The latter was obtained by log-linear regression of the urinary or fecal excretion rate of radioactivity vs. time.
Results

Demographic, Safety, and Tolerability Data. Four healthy, male Caucasian subjects were enrolled and completed the study. They were of a mean age of 40 years (range 35 - 45 years), of a height of 184 ± 5 cm (mean ± S.D.; range 179 - 191 cm) and weighed 82.0 ± 4.7 kg (mean ± S.D.; range 75.1 - 85.2 kg). Fingolimod was generally well tolerated. However, one subject experienced severe sinus bradycardia, starting at 4 h postdose, associated with temporary hypotension and syncope, while no abnormalities were observed in the twelve-lead ECG evaluation other than sinus bradycardia. After treatment with atropine (twice 0.5 mg), the subject's heart rate recovered to baseline conditions at approximately 24 h postdose. Another subject experienced moderate fatigue of 8.5 h duration during the first day after the administration of fingolimod. No concomitant medication was administered in this case. The safety laboratory parameters showed no clinically significant abnormalities or changes in any of the four subjects that would suggest a relationship to the study drug.

Blood Concentrations of Radioactivity, Fingolimod, and Fingolimod Phosphate. The blood concentrations of total radiolabeled components (radioactivity) and fingolimod increased slowly after dosing, reaching a first maximum around 12 h, followed by a shallow dip and a second maximum at 36 h. Thereafter, the concentrations decreased with estimated terminal half-lives of 382 h (15.9 days) and 137 h (5.7 days) for radioactivity and fingolimod, respectively. The blood concentrations of fingolimod phosphate, derived from the metabolite profiles, reached their maximum at 12 h and decreased thereafter with a terminal half-life of about 166 h (6.9 days), hence approximately in parallel to fingolimod (Tables 1 and 2; Fig. 2). About 15% of the AUC$_{0-\infty}$ of radioactivity accounted for fingolimod, 8% for fingolimod phosphate while the rest was due to metabolites other than fingolimod phosphate (see below).
Metabolite Structures. The metabolites M1 to M4 and fingolimod phosphate as well as fingolimod itself were assigned to peaks in the radiochromatograms of blood, urine, and feces (Fig. 3) by comparison of their retention times with those of the respective authentic standards. In addition, the mass spectra of M1 to M4, fingolimod phosphate, and fingolimod, obtained after isolation of the compounds from biological samples, matched those of the respective standards. Examples of mass spectra are shown in Fig. 4 A to C; structures of the metabolites are given in Fig. 5.

Metabolite M22 was identified as a glucuronide of metabolite M3, based on the product ion mass spectrum of its [M+H]^+ ion, obtained by nano-LC-MS/MS. Protonated M22 at m/z 458 underwent a prominent neutral loss of 176 mass units (anhydroglucuronic acid), typical for glucuronides, resulting in a fragment at m/z 282. The latter underwent further fragmentation very similar to protonated metabolite M3 (data not shown). The structure of M3 contains four potential sites of glucuronidation: two hydroxy groups, a primary amino group, and a carboxyl group. An acyl glucuronide structure could be excluded because isolated M22 was stable in alkaline solution (pH 10, 2 h at room temperature). The remaining possibilities could not be differentiated on the basis of the available data.

The structures of the nonpolar metabolites M27 to M30 shown in Fig. 5 were assigned primarily on the basis of retention times, in comparison with metabolites previously characterized by mass spectrometry and comparison with reference compounds (M29, M30) in animals (Novartis, unpublished data). As an example, the product ion spectrum of protonated M30, obtained after isolation from monkey blood, is shown in Fig. 4 D. All four metabolites are proposed to be products of acylations with endogenous fatty acids at the amino group of fingolimod, i.e. analogs of endogenous ceramides. M27 is proposed to be an amide of an octadecadienoic acid (possibly linoleic acid), M28 an amide of palmitic acid, M29 an amide of
2-hydroxystearic acid, and M30 an amide of stearic acid. The low concentrations of these metabolites in human blood did not allow to characterize them by mass spectrometry. To further support the structures of M27 to M30 in human blood, the four metabolites were isolated by HPLC all together in one fraction (including additional nonpolar trace metabolites), subjected to alkaline hydrolysis as described in the experimental part, and re-analyzed by HPLC. The radiochromatogram showed three peaks: a front-peak (natural radioactivity of \( ^{40}\text{K}^+ \) in the KOH used for hydrolysis), \([^{14}\text{C}]\)fingolimod, and a trace of \([^{14}\text{C}]\)fingolimod phosphate (resulting from trace metabolites other than M27 to M30). Fingolimod phosphate was stable under the hydrolysis conditions (data not shown). The alkaline hydrolysis of M27 to M30 to fingolimod is fully in line with the proposed structures of the four metabolites.

In contrast to fingolimod, its phosphate has an asymmetric center and can, therefore, exist as one of two enantiomers or a mixture of the two. As described previously (Albert et al., 2005), only the \((S)\)-enantiomer was detected in human blood, both at early (12 h) and later times after dosing (72 h). The \((R)\)-enantiomer was not detected (limit of detection: 3% of total fingolimod phosphate).

**Metabolite Profiles in Blood, Urine, and Feces.** Fingolimod-related components in blood covered a wide range of polarities and had, therefore, to be analyzed in two fractions: a polar fraction containing the major part and a nonpolar fraction containing a minor part of the total radioactivity. Some compounds were observed in both fractions. Attempts to analyze the two fractions in a single HPLC run resulted in poor chromatographic peak-shapes and were not pursued. Because of limitations in sample size, blood had to be pooled across subjects for some of the analyses. The recovery of radioactivity after sample preparation was 87-98% for blood sampled between 12 h and 480 h (sum of polar and nonpolar fractions) but dropped to 63% at 816 h postdose. All radioactivity injected onto the HPLC column was recovered in the column
effluent. The polar fraction contained fingolimod, fingolimod phosphate, and M3 as the major components while M2 and the nonpolar ceramide analogs M28, M29, and M30 were minor. The latter were recovered predominantly in the nonpolar fraction, together with M27. Numerous polar and nonpolar trace components of unknown structure, eluting mainly between 0 and 7 min and between 60 and 85 min, respectively, were observed as well. Quantitative data are given in Table 2, and examples of radiochromatograms are shown in Fig. 3 A to D. In line with the results of the quantitative LC-MS/MS analysis, the blood concentrations of fingolimod derived from the metabolite profiles showed essentially a plateau between 12 h and 36 h and decreased slowly thereafter. The concentrations of M2, M3, and fingolimod phosphate maximized at 12 h, followed by a slow decrease. After 36 h postdose, the concentrations of fingolimod, M2, M3, and fingolimod phosphate declined approximately in parallel. The ceramide analogs M27 to M30 showed very shallow time profiles. Between 480 h and 816 h postdose, M29 and M30 decreased very slowly with apparent half-lives of approximately 400 h (17 days), close to the half-life of radioactivity and longer than the half-lives of fingolimod and the more polar metabolites. The two minor ceramide analogs M27 and M28 were detected only sporadically.

Metabolite profiles in the 0-240 h urine and feces pools were analyzed for each of the four subjects individually. The recovery of radioactivity after sample preparation was 100% in case of urine and 63% in case of feces. The recovery of radioactivity in the HPLC effluent, relative to the amount injected, was 100% for urine and 94% for feces. Quantitative data on the fingolimod-related components in urine and feces are given in Table 3; Fig. 3 E and F show examples of radiochromatograms. In urine, M3 was the major metabolite, accompanied by M2 and small amounts of M4 and M22. Other, unidentified metabolites were present in traces only. Neither fingolimod, nor fingolimod phosphate nor the ceramide analogs M27 to M30 were detected in urine. The radiolabeled material in the feces extracts consisted mainly of fingolimod and
fingolimod phosphate with smaller contributions of M1, M2, M3, and a few unidentified metabolites of very low abundance. The ceramide analogs M27 to M30 were not detected in the feces extracts.

**Excretion of Radioactivity in Urine and Feces.** Urine and feces were collected quantitatively between 0 h and 240 h postdose. Additional urine fractions were collected during the time periods 288-312 h, 456-480 h, and 792-816 h. All excreta portions were assayed for radioactivity (Table 4). About one third of the administered radioactivity appeared in urine and feces within the first three days after administration. After 10 days, on average 62% of the dose was recovered in the excreta: 52% in urine and 10% in feces. Small amounts of radioactivity in the additional urine fractions, collected after 240 h, indicated that the excretion of radioactivity was still continuing. The excretion rate of radioactivity in urine between 96 h and 816 h and in feces between 96 h and 240 h decreased close to mono-exponentially with apparent half-lives of about 173 h (7.2 days) in urine and about 65 h (2.7 days) in feces (Fig. 6). Based on these values, the urinary and fecal excretion of radioactivity between time zero and infinity was estimated at 81% and 11% of dose, respectively.
Discussion

Fingolimod was absorbed slowly after oral administration. The blood concentrations showed maxima at 12 h and 36 h with a small dip in between, followed by a slow decrease (Fig. 2). Similar time profiles had been found previously (Budde et al., 2002; Kovarik et al., 2004). The extent of absorption was estimated ≥ 84% of dose, based on the amount of radioactivity excreted in urine (81% of dose; Table 4) and the amount of metabolites excreted in feces (≥ 3% of dose; Table 3). The absolute bioavailability (F) could not be determined in the present study but can be assumed to be high (Kovarik et al., 2007a). The low apparent clearance of fingolimod (CL/F, 8.7 ± 3.7 l/h), its high apparent terminal volume of distribution (Vₜ/F, 1509 ± 225 l), and its long terminal half-life (137 ± 55 h; Table 1) are in line with data from previous studies (Kovarik et al., 2004; Kovarik et al., 2006).

The structures of the fingolimod metabolites in blood and excreta indicate three biotransformation pathways: (i) formation of (S)-fingolimod phosphate, (ii) ω-hydroxylation at the octyl chain (metabolite M12, not observed in vivo), followed by rapid further oxidation to the carboxylic acid metabolite M1 and subsequent β-oxidation, and (iii) formation of nonpolar ceramide analogs (Fig. 5). In addition, a glucuronide of one of the β-oxidation products was observed as a trace metabolite in urine.

Fingolimod phosphate has been demonstrated to be the active principle (Brinkmann et al., 2002; Mandala et al., 2002) while fingolimod itself showed negligible pharmacological activity. Of the two enantiomers of fingolimod phosphate, the (S)-form is pharmacologically active while the (R)-form shows only marginal activity as an agonist at sphingosine 1-phosphate receptors or as an inhibitor of lymphocyte migration (Hale et al., 2004; Albert et al., 2005; Kiuchi et al., 2005). Only the (S)-enantiomer of fingolimod phosphate was observed in blood (Albert et al., 2005). According to data from in vitro experiments, (S)-fingolimod phosphate does not undergo
direct oxidative biotransformation. Only dephosphorylation back to fingolimod was observed in incubates with human liver microsomes, human liver S9 fractions, and human liver cytosol fractions. The intrinsic clearance of (S)-fingolimod phosphate by hydrolysis in human liver microsomes was low (12 µl·min⁻¹·mg microsomal protein⁻¹; Novartis, unpublished data) and in a similar order of magnitude as the intrinsic clearance of fingolimod by oxidation in human liver microsomes (10 µl·min⁻¹·mg microsomal protein⁻¹; Jin et al., 2010). Hence, the phosphorylation of fingolimod is reversible, in line with the approximately constant concentration ratio of fingolimod to fingolimod phosphate in blood during the elimination phase (Fig. 2, inset), observed also in a previous study (Kovarik et al., 2007b). The phosphorylation of fingolimod is catalyzed predominantly by sphingosine kinase type 2 with a smaller contribution by sphingosine kinase type 1 (Billich et al., 2003; Paugh et al., 2003). The dephosphorylation of fingolimod phosphate seems to be catalyzed mainly by the lipid phosphate phosphohydrolases LPP1a and LPP3 and to a smaller extent by the specific sphingosine 1-phosphate phosphatase SPP1 (Yamanaka et al., 2008; Mechtcheriakova et al., 2007).

The oxidative biotransformation of fingolimod occurred predominantly or exclusively by hydroxylation at the methyl terminal of the octyl chain. The resulting primary metabolite M12 was not observed in vivo due to rapid further oxidation to the carboxylic acid metabolite M1. However, in incubates with human liver microsomes, M12 was the major metabolite of fingolimod, formed predominantly by CYP4F2 and possibly other enzymes of the CYP4F subfamily with only minor contributions by other cytochrome P450 enzymes (Jin et al., 2010). Metabolite M1 underwent further biotransformation by sequential losses of two-carbon units, forming the carboxylic acid metabolites M2, M3, and M4 through β-oxidation, analogous to the degradation of endogenous fatty acids, known to occur in mitochondria and peroxisomes (Poirier et al., 2006).
Among the nonpolar metabolites in blood, the two major ones (M29 and M30) and two minor ones (M27 and M28) were characterized as ceramide analogs of fingolimod, possibly formed by the action of (dihydro)ceramide synthase and potentially also by ceramidase (reverse reaction), in analogy to the biosynthesis of endogenous dihydroceramide and ceramide (Merrill, 2002). The formation of the ceramide analogs of fingolimod might be reversible due to the action of ceramidase. However, this was not investigated.

These biotransformation pathways of fingolimod are all quite unusual for a xenobiotic and rather follow metabolic pathways known for structurally related endogenous compounds. The phosphorylation of fingolimod and the formation of ceramide analogs can be explained with the similarity between fingolimod and sphingosine or sphinganine. The β-oxidation must be triggered by the similarity of M1 to endogenous fatty acids. Even the initial hydroxylation of fingolimod at the methyl group appears to follow endogenous pathways as it is catalyzed mainly by CYP4F, a subfamily of enzymes poorly known for involvement in drug metabolism but catalyzing the hydroxylation of saturated and unsaturated fatty acids with a high preference for the ω-position (Ortiz de Montellano, 2008).

The major fingolimod-related components in the systemic circulation, as judged from their AUC values (Table 2), were fingolimod itself, fingolimod phosphate, M3, M29, and M30. M2, M27, and M28 were present in traces only. In terms of maximum concentration (C_{max}), fingolimod, fingolimod phosphate, and M3 were the major circulating components. M29 and M30 appeared to be formed very slowly, as suggested by their shallow concentration-time profiles with low blood concentrations at all time points analyzed. However, the half-lives of M29 and M30 (approximately 400 h) were longer than those of fingolimod (137 h on average) and fingolimod phosphate (approximately 166 h) and similar to that of radioactivity (382 h on
average), resulting in a substantial contribution of M29 and M30 to the AUC$_{0-\infty}$ of total radioactivity.

The elimination of fingolimod occurred almost exclusively by metabolism, as opposed to direct excretion. No fingolimod was excreted in urine and only 2-3% of the dose appeared in the feces (0-240 h) in the form of unchanged compound. Among the primary biotransformation pathways (phosphorylation, hydroxylation to M12, and formation of ceramide analogs), the hydroxylation was clearly the major contributor to the elimination of fingolimod. Elimination due to phosphorylation was a minor process because only 1-2% of dose was recovered in the feces in the form of fingolimod phosphate, and no fingolimod phosphate was found in urine. Reversible phosphorylation of fingolimod is not an elimination pathway but rather a distribution-type process. The formation of the ceramide analogs does not seem to be a major elimination pathway of fingolimod either, because no ceramide analogs were detected in urine or feces. However, excretion of a minor amount of ceramide analogs in the feces cannot be excluded as these nonpolar components might not have been recovered efficiently from the fecal matrix. The elimination of fingolimod phosphate occurred predominantly by dephosphorylation back to fingolimod, followed by oxidation of the latter. Direct oxidation of fingolimod phosphate does not seem to occur, as mentioned above, and direct excretion (1-2% of dose detected in feces 0-240 h, no fingolimod phosphate detected in urine) appeared to be a minor process.

The excretion of radioactivity was slow and still incomplete after 10 days of sample collection (approximately 52% and 10% of dose in urine and feces, respectively). However, by extrapolation an estimate of total excretion up to infinity of 92% of dose was obtained (Table 4), indicating essentially complete excretion of fingolimod-related material. The carboxylic acid metabolite M3 accounted for a major part (approximately 70%) of the radioactivity excreted in urine 0-240 h, accompanied by smaller amounts of M2, M4, M22, and some unidentified trace
metabolites. The radioactivity in feces 0-240 h represented mainly fingolimod and fingolimod phosphate with smaller contributions by M1, M2, M3, and a few trace metabolites of unknown structure (Table 3).

In conclusion, orally administered fingolimod is absorbed slowly but almost completely, followed by extensive tissue distribution. The pharmacologically active principle is formed by reversible phosphorylation to (S)-fingolimod phosphate. Conjugation of fingolimod with endogenous fatty acids results in ceramide analogs. The elimination of fingolimod occurs predominantly by CYP4F-catalyzed ω-hydroxylation at the octyl chain, followed by further oxidation to a carboxylic acid and subsequent β-oxidation. All these biotransformations are rather unusual in drug metabolism but follow metabolic processes of structurally related endogenous compounds. The excretion of fingolimod-related material was very slow but essentially complete with the major portion appearing in the urine.
Acknowledgments. The authors wish to thank Jean-Pierre Baldeck, Christine Douglas von Däniken, and Hubert Borell (Drug Metabolism and Pharmacokinetics, Novartis) for their technical support, Dr. Rhys Salter (Isotope Lab, Novartis) for the preparation of the radiolabeled compound, Dr. Robert L. Schmouder (Translational Medicine, Novartis) and Dr. Felix Waldmeier (Drug Metabolism and Pharmacokinetics, Novartis) for their contributions to the study design and protocol, Dr. Gilles-Jacques Rivière and Nathalie Gauducheau (Drug Metabolism and Pharmacokinetics, Novartis) for bioanalytical support, Dr. Urs Keller and Dr. Michael Seiberling (Swiss Pharma Contract Ltd.) for their support of the clinical part of the study, and Drs. Pieter J. Swart and Olivier Kretz for critical review of the paper.
Authorship Contributions

Participated in research design: Zollinger, Gschwind, Jin, and Hartmann

Conducted experiments: Jin and Sayer

Performed data analysis: Zollinger, Gschwind, Jin, Sayer, and Hartmann

Wrote or contributed to the writing of the manuscript: Zollinger, Gschwind, and Hartmann

Other: Zécri synthesized fingolimod metabolites
References


Footnotes.

Part of this work has been presented at the 10th European Regional Meeting of the International Society for the Study of Xenobiotics (ISSX), Vienna, Austria, May 18-21, 2008.

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Legends for figures.

**Fig. 1.** Structure of [14C]fingolimod.

**Fig. 2.** Time profiles of total radiolabeled components (radioactivity), fingolimod, and fingolimod phosphate in blood of four healthy male subjects treated with a single oral dose of 4.5 mg [14C]fingolimod. Concentrations of fingolimod were determined by LC-MS/MS; concentrations of fingolimod phosphate were derived from metabolite profiles. Inset: semilogarithmic plot up to 480 h; fingolimod phosphate concentration at 816 h could not be determined with sufficient accuracy.

**Fig. 3.** Representative metabolite profiles in blood (A-D) and excreta (E-F) of healthy male subjects treated with a single oral dose of 4.5 mg [14C]fingolimod. For technical reasons, blood extracts had to be analyzed in two fractions, a polar and a nonpolar fraction.

**Fig. 4.** Mass spectra of fingolimod (A) and selected metabolites (B-D).

**Fig. 5.** Metabolic pathways of fingolimod in man.

**Fig. 6.** Cumulative excretion of radioactivity in healthy male subjects treated with a single oral dose of 4.5 mg [14C]fingolimod. Measured values are given as mean ± S.D. of n = 4. Extrapolations are described in the experimental section.
### Table 1

**Pharmacokinetic parameters of total radiolabeled components (radioactivity) and fingolimod in blood**

Data from healthy male subjects treated with a single oral dose of 4.5 mg \(^{14}\text{C}\)fingolimod; mean ± S.D. of \(n = 4\); ranges in parentheses. Radioactivity and fingolimod were determined by liquid scintillation counting and LC-MS/MS, respectively.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Radioactivity</th>
<th>Fingolimod</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C_{\text{max}}) (pmol/ml)</td>
<td>33.3 ± 1.1 (32.1 - 34.3)</td>
<td>9.5 ± 1.6 (7.5 - 11.1)</td>
</tr>
<tr>
<td>(C_{\text{max}}) (ng/ml)</td>
<td>2.9 ± 0.5 (2.3 - 3.4)</td>
<td></td>
</tr>
<tr>
<td>(t_{\text{max}}) (^a)</td>
<td>median: 36 (36 - 36)</td>
<td>median: 24 (8 - 36)</td>
</tr>
<tr>
<td>(\text{AUC}_{0-\infty}) (nmol·h/ml)</td>
<td>12.7 ± 2.5 (9.5 - 15.4)</td>
<td>1.85 ± 0.59 (1.01 - 2.39)</td>
</tr>
<tr>
<td>(\text{AUC}_{0-\infty}) (ng·h/ml)</td>
<td>568 ± 181 (312 - 734)</td>
<td></td>
</tr>
<tr>
<td>CL/F (^b) (l/h)</td>
<td>8.7 ± 3.7 (6.3 - 14.1)</td>
<td></td>
</tr>
<tr>
<td>(V_z/F) (^b) (l)</td>
<td>1509 ± 225 (1290 - 1790)</td>
<td></td>
</tr>
<tr>
<td>(t_{1/2}) (h)</td>
<td>382 ± 43 (319 - 411)</td>
<td>137 ± 55 (63 - 196)</td>
</tr>
</tbody>
</table>

\(^a\) Time of maximum concentration.

\(^b\) Clearance (CL) and terminal volume of distribution (\(V_z\)), divided by absolute bioavailability (F; not determined).
Table 2

Pharmacokinetic parameters of fingolimod and metabolites in blood, derived from metabolite profiles

Data from healthy male subjects treated with a single oral dose of 4.5 mg [14C]fingolimod; mean values of 3 to 4 subjects; components listed in the order of elution.

<table>
<thead>
<tr>
<th>Component</th>
<th>C_{max} in pmol/ml (ng/ml in parentheses)</th>
<th>t_{max} (h)</th>
<th>AUC_{0-8.16 h} in nmol·h/ml (ng·h/ml in parentheses)</th>
<th>AUC_{0-\infty} in nmol·h/ml (ng·h/ml in parentheses)</th>
<th>t_{1/2} (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3</td>
<td>3.8 (1.1)</td>
<td>12</td>
<td>0.776 (218)</td>
<td>0.808 (227)</td>
<td>170</td>
</tr>
<tr>
<td>M2</td>
<td>0.6 (0.2)</td>
<td>12</td>
<td>0.140 (43)</td>
<td>0.140 (43)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fingolimod</td>
<td>9.2 (2.8)</td>
<td>36</td>
<td>2.18 (669)</td>
<td>2.25 (691)</td>
<td>162</td>
</tr>
<tr>
<td>Fingolimod phosphate</td>
<td>8.4 (3.3)</td>
<td>12</td>
<td>0.965 (374)</td>
<td>0.999 (387)</td>
<td>166</td>
</tr>
<tr>
<td>M27</td>
<td>0.2 (0.1)</td>
<td>216</td>
<td>0.072 (41)</td>
<td>0.072 (41)</td>
<td>n.d.</td>
</tr>
<tr>
<td>M28</td>
<td>0.5 (0.3)</td>
<td>36</td>
<td>0.046 (25)</td>
<td>0.046 (25)</td>
<td>n.d.</td>
</tr>
<tr>
<td>M29</td>
<td>1.2 (0.7)</td>
<td>36</td>
<td>0.834 (492)</td>
<td>1.19 (704)</td>
<td>404</td>
</tr>
<tr>
<td>M30</td>
<td>1.6 (0.9)</td>
<td>72</td>
<td>0.684 (393)</td>
<td>0.927 (532)</td>
<td>410</td>
</tr>
<tr>
<td>Sum of additional</td>
<td>n.d.</td>
<td>n.d.</td>
<td>2.78</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>metabolites detected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total radioactivity</td>
<td>33.3</td>
<td>36</td>
<td>9.35</td>
<td>12.7</td>
<td>382</td>
</tr>
</tbody>
</table>
n.d.: not determined.  

a Sum of polar and nonpolar fractions (cf. experimental section), analyzed individually or as pools of 3 or 4 subjects, depending on available sample size.  
b Time of maximum concentration.  
c Differences to values in Table 1 are due to the different methods of analysis and different numbers of time points analyzed.  
d Detected only at 2 time points.  
e Concentrations showed a plateau between 36 h and 480 h postdose.  
f Estimated values, based on two time points (480 h and 816 h).  
g Numerous polar as well as nonpolar trace components, all below 2.2% of AUC0-816 h of total radiolabeled components.  
h No material lost during chromatography.
Table 3

*Amounts of fingolimod and metabolites excreted in urine and feces, derived from metabolite profiles*

Data from healthy male subjects treated with a single oral dose of 4.5 mg [14C]fingolimod; mean ± S.D. of n = 4; components listed in the order of elution.

<table>
<thead>
<tr>
<th>Component</th>
<th>Urine (0-240 h)</th>
<th>Feces (0-240 h)</th>
<th>Total excretion (0-240 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4</td>
<td>3.1 ± 0.9</td>
<td>-</td>
<td>3.1 ± 0.9</td>
</tr>
<tr>
<td>M22</td>
<td>1.3 ± 0.1</td>
<td>-</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>M3</td>
<td>36.6 ± 5.0</td>
<td>0.3 ± 0.1</td>
<td>36.8 ± 5.1</td>
</tr>
<tr>
<td>M2</td>
<td>7.1 ± 1.2</td>
<td>0.5 ± 0.2</td>
<td>7.6 ± 1.2</td>
</tr>
<tr>
<td>M1</td>
<td>-</td>
<td>0.2 ± 0.01</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>Fingolimod</td>
<td>-</td>
<td>2.4 ± 0.5</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>Fingolimod phosphate</td>
<td>-</td>
<td>1.7 ± 0.4</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Sum of additional metabolites detected</td>
<td>4.2 ± 0.5</td>
<td>0.7 ± 0.1</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>Material lost during sample preparation</td>
<td>-</td>
<td>3.7 ± 0.8</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>Material lost during chromatography</td>
<td>-</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Total radioactivity</td>
<td>52.3 ± 6.3</td>
<td>9.9 ± 1.7</td>
<td>62.1 ± 7.6</td>
</tr>
</tbody>
</table>

-: not detected.
Table 4

*Cumulative excretion of radioactivity in urine and feces*

Data from healthy male subjects treated with a single oral dose of 4.5 mg [\(^{14}\)C]fingolimod; mean ± S.D. of n = 4.

<table>
<thead>
<tr>
<th>Time Period</th>
<th>Urine Excretion (%) of Dose</th>
<th>Feces Excretion (%) of Dose</th>
<th>Total Excretion (%) of Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-72 h</td>
<td>22.3 ± 5.3</td>
<td>5.4 ± 1.1</td>
<td>27.7 ± 5.3</td>
</tr>
<tr>
<td>0-240 h</td>
<td>52.3 ± 6.3</td>
<td>9.9 ± 1.7</td>
<td>62.1 ± 7.6</td>
</tr>
<tr>
<td>288-312 h</td>
<td>2.0 ± 0.5</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>456-480 h</td>
<td>1.0 ± 0.4</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>792-816 h</td>
<td>0.3 ± 0.2</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>0-816 h (estimated)</td>
<td>78 ± 3</td>
<td>11 ± 2</td>
<td>89 ± 3</td>
</tr>
<tr>
<td>0-infinity (estimated by extrapolation)</td>
<td>81 ± 3</td>
<td>11 ± 2</td>
<td>92 ± 2</td>
</tr>
</tbody>
</table>

n.s.: not sampled
Fig. 1

![Chemical structure](image_url)
Fig. 2

- Radioactivity (mean ± S.D. of n=4)
- Fingolimod (mean ± S.D. of n=4)
- Fingolimod phosphate (mean of n=4)
Fig. 3

A. Blood 36 h, polar fraction, subject 1

B. Blood 36 h, nonpolar fraction, pool of subjects 1-4

C. Blood 240 h, polar fraction, subject 1

D. Blood 480 h, nonpolar fraction, pool of subjects 1-4

E. Urine 0-240 h, subject 1 (profile represents 52% of dose)

F. Feces, 0-240 h, subject 1 (profile represents 5.0% of dose)
Fig. 4

A  MS/MS product ion spectrum of protonated fingolimod; authentic standard
Q-Tof 2 mass spectrometer, 20 V collision offset

B  MS/MS product ion spectrum of protonated M3; metabolite isolated from human blood
Q-Tof 2 mass spectrometer, 20 V collision offset

C  MS/MS product ion spectrum of protonated fingolimod phosphate; metabolite isolated from human blood
Q-Tof 2 mass spectrometer, 15 V collision offset

D  MS/MS product ion spectrum of protonated M30; metabolite isolated from blood of cynomolgus monkeys treated with a
single oral dose of 3 mg/kg fingolimod; LTQ Orbitrap mass spectrometer, 30% normalized collision energy
**Fig. 5**

This diagram illustrates the metabolism of Fingolimod and its metabolites. The primary pathway begins with Fingolimod, which is metabolized by CYP4F to M12. M12 is then glucuronidated to M22, which is excreted in urine. M12 can also be metabolized to M1, which is excreted in feces. M1 is further metabolized to M2, which is excreted in blood, urine, and feces. M2 is then further metabolized to M3, which is also excreted in blood, urine, and feces. M3 is then metabolized to M4, which is excreted in urine. The pathway is further complicated by the presence of Fingolimod phosphate, which is converted to Fingolimod by sphingosine kinase type 2. M29 and M30 are also observed in blood, with M29 being predominantly metabolized to M29 and M30 being predominantly metabolized to M30.
Fig. 6

Cumulative excretion of radioactivity (% dose)

- Total (observed)
- Urine (observed)
- Feces (observed)
- Calculated

Time (h)