Absorption, Metabolism, and Excretion of Darunavir, a New Protease Inhibitor, Administered Alone and with Low-Dose Ritonavir in Healthy Subjects

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ABSTRACT:

Absorption, metabolism, and excretion of darunavir, an inhibitor of human immunodeficiency virus protease, was studied in eight healthy male subjects after a single oral dose of 400 mg of [14C]-darunavir given alone (unboosted subjects) or with ritonavir [100 mg b.i.d. 2 days before and 7 days after darunavir administration (boosted subjects)]. Plasma exposure to darunavir was 11-fold higher in boosted subjects. Total recoveries of radioactivity in urine and feces were 93.9 and 93.5% of administered radioactivity in unboosted and boosted subjects, respectively. The most radioactivity was recovered in feces (81.7% in unboosted subjects and 79.5% in boosted subjects, compared with 12.2 and 13.9% recovered in urine, respectively). Darunavir was extensively metabolized in unboosted subjects, mainly by carbamate hydrolysis, isobutyl aliphatic hydroxylation, and aniline aromatic hydroxylation and to a lesser extent by benzylic aromatic hydroxylation and glucuronidation. Total excretion of unchanged darunavir accounted for 8.0% of the dose in unboosted subjects. Boosting with ritonavir resulted in significant inhibition of carbamate hydrolysis, isobutyl aliphatic hydroxylation, and aniline aromatic hydroxylation but had no effect on aromatic hydroxylation at the benzylic moiety, whereas excretion of glucuronide metabolites was markedly increased but still represented a minor pathway. Total excretion of unchanged darunavir accounted for 48.8% of the administered dose in boosted subjects as a result of the inhibition of darunavir metabolism by ritonavir. Unchanged darunavir in urine accounted for 1.2% of the administered dose in unboosted subjects and 7.7% in boosted subjects, indicating a low renal clearance. Darunavir administered alone or with ritonavir was well tolerated.

Darunavir (TMC114, Prezista; Tibotec BVBA, Mechelen, Belgium) is an inhibitor of the human immunodeficiency virus (HIV) protease (Fig. 1). Its molecular formula is C27H37N3O7S and molecular weight is 593.73. Darunavir binds tightly to HIV protease with a dissociation constant (Kd) of 4.5 × 10⁻¹² M (King et al., 2004) and is highly active against wild-type and resistant strains of the virus (De Meyer et al., 2005), inhibiting dimerization (Koh et al., 2007) and catalytic activity of HIV-1 protease. It selectively inhibits the cleavage of HIV-encoded gag-pol polyproteins in virus-infected cells, preventing the formation of mature infectious virus particles.

Darunavir is metabolized mainly by cytochrome P450 isozyme 3A (CYP3A) (Mamidi et al., 2005). As observed with other protease inhibitor (PIs) that are CYP3A4 substrates (Cooper et al., 2003; Zeldin and Petruschke, 2004), administration of darunavir with low-dose ritonavir as a pharmacokinetic booster results in clinically relevant increases in the systemic exposure to darunavir. Ritonavir is a potent CYP3A4 inhibitor, and inhibition of this isozyme in the intestinal tract and liver, where CYP3A4 exerts its effect on first-pass metabolism, reduces the metabolism of the parent drug, with a consequent increase in exposure to the unchanged drug.

Darunavir is therefore administered in combination with low-dose ritonavir, and a dosing regimen of 600/100 mg b.i.d., used together with other antiretroviral agents, has been shown to be effective in decreasing the HIV-1 viral load in antiretroviral treatment-experienced adults, such as those with HIV-1 strains resistant to more than one PI (Cloot et al., 2007). On this basis, darunavir has received regulatory approvals for the treatment of HIV infection in this indication (Prezista, U.S. prescribing information).

In this study, we report on the absorption, metabolism, and excretion of darunavir in the absence (unboosted) and presence (boosted) of low-dose ritonavir in healthy male subjects after a single, 400-mg oral dose of [14C]-darunavir solution. The objective of the study was to characterize the effect of low-dose ritonavir on the excretion and metabolism of darunavir and to elucidate the metabolic pathways and structures of the metabolites.

This work was supported by Tibotec BVBA.

ABBREVIATIONS: darunavir, TMC114; [3-[4-amino-benzenesulfonyl]-isobutylaminio]-1-benzyl-2-hydroxypropyl]-carbamic acid hexahydro-furo-[2,3-b]furan-3-yl ester·ethanolate; HIV, human immunodeficiency virus; PI, protease inhibitor; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS/MS, tandem mass spectrometry; AUC, area under the plasma concentration-time curve; ESI, electrospray ionization; P-gp, P-glycoprotein.
Materials and Methods

Darunavir and Reference Compounds. Unlabeled darunavir (R319064) was synthesized according to Good Manufacturing Practice regulations by the chemical and manufacturing department of Johnson & Johnson Pharmaceutical Research and Development (Beerse, Belgium). [14C]Darunavir, a radioisotope synthesized according to Good Laboratory Practices by the carbon-14 custom preparations group of GE Healthcare (Little Chalfont, Buckinghamshire, UK), was randomly labeled with 14C in the aniline sulfonate moiety of the molecule (Fig. 1). [14C]Darunavir was mixed with unlabeled darunavir in a polyethylene glycol-400 solution, together with α-tocopheryl polyethylene glycol-1000 succinate, sodium saccharinate, and Aurantii Cortici Tinctura Fortis to yield a solution with a specific activity of 3.80 kBq/mg base-Eq and a radiochemical purity of 99.3% (radio high-performance liquid chromatography (HPLC)).

The following nonradiolabeled authentic substances, postulated as metabolites of darunavir after cross-species in vitro and in vivo metabolite identification studies, were synthesized by the radiochemistry group of Johnson & Johnson Pharmaceutical Research and Development: R109348, R330511 (TMC183240), R330470 (TMC143202), R330701 (TMC189613), R330576 (TMC182490), R374699 (TMC73285), R330689 (TMC189373), and R330326 (TMC79894) (Fig. 1). Two other authentic substances, R426855 and R426857 (Fig. 1), were synthesized after NMR identification of metabolites in the course of the present study. The chemical structures and purity of the authentic substances were confirmed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Solvents and reagents were all of analytical grade and were purchased from commercial sources.

Subjects and Dosing. The clinical part of the study was conducted at the Johnson & Johnson Pharmaceutical Research and Development Clinical Pharmacology Unit in the Jan Palfijn Hospital (Merksem, Belgium) in accordance.
with Good Clinical Practice guidelines, the Declaration of Helsinki (1964 and subsequent revisions), and the Administration of Radioactive Substances Advisory Committee. The protocol was approved by an independent ethics committee. All participating subjects gave written informed consent before participation.

Eight healthy white men between 40 and 55 years of age, with a body mass index of 24 to 27 kg/m², were enrolled in this single-center, single-dose, open-label study. Subjects were healthy based on medical history, a pre-study physical examination, electrocardiogram, vital signs, and clinical laboratory tests. Subjects were nonsmokers or light smokers and had no history of alcohol or drug abuse. No metabolizer phenotyping was done. No medication other than the study drug and acetaminophen was allowed from 14 days before dosing with study drug(s) until the end of the study, with the exception of emergency medication to treat adverse events. Subjects remained in the study center from at least 10 h before administration of darunavir until up to 12 days thereafter. Subjects fasted from at least 10 h before administration of darunavir until 4 h thereafter.

All subjects received a single 400-mg dose of darunavir administered as 20 ml of an oral solution containing 14C-labeled darunavir and unlabeled darunavir at a total darunavir concentration of 20 mg/ml (with a total radioactivity of ~40.5 µCi), after which the flask was rinsed twice with approximately 20 ml of water that was also ingested. Four of the eight subjects also received 100 mg of ritonavir b.i.d. (administered at 12-h intervals) from 2 days before until 7 days after administration of darunavir. When only ritonavir was administered, it was taken under fed conditions, within 15 min after completing a meal (product label, Norvir; Abbott Laboratories, Chicago, IL). When ritonavir was coadministered with darunavir, it was taken under fasted conditions within 5 min after administration of darunavir.

Sample Collection. Blood samples (5 ml to yield 2 ml of plasma for determination of darunavir and/or ritonavir) were obtained before administration of darunavir and at 0.5, 1, 1.5, 2, 3, 4, 6, 9, 12, 24, 36, 48, 72, 96, 120, 144, and 168 h thereafter. In addition, blood samples (20 ml) were collected for determination of radioactivity in whole blood and for metabolic profiling at 1, 2, 4, 9, 12, 24, and 48 h after dosing. Urine samples for determination of radioactivity and for metabolic profiling were collected from 0 to 4, 4 to 9, 9 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, 120 to 144, and 144 to 168 h after dosing. Feces samples for determination of radioactivity and for metabolic profiling were collected per stool from 1 day before administration of darunavir until 168 h thereafter. All samples were stored at ≈18°C until analysis.

Determination of Radioactivity. Radioactivity in blood, plasma, urine, and feces samples was measured using a Packard Tri-Carb 1900TR or 2100TR liquid scintillation spectrometer (PerkinElmer Life and Analytical Sciences, Waltham, MA).

Blood and plasma. Blood radioactivity concentrations were measured after combustion of quadruplicate dried 0.2-ml aliquots in a Packard sample oxidizer (model 307; PerkinElmer Life and Analytical Sciences). Carbosorb (9 ml; PerkinElmer Life and Analytical Sciences) was used to absorb the 14CO₂, precipitated proteins. Subsequently, the supernatants were collected and evaporated to dryness under nitrogen. The evaporation residues were redissolved in 500 µl of dimethyl sulfoxide-water (1:1, by volume) and 800-µl aliquots of these samples were injected onto the radio-HPLC system.

Urine. Duplicate 0.25-ml aliquots of urine were diluted with distilled water up to 1 ml and mixed with 10 ml of Ultima Gold as a scintillation cocktail. The total radioactivity levels were expressed as nanogram-equivalents per milliliter using the specific activity of [14C]darunavir in the drug formulation. The lower limit of quantification in plasma was 90 dpm/ml (394 ng-Eq/ml).

Urine. Duplicate 0.25-ml aliquots of urine were diluted with distilled water up to 1 ml and mixed with 10 ml of Ultima Gold as a scintillation cocktail.

Feces. Feces samples were homogenized in methanol with an Ultra Turrax (IKA, Staufen, Germany) and centrifuged, and residues were extracted another two times with methanol, followed by filtration of the suspensions through a Büchner funnel. In total, on average 7 ml of methanol was used per gram of feces for the extraction. The methanolic extracts of each feces sample were combined, and duplicate 0.25-ml aliquots were diluted with distilled water up to 1 ml and mixed with 10 ml of Ultima Gold as a scintillation cocktail. The feces residues were air-dried, and the weight of the residues was measured. The dried residues were ground to a fine powder in an Ultra Centrifugal Mill ZM100 (Retsch, Haan, Germany). Four weighed subsamples of approximately 100 mg of each residue sample were combusted, and radioactivity was measured in a manner similar to that for the blood samples. Feces samples with low radioactivity levels were lyophilized using a Genesis Super 12 ES freeze dryer (VirTis, Gardiner, NY) before combustion and measurement of radioactivity.

Determination of Darunavir and Ritonavir in Plasma. Under basic conditions (pH = 4.5), darunavir, ritonavir, and internal standard were extracted from plasma (100 µl) with tertiary butylmethyl ether. After evaporation and reconstitution in injection solvent, these analytes were quantified using a validated LC-MS/MS assay. With use of a Waters Alliance 2790 HPLC system (Waters, Milford, MA), 10-µl aliquots of the processed samples were injected on a Hypersil 3-µm C18 BDX column (100 x 4.6 mm i.d.) (Phenomenex, Torrance, CA) maintained at 40°C. The mobile phase was a mixture (35:65) of 20 mM ammonium acetate (adjusted to pH 4.5 with acetic acid) and acetoni- trile delivered at 0.65 ml/min. The eluent was split in a ratio of 1:11 to introduce 0.1 ml/min into the mass spectrometer. Quantification was achieved by MS/MS detection in the positive ion mode, using a Sciex API 3000 mass spectrometer (PerkinElmer Life and Analytical Sciences) equipped with a TurbolonSpray interface. The transitions monitored were m/z 565.60 to 392.10 for darunavir (the ammonium adduct of darunavir), m/z 721.00 to 296.04 for ritonavir, and m/z 599.26 to 426.02 for internal standard. The effective linear range of quantification for undiluted samples was 10.0 to 10,000 ng/ml for darunavir and 5.00 to 5000 ng/ml for ritonavir, with acceptable accuracy and precision (~15%).

Pharmacokinetic Analysis. The levels of radioactivity in blood and plasma are presented as nanogram-equivalents per milliliter. The blood to plasma concentration ratio of the radioactivity was calculated. The amounts of radioactivity excreted in urine or feces are expressed as a percentage of the administered radioactive dose. The amount of radioactivity in the feces samples was calculated as the sum of the amount of radioactivity in the methanolic extracts and the residues prepared from these samples. The excretion half-life of 14C in urine was determined by log-linear regression of the excretion rate-time data. The relative abundance of unchanged darunavir and its major metabolites in pooled urine samples and in pools of methanolic feces extracts is expressed as a percentage of the administered dose.

Pharmacokinetic parameters were calculated using noncompartamental analysis. Actual blood sampling times and target urine sampling times were used. Values are given as mean ± S.D., except for the time of maximum plasma concentration (tmax), for which medians and range are given. The area under the plasma concentration-time curve (AUC) values were calculated by linear trapezoidal summation and extrapolation to infinity, calculated as AUC(t) = AUC(last) + C(last) t), where AUC(last) is the last measurable concentration and t is the elimination rate constant, estimated by linear regression of the terminal points of the ln-plasma concentration-time curve. The terminal half-life (t1/2) was calculated as 0.693 AUC.
dimethyl sulfoxide. Aliquots of 150 μl (approximately 4400–36,900 dpm) of these samples were injected onto the radio-HPLC system.

The HPLC apparatus consisted of a Waters Alliance 2695 system equipped with an automatic injector. The samples were chromatographed on a stainless steel column (25 cm × 4.6 mm i.d.) packed with Kromasil C18 (5 μm; Akzo Nobel, EKA, Bohus, Sweden). The columns were packed by a balanced density slurry procedure [Haskel DSTV 122-C pump (Haskel Inc., Burbank, CA), 7 × 10^3 Pa]. UV detection was performed at 280 nm using a Waters 996 diode array detector. On-line radioactivity detection of HPLC eluates was performed with a Berthold Radioactivity Monitor LB 509 system equipped with a flow-through cell of 1000 μl (Berthold Technologies, Bad Wildbad, Germany). The eluates were mixed with Ultima-Flo AP (PerkinElmer Life and Analytical Sciences) as a scintillation cocktail delivered by a Berthold LB 5035-3 pump at a flow rate of 4.0 mL/min. Detector outputs were connected to a Millennium (Waters) chromatography data system. Elution was started with a linear gradient at a flow rate of 1 mL/min from 100% of an aqueous solution of 0.1 M ammonium acetate adjusted to pH 6.0 (solvent system A) to 40% of solvent system A and 60% of solvent system B, consisting of an aqueous solution of 1 M ammonium acetate (adjusted to pH 6.0)-methanol-acetonitrile (10:45:45, by volume) over 60 min. This solvent composition was held for 10 min. Subsequently, a linear gradient over 2 min to 100% of solvent system B was applied, which was then maintained for 10 min before returning to the starting conditions.

The concentrations of darunavir and its major metabolites in plasma, urine, and feces extracts were calculated on the basis of the recovery of the radioactivity in the samples and on the areas of the radioactivity peaks obtained after reverse-phase radio-HPLC of appropriate aliquots of these samples.

Structural Characterization of Metabolites. Metabolites were identified by LC-MS/MS and by HPLC cochromatography of a mixture of the parent compound and a number of authentic compounds postulated as metabolites (see Fig. 1 for structures). A qTOF Ultima mass spectrometer (Waters) was used, equipped with a dual electrospray ionization probe and operated in the positive ion mode. Data were acquired and processed using MassLynx 4.0 software (Waters). Three metabolites were isolated and identified by NMR spectroscopy.

Glucuronic acid and sulfate conjugates excreted in urine were characterized by comparison of radio-HPLC chromatograms of samples with and without treatment with β-glucuronidase/aryl sulfatase from Helix pomatia (10 μL/mL acetate-buffered sample; pH 5.0; Roche Diagnostics, Mannheim, Germany), β-glucuronidase from Escherichia coli (10 μL/mL phosphate-buffered sample, pH 7.0; Roche Diagnostics), and arylsulfatase from Aerobacter aerogenes (10 μL/mL phosphate buffer, pH 7.0; Sigma-Aldrich, St. Louis, MO). Incubations were performed for 12 to 24 h at 37°C. D-Saccharo-1,4-lactone (10 mCi/ml phosphate buffered sample, pH 7.0) was used, equipped with a dual electrospray ionization probe and operated in the positive ion mode. Data were acquired and processed using MassLynx 4.0 software (Waters). Three metabolites were isolated and identified by NMR spectroscopy.

Results

Demographics, Safety, and Tolerability. Eight healthy, male white subjects received the study drug(s) (darunavir only in four subjects and darunavir/ritonavir in four subjects) and completed the study. Subject age ranged from 40 to 55 years (median, 51.0 years), body weight ranged from 69 to 90 kg (median, 79.5 kg), and body mass index ranged from 24 to 27 kg/m² (median, 25.6 kg/m²). One subject (treated with darunavir only) used unallowed concomitant medication during the study (diclofenac sodium). Darunavir and darunavir/ritonavir were well tolerated, no serious adverse events occurred, and no subjects discontinued the study because of an adverse event. Electrocardiograms, clinical laboratory tests, and vital signs showed no clinically relevant changes.

Pharmacokinetics and Radioactivity of Darunavir in Plasma. The mean plasma concentration-time profiles and pharmacokinetic parameters of total radioactivity and unchanged darunavir after a single oral dose of 400 mg of [14C]darunavir alone (unboosted) or in combination with 100 mg of ritonavir b.i.d. (boosted) to four male healthy subjects each are shown in Fig. 2 and Table 1, respectively. Darunavir was absorbed rapidly after oral administration in unboosted and boosted subjects. The overall exposure to darunavir (AUC_0–∞) was approximately 11-fold higher in boosted subjects, compared with that in unboosted subjects. The t1/2 values of [14C]darunavir and unchanged darunavir were 4.1 and 29.4 h in unboosted subjects and 30.6 and 13.5 h in boosted subjects, respectively. The shorter [14C]-darunavir t1/2 relative to unchanged compound in unboosted subjects could be related to the difference in sensitivity of the bioanalytical assays (lower limit of quantification was 10 ng/ml for darunavir versus 394 ng-Eq/ml for [14C]darunavir). Based on the ratio of the AUC_0–∞ values for unchanged drug and total radioactivity, unchanged darunavir accounted for approximately 37 and 68% of the total radioactivity in plasma of unboosted and boosted subjects, respectively (Table 1). These results showing that unchanged drug accounted for a larger percentage of the total radioactivity in plasma of boosted subjects than in plasma of unboosted subjects indicate that ritonavir acts as an inhibitor of darunavir metabolism. The ratio of AUC_0–∞ (boosted) to AUC_0–∞ (unboosted) for total radioactivity was 5.9, indicating that the exposure to darunavir and its metabolites was notably higher in boosted than in unboosted subjects.

Pharmacokinetics of Ritonavir in Plasma. When ritonavir was coadministered with darunavir, the median t max for ritonavir was 3.0 h, and the mean maximum concentration (C max) and AUC_0–12 h values were 1747 ng/ml and 10,563 ng·h/ml, respectively. The interindividual variability (percent coefficient of variability) of ritonavir was 52% for C max and 53% for AUC_0–12 h.

Distribution of Darunavir in Blood. The mean blood to plasma concentration ratio of total radioactivity ranged from 0.68 to 0.74 in unboosted subjects and from 0.59 to 0.70 in boosted subjects, indicating that the radioactivity in blood was mainly distributed to plasma. The level of radioactivity was below the lower limit of detection in the 4-h postdose blood samples of unboosted subjects and in the 24-h postdose blood samples of boosted subjects.
Urinary and Fecal Excretion and Mass Balance. After a single oral dose of 400 mg of [14C]darunavir in healthy male subjects, alone or in combination with 100 mg of ritonavir b.i.d., the major part of the [14C]darunavir-related radioactive activity was excreted via the feces (Table 2). At 168 h after dosing, the mean percentage of administered radioactivity excreted via the feces was 81.7% (range, 72.8–93.4%) in unboosted subjects and 79.5% (range, 68.5–86.9%) in boosted subjects. The corresponding mean percentage of administered radioactive activity excreted in urine was 12.2% (range, 9.9–15.9%) in unboosted subjects and 13.9% (range, 10.2–16.4%) in boosted subjects. At 1 week after dosing, the total urinary and fecal excretions amounted to 93.9 and 93.5% of the administered radioactive activity in unboosted and boosted subjects, respectively. The coadministration of ritonavir seemed to have no substantial effect on the total excretion of [14C]-darunavir-related radioactive activity. From the 9 to 24 h collection period onwards, the urinary excretion rate of radioactivity declined, with a mean half-life of 20.2 h in unboosted subjects and 17.5 h in boosted subjects.

Metabolite Profile of Darunavir. Except stated otherwise, percentages quoted in the text below are mean values.

Plasma. Representative radio-HPLC chromatograms for overall pooled 1 h plasma samples from unboosted and boosted subjects are shown in Fig. 3. Later plasma samples contained only low amounts of radioactive activity. After deproteinization, the recovery of radioactivity in plasma pools collected at different time points ranged from 83.4 to 107.8% in unboosted subjects and from 83.9 to 90.7% in boosted subjects. The sample radioactivity in the plasma pools obtained up to 4 h after dosing was mainly unchanged darunavir, accounting for 25.9 to 50.0% of the injected sample radioactivity in unboosted subjects and 23.0 and 72.5% of the sample radioactivity when normalized to the percentage of the sum of unchanged drug and detected metabolites) in boosted subjects.

Besides unchanged darunavir, a few metabolites were present in human plasma samples. In unboosted subjects, metabolites 6 (4.8% of injected sample radioactive activity), 23 (7.7%), and 29 (2.4%) were detected in pooled 1-h plasma samples, together with some metabolites eluting between 53 and 56 min (6.0%). Metabolites 6, 23, and 29 were identified as a monohydroxylated carbamate hydroxylated metabolite (R426855), a monohydroxylated metabolite at the isobutyl moiety (R426857), and a monohydroxylated metabolite at the aniline moiety (R330326), respectively (see Metabolite Identification for identification data, and Fig. 7 for the chemical structures). In addition, LC-MS/MS data revealed that the metabolites eluting between 53 and 56 min were formed by carbamate hydrolysis, carbamate hydrolysis in combination with monohydroxylation, glucuronidation, and glucuronidation in combination with monohydroxylation of the parent drug. In boosted subjects, none of the metabolites detected in the plasma of unboosted subjects was present, except for some metabolites eluting between 53 and 56 min (7.9%). However, no LC-MS/MS analysis was performed to identify these metabolites. In 4-h plasma samples of unboosted and boosted subjects, only unchanged darunavir could be detected.

Urine. The mass balance of unchanged darunavir and its metabolites in the urine of unboosted and boosted subjects is summarized in Table 3. Representative radio-HPLC chromatograms of individual urine pools for 0 to 9 h after dosing for unboosted and boosted subjects are shown in Fig. 4. The recovery of radioactive activity after radio-HPLC analysis (detected radioactive activity relative to injected radioactive activity) amounted to 66.5 ± 4.1% (mean ± S.D.) in urine samples of unboosted subjects and to 87.9 ± 5.7% in urine samples of boosted subjects. The structures of the metabolites are shown in the metabolic scheme (Fig. 7).

Unchanged darunavir accounted for 1.15 and 7.65% of the dose (23.0 and 72.5% of the sample radioactive activity when normalized to the percentage of the sum of unchanged drug and detected metabolites) in the urine of unboosted and boosted subjects, respectively.

Besides unchanged darunavir, several metabolites in urine could be identified by LC-MS/MS and NMR. In the urine of unboosted subjects, identified biotransformation products included metabolites 6, 11, 15, 17, 18, 19, 20, 23, and 29. Metabolites 15, 17, and 18 eluted close together in the radio-HPLC chromatography. Identification data for the metabolites are summarized in Table 4. The identified metabolites each represented relatively small amounts (<1.02%) of the dose (Table 3).

In the urine of boosted subjects, a combined peak of metabolites 17, 18, and 19 accounted for 1.57% of the dose. Certain metabolites that were detected in the urine of unboosted subjects, including metabolites 6, 11, and 15, could not be detected in the urine of boosted subjects. Metabolites 20, 23, and 29, which were only detected in the

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Darunavir (Unboosted)</th>
<th>Total Radioactivity</th>
<th>Darunavir/Ritonavir (Boosted)</th>
<th>Total Radioactivity</th>
</tr>
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<tbody>
<tr>
<td>$t_{\text{max}}$ (h)</td>
<td>0.5 (0.5–1.0)</td>
<td>0.5 (0.5–1.0)</td>
<td>0.75 (0.5–1.5)</td>
<td>0.75 (0.5–1.0)</td>
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<tr>
<td>C$_{\text{max}}$ (ng or ng-Eq/ml)</td>
<td>2730 ± 648</td>
<td>4783 ± 1110</td>
<td>5125 ± 906</td>
<td>6812 ± 1585</td>
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<td>AUC$_{0\rightarrow t}$ (ng or ng-Eq · h/ml)</td>
<td>4291 ± 1956</td>
<td>10,349 ± 5011</td>
<td>50,389 ± 15,631</td>
<td>53,301 ± 21,270</td>
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<tr>
<td>AUC$_{0\rightarrow \infty}$ (ng or ng-Eq · h/ml)</td>
<td>4746 ± 1647</td>
<td>12,779 ± 5361</td>
<td>50,760 ± 15,600</td>
<td>74,966 ± 19,090</td>
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<tr>
<td>$t_{1/2, \text{res}}$ (h)</td>
<td>29.4 ± 45.3</td>
<td>4.1 ± 0.9</td>
<td>13.5 ± 1.83</td>
<td>30.6 ± 20.6</td>
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### Table 2

<table>
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<tr>
<th>Matrix</th>
<th>Collection Interval</th>
<th>Dose Recovered</th>
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<tr>
<td></td>
<td>h</td>
<td>%</td>
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<tr>
<td>Urine</td>
<td>0–4</td>
<td>4.66 ± 1.39</td>
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<tr>
<td></td>
<td>4–9</td>
<td>2.01 ± 0.57</td>
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<tr>
<td></td>
<td>9–24</td>
<td>2.32 ± 0.29</td>
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<td></td>
<td>24–48</td>
<td>1.95 ± 0.44</td>
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<td></td>
<td>48–72</td>
<td>0.83 ± 0.17</td>
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<td></td>
<td>72–96</td>
<td>0.38 ± 0.11</td>
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<tr>
<td></td>
<td>96–120</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>120–144</td>
<td>N.D.</td>
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<tr>
<td></td>
<td>144–168</td>
<td>N.D.</td>
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<td></td>
<td>0–168</td>
<td>12.22 ± 2.82</td>
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<tr>
<td>Feces</td>
<td>MeOH extract</td>
<td>68.31 ± 7.04</td>
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<td></td>
<td>Residue</td>
<td>13.37 ± 3.16</td>
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<td></td>
<td>MeOH extract + residue</td>
<td>81.68 ± 8.57</td>
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<tr>
<td>Urine and feces</td>
<td>0–168</td>
<td>93.90 ± 8.62</td>
</tr>
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</table>

N.D., not detected.
48 h after dosing, feces was analyzed for up to 64 to 84 h (unboosted subjects) and for up to radioactivity after radio-HPLC analyses (detected radioactivity relative to injected radioactivity) was 65.2 ± 3.6% (mean ± S.D.) in feces samples of unboosted subjects and 85.2 ± 7.0% in feces samples of boosted subjects. The structures of metabolites detected in feces samples are shown in the metabolic scheme (Fig. 7). The mass balance of unchanged darunavir and identified metabolites in methanolic fecal extracts from unboosted and boosted subjects is summarized in Table 3.

Unchanged darunavir accounted for 6.83 and 41.2% of the dose (25.6 and 88.4% of the sample radioactivity when normalized to the percentage of the sum of unchanged drug and detected metabolites) in methanolic fecal extracts of unboosted and boosted subjects, respectively.

Several of the metabolites detected in urine were also present in the methanolic fecal extracts, in general in higher percentages. In the extracts of unboosted subjects, metabolites 6, 15, 19, 33, 23, and 29 were detected, each accounting for 1.60 to 4.38% of the dose (Table 3). Metabolites 6, 15, 23, and 29 were the most common biotransformation products in feces. Identification data of the metabolites are summarized in Table 4. Metabolites 17 and 18, coeluting with metabolite 15 in urine samples, were not recovered in feces.

In the methanolic fecal extracts of boosted subjects, only four metabolites could be identified: metabolites 19, 33, 23, and 29. Except for metabolite 33, the abundance of these metabolites in fecal extracts of boosted subjects was lower than in unboosted subjects, each accounting for 2.01% of the dose at the most.

In addition, several unidentified metabolites were detected, accounting in total for at least 17.1 to 23.9% of the dose in fecal extracts of unboosted subjects and for at least 6.5 to 8.7% of the dose in fecal extracts of boosted subjects. The multitude of unidentified metabolites, in particular when relatively low amounts of radioactivity were injected, had an effect on the recovery of the radioactivity.

**Metabolic Identification.** The metabolites of darunavir were given a code number based mainly on their retention times, in accordance with code numbers used in previous in vitro and in vivo studies in rats. Unchanged darunavir and its major metabolites were identified by LC-MS/MS. The characteristic product ions used for structural identification of darunavir metabolites are listed in Table 4. The identity of several metabolites was also confirmed by HPLC cochromatography with known authentic substances (see Fig. 1 for structures of the authentic substances) and, for some metabolites, also by NMR. The elemental composition of the unchanged drug and all of its metabo-
lites was confirmed by accurate mass measurement. All measured masses of the pseudomolecular ions were within a 5-ppm error relative to the corresponding calculated masses. The metabolites identified and the means of identification are summarized in Table 4, and the structures are shown in the metabolic scheme (Fig. 7). Further evidence for the identity of certain glucuronide metabolites in urine came from hydrolysis experiments with \( \text{H}9252\)-glucuronidase and with \( \text{H}9252\)-glucuronidase from \( \text{H. pomatia} \) and with \( \text{H}9252\)-glucuronidase from \( \text{E. coli} \). As an example, when the 0 to 9 h pooled urine sample from a boosted subject was hydrolyzed with \( \beta \)-glucuronidase/arylsulfatase, the combined peak containing metabolite 19 and the glucuronide metabolites 17 and 18, as well as metabolite 20, seen in the chromatogram for the untreated sample was substantially reduced after enzymatic hydrolysis.

The MS spectrum of the drug substance R319064 (darunavir) showed a protonated molecular ion at \( m/z \) 548. MS/MS fragmentation of the pseudomolecular ion was characterized by the diagnostic fragment ions \( m/z \) 392, 241, 202, 156, and 113 (Fig. 6). The fragmentation

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Identification Method</th>
<th>LC Retention Time</th>
<th>([M + H]^+) (_{\text{exp}})</th>
<th>([M + H]^+) (_{\text{calc}})</th>
<th>Characteristic Product Ions (_{m/z})</th>
<th>Identification</th>
<th>Plasma</th>
<th>Urine</th>
<th>Feces</th>
</tr>
</thead>
<tbody>
<tr>
<td>UD</td>
<td>LC-MS/MS coelution</td>
<td>67.0–68.0</td>
<td>548.2410(^a)</td>
<td>548.2430</td>
<td>392, 241, 202, 156, 113</td>
<td>Unchanged drug, darunavir (R319064; TMC114)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>LC-MS/MS NMR coelution</td>
<td>45.6–46.4</td>
<td>408.1938(^b)</td>
<td>408.1957</td>
<td>390, 156</td>
<td>Carbamate hydrolysis and monohydroxylation at the isobutyl function towards a tertiary alcohol function (R426855)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>LC-MS/MS</td>
<td>50.1–50.4</td>
<td>584.2255(^c)</td>
<td>584.2278</td>
<td>348, 257, 172</td>
<td>Glucuronidation of metabolite 15</td>
<td>N.D.</td>
<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>LC-MS/MS</td>
<td>52.6–53.4</td>
<td>408.1956(^b)</td>
<td>408.1957</td>
<td>172</td>
<td>Carbamate hydrolysis and monohydroxylation of the aniline moiety</td>
<td>N.D.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>LC-MS/MS</td>
<td>52.6–53.4</td>
<td>740.2697(^c)</td>
<td>740.2701</td>
<td>584, 113</td>
<td>Monohydroxylation and glucuronidation at the (4-aminobenzenesulfonyl)-isobutylamino-1-benzyl-2-hydroxypropyl moiety</td>
<td>N.D.</td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>18</td>
<td>LC-MS/MS</td>
<td>52.6–53.4</td>
<td>724.2742(^c)</td>
<td>724.2751</td>
<td>568, 332</td>
<td>N-Glucuronidation of parent drug at the aniline moiety</td>
<td>N.D.</td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>19</td>
<td>LC-MS/MS NMR coelution</td>
<td>54.5–55.3</td>
<td>392.2026(^c)</td>
<td>392.2008</td>
<td>241, 156</td>
<td>Carbanate hydrolysis (R374699)</td>
<td>N.D.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>LC-MS/MS</td>
<td>55.7–56.2</td>
<td>724.2734(^c)</td>
<td>724.2751</td>
<td>568</td>
<td>Glucuronidation of parent drug at the (4-aminobenzenesulfonyl)-isobutylamino-1-benzyl-2-hydroxypropyl moiety</td>
<td>N.D.</td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>33</td>
<td>LC-MS/MS coelution</td>
<td>56.4–57.1</td>
<td>564.2357(^b)</td>
<td>564.2380</td>
<td>408, 241, 218, 156, 113</td>
<td>Monohydroxylation at the benzyl function (R330689)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td>LC-MS/MS NMR coelution</td>
<td>57.9–58.7</td>
<td>564.2353(^b)</td>
<td>564.2380</td>
<td>408, 390, 241, 156, 113</td>
<td>Monohydroxylation at the isobutyl function toward a tertiary alcohol function (R426857)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>29</td>
<td>LC-MS/MS NMR coelution</td>
<td>64.8–66.0</td>
<td>564.2354(^b)</td>
<td>564.2380</td>
<td>408, 257, 202, 172, 113</td>
<td>Monohydroxylation at the ortho position with respect to the amine function of the aniline moiety (R330326)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

N.D., not detected; UD, unchanged drug; \([M + H]^+\) \(_{\text{exp}}\), calculated/theoretical accurate mass; \([M + H]^+\) \(_{\text{calc}}\), measured accurate mass.

\(^a\)Characteristic product ions for identified metabolites and for authentic standards (R319064, R426855, R374699, R330689, R426857, and R330326).

\(^b\)Obtained in the analysis of feces.

\(^c\)Obtained in the analysis of urine.
behaviors of the reference compound R319064 and of unchanged drug were identical. Therefore, unchanged drug was identified as the parent drug R319064 (darunavir).

The protonated molecular ion at \( m/z \) 408 on the electrospray ionization (ESI) mass spectrum of metabolite 6 showed a shift of 16 mass units relative to the authentic reference compound R374699 (\( m/z \) 392), indicating hydroxylation of the molecule. The fragment ion \( m/z \) 390, corresponding to a facile loss of water, suggested an aliphatic hydroxylation. The unchanged fragment ion at \( m/z \) 156 indicated that the hydroxylation did not take place at the aniline moiety. After isolation and purification from a human feces sample, NMR indicated that the hydroxylation took place at the isobutyl function of the molecule toward a tertiary alcohol. The proposed structure was synthesized, and the retention time and MS/MS spectra were compared. The fragmentation pattern and HPLC retention times of metabolite 6 and R426855 were identical.

The protonated molecular ion of metabolite 11 [\( m/z \) 584, a shift of 192 mass units (176 + 16), compared with metabolite 19] suggested a glucuronidation and hydroxylation of metabolite 19. The same shift of 192 mass units was seen for the ion at \( m/z \) 348 (156 + 192), indicating that the glucuronidation and hydroxylation took place at the aniline part of the molecule. Other ions in the MS/MS spectrum had shifts of 16 mass units (\( m/z \) 156 to 172 and \( m/z \) 241 to 257), pointing to an aromatic hydroxylation rather than an hydroxylamine.

The protonated molecular ion of metabolite 15 (\( m/z \) 408) had a shift of 140 mass units compared with the parent drug and a shift of 16 mass units compared with metabolite 19, suggesting carbamate hydrolysis in combination with hydroxylation. The base peak in the
MS/MS spectrum was also shifted 16 mass units (m/z 156 to 172), indicating that an aromatic hydroxylation took place at the aniline part of the molecule. Metabolite 15 might be formed by carbamate hydrolysis of metabolite 29 as can be derived from the similarity in the MS² spectra of both metabolites. However, the two possible hydroxylation positions on the aniline moiety can not be differentiated by mass spectrometry.

The protonated molecular ion at m/z 740 in the ESI mass spectrum of metabolite 17 and the base peak in the MS/MS spectrum at m/z 584 had a shift of 192 mass units (176 + 16) compared with the drug substance fragmentation (m/z 548 and 392, respectively), suggesting combined glucuronidation and hydroxylation at the [4-(aminobenzenesulfonyl)-isobutyl-amino]-1-benzyl-2-hydroxy-propyl moiety. Fragment m/z 113 was unchanged, confirming that the hexahydro-furo[2,3]furan moiety is unchanged.

Metabolites in Plasma between 53 and 56 Min. At retention times between 53 and 56 min, protonated molecular ions at m/z 724, 392, 740, and 408 were found in the ESI mass spectra of directly injected human plasma. Metabolites were identified on the basis of retention time and accurate mass and originated from carbamate hydrolysis (m/z 392; metabolite 19), carbamate hydrolysis plus monoxygenation (m/z 408; metabolite 15), glucuronidation (m/z 724; metabolites 18 and 20), and glucuronidation plus monoxygenation of the parent drug (m/z 740; metabolite 17).

Discussion

The aim of the current study was to characterize the excretion and metabolism of a single 400-mg dose of [14C]darunavir in human male subjects in the absence (unboosted) and presence (boosted) of low-dose ritonavir (100 mg b.i.d.) and to elucidate metabolic pathways and structures of the metabolites formed.

Darunavir was rapidly absorbed in unboosted and boosted subjects, with no relevant difference in t_max, indicating that any acute inhibition of the efflux transporter P-glycoprotein (P-gp) by coadministered ritonavir, a known P-gp inhibitor (Kugeyama et al., 2005), had little effect on the transport of darunavir across the gastrointestinal lumen. This in vivo finding concurs with in vitro findings from a CaCo-2 cell model in which P-gp was found to modulate darunavir permeation but with a limited effect at higher concentrations of darunavir (i.e., >100 μM or 55 μg/ml) (Lachau-Durand et al., 2006) that are clinically relevant.

The coadministration of ritonavir had a marked effect on the systemic exposure to darunavir, compared with the administration of darunavir alone, with an approximately 11-fold higher exposure to darunavir (AUC_0–τ) in plasma of boosted subjects. The exposure to total radioactivity in plasma was 5.9-fold higher in boosted subjects, indicating that the combined exposure to darunavir and its metabolites in plasma was also notably higher in these subjects.

Ritonavir had no substantial effect on the total excretion of [14C]-darunavir-related radioactivity. At 1 week after dosing, the mean total recovery of radioactivity in urine and feces was approximately 94% of the administered radioactivity in unboosted and boosted subjects. In both cases, most of the radioactivity was recovered in feces (approximately 80%), and only small proportions of darunavir and its metabolites were excreted in urine (up to approximately 14%). In total, the mass balance of unchanged darunavir and identified metabolites was low, particularly in unboosted subjects. However, the recovery of radioactivity was almost complete after extraction from the feces samples in which the major fraction of radioactivity (>80%) was found. Because darunavir is extensively metabolized specially when it is unboosted, relatively low percentages of the urine and feces radioactivity were profiled due to the presence of several unidentified metabolites, each accounting for a small amount of the dose. Because the analyzed feces samples contained on average 95.1% of the total radioactivity excreted via the feces, it is unlikely that a major darunavir metabolite remained unidentified.

Darunavir is almost exclusively metabolized by CYP3A4 in human hepatocytes and subcellular fractions (Mamidi et al., 2005). As with other HIV drugs that are CYP3A4 substrates (Bu et al., 2004; Zeldin and Petruschke, 2004), the strong inhibition of CYP3A4 by coadministered ritonavir reduced the metabolism of darunavir, with a corresponding increase in the systemic bioavailability of the parent drug. Darunavir administered alone was extensively metabolized, with excretion of the unchanged drug accounting for 8.0% of the dose. In contrast, when coadministered with low-dose ritonavir, darunavir metabolism was considerably reduced. The excretion of unchanged drug accounted for 48.8% of the dose in boosted subjects due to...
inhibition of darunavir metabolism by ritonavir. Unchanged darunavir in urine accounted for 1.2% of the dose in unboosted subjects and 7.7% in boosted subjects, indicating that only a low proportion of the parent drug was excreted renally. The exact mechanism by which ritonavir inhibited the metabolism of darunavir is unknown. The direct inhibition of CYP3A4 is very likely, although other mechanisms, such as the inhibition of sodium-dependent and -independent hepatocyte uptake (McRae et al., 2006), cannot be ruled out. Any ritonavir-induced reduction in hepatocyte uptake of darunavir would decrease the availability of darunavir for CYP3A metabolism, which would in
In vitro studies using hepatocytes and liver subcellular fractions from mice, rats, rabbits, dogs, and humans confirmed three major phase I metabolic pathways for darunavir: carbamate hydrolysis, isobutyl aliphatic hydroxylation, and aniline aromatic hydroxylation (Vermeir et al., 2006). In general agreement, in vivo metabolic pathways of darunavir in the current study in humans included carbamate hydrolysis, aliphatic and aromatic hydroxylation, and glucuronidation (Fig. 7). The main effect of boosting with ritonavir was inhibition of carbamate hydrolysis, isobutyl aliphatic hydroxylation, and aniline aromatic hydroxylation, resulting in increased exposure to unchanged drug (Fig. 8). Glucuronidation, which was a minor metabolic pathway in vitro and in vivo, was stimulated in boosted subjects.

In vivo, in the absence of ritonavir, the metabolism of darunavir in animals was qualitatively similar to the metabolism observed in unboosted humans. In rats and dogs, as in humans and as seen in vitro, three major phase I metabolic reactions were identified: carbamate hydrolysis, isobutyl aliphatic hydroxylation, and aniline aromatic hydroxylation (M. Vermeir, S. Cameron, and P. Annaert, unpublished data). In dogs, as in humans, the major phase I metabolic pathway was carbamate hydrolysis, whereas in rats isobutyl aliphatic hydroxylation toward a tertiary alcohol as well as toward a primary or secondary alcohol was a major biotransformation. Phase II glucuronidation was a minor pathway in all species. The qualitative similarity of the major metabolic pathways of darunavir in rats, dogs, and humans indicates that these animal species are suitable for assessing aspects of darunavir safety in humans.

Although similar major metabolic pathways were observed in rats, dogs, and humans, some additional metabolic conversions in rats were not observed in vivo in humans although they were seen in vitro, namely alicyclic hydroxylation at and oxidative ring opening of the hexahydrofuran fused rings.

The main route of excretion for [14C]darunavir was via the feces in rats (94% of the dose) and dogs (86%), as also seen in unboosted humans (82%). Urinary excretion was only approximately 4% of the administered dose in rats and dogs, compared with 12.2% in humans. Unchanged darunavir was excreted mainly via feces and accounted for up to 12.3% of the administered dose in rats and 26% in dogs, compared with 6.8% in humans. In rats, biliary excretion played a major role in the elimination of [14C]darunavir (54% of the radioactive dose excreted in the bile with unchanged compound represented approximately 1% of the dose).

It is interesting to note that coadministered ritonavir had limited or no effect on in vivo bioavailability of darunavir in mice, rats, and dogs. This contrasts with the marked reduction in metabolism of darunavir, and consequent increase in its oral bioavailability, when coadministered with ritonavir in humans. In repeated dose toxicity studies, the increase in darunavir exposure in the presence of ritonavir was highest in rabbits (15-fold), modest in mice (2-fold) and rats (4-fold), and nonexistent in dogs (Lachau-Durand et al., 2005; unpublished data). The reasons for such a discrepancy between rabbits and dogs are not clear. However, in mice and rats, darunavir induces hepatic microsomal CYP3A, which probably played a key role in masking the overall effect of ritonavir on darunavir exposure. In line with its CYP3A effect, ritonavir had inhibitory effects on various metabolic pathways in rats, including aromatic hydroxylation oxidative ring opening and carbamate hydrolysis. No difference in the excretion of radioactivity was seen in rats after administration of [14C]darunavir in the absence or presence of coadministered ritonavir.

The metabolic disposition of [14C]darunavir in the current study fell within the ranges reported for other PIs administered to healthy subjects. After oral administration of 14C-labeled amprenavir, saquinavir, indinavir, ritonavir, or nelfinavir, the recovery of radioactivity ranged from 75 to 88% in feces and from 1 to 19% in urine (Sadler et al., 2001).

Amprenavir, administered as the produg fosamprenavir, is the PI with the closest structural resemblance to darunavir. As with darunavir, amprenavir is almost exclusively metabolized via CYP3A4 (Decker et al., 1998), and the systemic exposure to amprenavir can therefore be boosted by coadministration of ritonavir (Wier et al., 2006). The pattern of radiocarbon recovery after administration of unboosted [14C]amprenavir was comparable to the recovery after administration of [14C]darunavir, with 75% of the administered dose recovered in feces and 14% in urine (Sadler et al., 2001). However, amprenavir is metabolized to a greater extent than darunavir, with unchanged amprenavir being below the limit of quantification in feces and urine. Despite their structural similarity, the metabolism of amprenavir differs from that of darunavir. Two primary metabolites of [14C]amprenavir accounted for 94% of the radioactivity recovered in feces, resulting from dioxidation of the tetrahydrofuran ring and subsequent oxidation of the p-aniline sulfonate group (Sadler et al., 2001). As seen with darunavir, there was some variability in the metabolism of amprenavir between humans and animals (dogs and rats).

In conclusion, darunavir was extensively metabolized in unboosted subjects, mainly by carbamate hydrolysis, isobutyl aliphatic hydroxylation, and aniline aromatic hydroxylation, to a lesser extent by benzyclic aromatic hydroxylation, and to only a minor extent by glucuronidation. In contrast to some animal species, pharmacokinetic boosting of darunavir with low-dose ritonavir resulted in a marked reduction in the metabolism of darunavir due to CYP3A inhibition and possibly also inhibition of hepatocyte uptake, by ritonavir. These effects led to an increase in the systemic exposure of darunavir. The effects of coadministered ritonavir on the metabolism of darunavir included significant inhibition of carbamate hydrolysis, isobutyl aliphatic hydroxylation, and aniline aromatic hydroxylation, with no effect on benzyclic aromatic hydroxylation, whereas excretion of gluc-
uroside metabolites was stimulated while still representing only a
minor pathway.

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