IN VIVO METABOLISM OF [14C]RUBOXISTAURIN IN DOGS, MICE, AND RATS FOLLOWING ORAL ADMINISTRATION AND THE STRUCTURE DETERMINATION OF ITS METABOLITES BY LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY AND NMR SPECTROSCOPY

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ABSTRACT:
Ruboxistaurin (LY333531), a potent and isomor-selective protein kinase C \( \beta \) inhibitor, is currently undergoing clinical trials as a therapeutic agent for the treatment of diabetic microvascular complications. The present study describes the disposition and metabolism of \[14C\]ruboxistaurin following oral administration of an oral dose to dogs, mice, and rats. The study revealed that ruboxistaurin was highly metabolized in all species. Furthermore, the results from the bile duct-cannulated study revealed that ruboxistaurin was well absorbed in rats. The primary route of excretion of ruboxistaurin and its metabolites was through feces in all species. The major metabolite detected consistently in all matrices for all species was the \( N \)-desmethyl metabolite 1, with the exception of rat bile, in which hydroxy \( N \)-desmethyl metabolite 5 was detected as the major metabolite. Other significant metabolites detected in dog plasma were 2, 3, 5, and 6 and in mouse plasma 2, 5, and 19. The structures of the metabolites were proposed by tandem mass spectrometry with the exception of 1, 2, 3, 5, and 6, which were additionally confirmed either by direct comparison with authentic standards or by nuclear magnetic resonance spectroscopy. To assist identification by nuclear magnetic resonance spectroscopy, metabolites 3 and 5 were produced via biotransformation using recombinant human CYP2D6 and, likewise, metabolite 6 and compound 4 (regiosomer of 3 which did not correlate to metabolites found in vivo) were produced using a microbe, Mortierella zonata. The unambiguous identification of metabolites enabled the proposal of clear metabolic pathways of ruboxistaurin in dogs, mice, and rats.

Materials and Methods
Materials. Ruboxistaurin mesylate (Jirousek et al., 1996), two forms of \[14C\]ruboxistaurin mesylate (O’Bannon et al., 2004), compounds 1 and 2 (Jirousek et al., 1996), and compounds 6 and 7 were prepared at Eli Lilly and Company (Indianapolis, IN). \[14C\]Ruboxistaurin with two radiocarbons on the C-2 position of the two indole ring carbons (form 1; radiochemical purity 98.4% and specific activity 37.2 \( \mu \)Ci/mg) was used in the mouse and rat studies, and \[14C\]ruboxistaurin with radiocarbon on one of the carbonyl

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine-specific intracellular enzymes, which regulate a variety of cellular functions including cell growth, metabolism, and differentiation (Nishizuka, 1986, 1988; Farago and Nishizuka, 1990). Uncontrolled activation of PKC has been implicated in the progression of numerous disease processes (Bradshaw et al., 1993). Mammalian PKC, grouped into three subclasses, consists of approximately 13 isoforms (Mellor and Parker, 1998) that differ in structure and cofactor requirements (Newton, 2003). Studies have shown that \( \beta I \) and \( \beta II \) isoform-specific activation of PKC has been implicated in the development and progression of several diabetic microvascular complications, including diabetic retinopathy (Ishii et al., 1996; Suzuma et al., 2002; Yokota et al., 2003; Curtis and Schofield, 2004). In in vivo preclinical models, ruboxistaurin (LY333531) has been found to be an orally effective PKC\( \beta \)-selective inhibitor with nanomolar potency (Jirousek et al., 1996). Ruboxistaurin has demonstrated efficacy at nontoxic doses in preventing the development and progression of diabetic retinopathy in preclinical animal models (Ishii et al., 1996; Aiello et al., 1997; Danis et al., 1998; Nakamura et al., 1999), was well tolerated, and improved diabetes-induced retinal blood flow abnormalities in patients (Aiello et al., 1999). Ruboxistaurin is currently in phase III clinical trials for the treatment of diabetic microvascular complications.

Previously, the disposition of ruboxistaurin (LY333531) and its \( N \)-desmethyl metabolite 1 (major circulating and equally active as that of ruboxistaurin) in rats and beagle dogs following administration of a single oral dose was reported (Burkey et al., 2002). The study found, in general, that disposition of \[14C\]ruboxistaurin was comparable in rats and dogs. The primary route of excretion in both species was found to be fecal with a substantial biliary component. However, that report did not identify the various phase I and II metabolites detected in plasma and excreta. Therefore, the aim of the present study was to explore in detail the in vivo metabolism following oral administration of \[14C\]ruboxistaurin to dogs, mice, and rats.

ABBREVIATIONS: PKC, protein kinase C; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; P450, cytochrome P450; DQCOsy, double quantum-filtered correlation spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation.
carbons of the maleimide group (form II; radiochemical purity 98.8% and specific activity 97.3 μCi/mg) was used in the dog study. Figure 1 shows the structure of [14C]ruboxistaurin. The microorganism Mortierella zonata was obtained from the culture collection of Eli Lilly and Company. Recombinant human CYP2D6 and CYP2D6 reaction mixture were purchased from BioAnalytics Inc. (Pasadena, CA). All other reagents and solvents were either analytical grade or HPLC grade. Male Fischer 344 rats, male CD-1 mice, and female beagle dogs were obtained from Taconic Farms (Germantown, NY), Charles River Laboratories, Inc. (Wilmington, MA), and Marshall Farms (North Rose, NY), respectively.

**Dosing and Sample Collection.** A single dose of [14C]ruboxistaurin mesylate (form I), suspended in 10% acacia in water, was administered to mice (75 mg/kg; n = 24), rats (5 mg/kg; n = 9), and bile duct-cannulated rats (5 mg/kg; n = 6) by oral gavage. A single dose of [14C]ruboxistaurin mesylate (form II), suspended in 10% acacia and 0.05% Dow Corning Antifoam 1510-US in water, was administered to female beagle dogs (10 mg/kg; 214 BARBUCH ET AL.)

**Metabolite Profiling.** The microorganism Mortierella zonata was obtained from the culture collection of Eli Lilly and Company. Recombinant human CYP2D6 and CYP2D6 reaction mixture were purchased from BioAnalytics Inc. (Pasadena, CA). All other reagents and solvents were either analytical grade or HPLC grade. Male Fischer 344 rats, male CD-1 mice, and female beagle dogs were obtained from Taconic Farms (Germantown, NY), Charles River Laboratories, Inc. (Wilmington, MA), and Marshall Farms (North Rose, NY), respectively.

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**Analysis of Radioactivity.** Radioactivity in plasma, bile, and urine was determined by liquid scintillation counting on a Tri-Carb model 2300 TR liquid scintillation spectrometer (PerkinElmer Life and Analytical Sciences, Boston, MA) by adding Ultima Gold scintillation fluid to a known amount of the sample. Radioactivity in feces was determined by combustion of a known amount of fecal homogenate with a PerkinElmer Sample Oxidizer model 307 (PerkinElmer Life and Analytical Sciences) followed by liquid scintillation counting. Determination of radioequivalents, data acquisition and storage, and statistical analysis were performed using ADME/WinPET and ADME/LIMS systems (Eli Lilly and Company).

**LC/Radioactivity Analysis.** Chromatography was performed on a Waters 2690 Separations Module using either a YMC Basic 55 column (4.6 × 150, 5-μm particle size) or a Phenomenex Synergi Polar-RP column (4.6 × 250 mm, 4-μm particle size; Phenomenex, Torrance, CA) with a flow rate of 1 ml/min. The solvent system consisted of 10 mM aqueous ammonium acetate (mobile phase A) and acetonitrile (mobile phase B), and the analytes were eluted using a gradient profile: 0 min/10% B, 50 min/60% B, 50.1 min/80% B, 52 min/80% B, or a similar gradient profile. Radiodetection was performed either using a Berthold LB590 Radioflow detector (Berthold Technologies, Oakridge, TN) by means of a 500-μl liquid flow cell with a flow rate of liquid scintillant (Ultra Flo AP; PerkinElmer Life and Analytical Sciences) to mobile phase ratio of 3:1, or by collecting the column effluent into a Deepwell Luma-96 solid scintillation-coated plate (PerkinElmer Life and Analytical Sciences), which then was dried and sealed with PerkinElmer Topseal A microplate film. The radioactivity from the plates was analyzed by a Topcount NXT microplate scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences). The HPLC column recovery of radioactivity ranged from 90 to 116% for all matrices.

**LC/MS Analysis.** The HPLC system consisted of a Shimadzu VP series (Shimadzu Scientific Instruments Inc., Columbia, MD) including a SIL-10AXL autosampler and a Surveyor photodiode array detector. Chromatographic separations were carried out either on a Phenomenex Synergi Polar-RP column (3.0 × 150 mm, 4-μm particle size) with a flow rate of 0.4 ml/min (or a Waters YMC Basic column (3.0 × 150 mm, 5-μm particle size). The mobile phase consisted of 10 mM aqueous ammonium acetate (mobile phase A) and acetonitrile (mobile phase B), and the analytes were eluted using a gradient profile: 0 min/10% B, 5 min/10% B, 50 min/75% B, 50.1 min/90% B, 52 min/100% B, or a similar gradient profile. Radiodetection was performed either using a Berthold LB590 Radioflow detector (Berthold Technologies, Oakridge, TN) by means of a 500-μl liquid flow cell with a flow rate of liquid scintillant (Ultra Flo AP; PerkinElmer Life and Analytical Sciences) to mobile phase ratio of 3:1, or by collecting the column effluent into a Deepwell Luma-96 solid scintillation-coated plate (PerkinElmer Life and Analytical Sciences), which then was dried and sealed with PerkinElmer Topseal A microplate film. The radioactivity from the plates was analyzed by a Topcount NXT microplate scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences). The HPLC column recovery of radioactivity ranged from 90 to 116% for all matrices.

**TABLE 1**

<table>
<thead>
<tr>
<th>Time</th>
<th>Dog</th>
<th>Mouse</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>Feces</td>
<td>Urine</td>
</tr>
<tr>
<td>0–24 h</td>
<td>0.74 ± 0.09</td>
<td>49.1 ± 19.7</td>
<td>2.48 ± 0.17</td>
</tr>
<tr>
<td>24–48 h</td>
<td>0.31 ± 0.07</td>
<td>30.8 ± 17.5</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>48–72 h</td>
<td>0.12 ± 0.02</td>
<td>6.9 ± 3.1</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>72–96 h</td>
<td>0.06 ± 0.01</td>
<td>2.35 ± 1.46</td>
<td>N.A.</td>
</tr>
<tr>
<td>96–120 h</td>
<td>0.03 ± 0.01</td>
<td>0.71 ± 0.41</td>
<td>N.A.</td>
</tr>
<tr>
<td>Matrix total</td>
<td>1.25 ± 0.27</td>
<td>89.9 ± 0.6</td>
<td>2.52 ± 0.17</td>
</tr>
<tr>
<td>Total percentage recovery</td>
<td>92.0 ± 0.6</td>
<td>91.4 ± 1.8</td>
<td>100.0 ± 1.5</td>
</tr>
</tbody>
</table>

N.A., not applicable.

* For dog, total percentage recovery includes the cage wash, and for mice and rats, it includes the cage wash and the carcass.
Mass spectrometric analysis was conducted on a Finnigan TSQ Quantum mass spectrometer (Thermo Electron Corporation, Waltham, MA) equipped with an electrospray ion source. Accurate mass determination was performed using a Waters Micromass Q-TOF II quadrupole/orthogonal time-of-flight mass spectrometer. The protonated ion \((m/z 311.0814)\) of sulfadimethoxine (Sigma-Aldrich, St. Louis, MO) was used as the lock mass in all accurate mass determinations.

Microbial Transformation of Ruboxistaurin. Frozen microbial stock culture of \(M.\ zonata\) (0.3 ml to each flask) was incubated at 25°C and 250 rpm for 4 days with 20 ml of Traders Fungal Veg medium (total three flasks), which consisted of 25 g of dextrose (Corn Products International, Bedford Park, IL) and 25 g of cottonseed flour (Traders Protein Southern Cotton Oil Co., Lubbock, TX) in 1 liter of tap water. At the end of this period, the seed culture (12 ml to each flask) was inoculated at 25°C and 250 rpm into three flasks, each containing 176 ml of biotransformation medium, which consisted of dextrose (20 g), soybean meal (5 g), NaCl (5 g), yeast extract (5 g), and \(K_2HPO_4\) (5 g) in 1 liter of distilled water. After 2 days of growth, ruboxistaurin (110 mg in 4.4 ml of DMSO) was added to the fermentation. After 10 days, the fermentation was harvested, and the cells were removed by centrifugation and extracted with 500 ml of methanol. The methanol extract was used for the purification of metabolites.

CYP2D6-Catalyzed Biotransformation of Ruboxistaurin. Ruboxistaurin (55 mg) was suspended in 2 ml of isopropanol. CYP2D6 Mix consisting of buffer, stabilizers, and the NADPH recycling system (35 g) was dissolved in 90 ml of water. CYP2D6 (100 nmol) was suspended in 20 ml of water. To the CYP2D6 Mix solution, 8 ml of the CYP2D6 suspension followed by 400 \(\mu l\) of compound suspension were added. The reaction mixture was shaken at 150 rpm at 30°C. Subsequent additions of CYP2D6 (4 ml each) and the compound (200

![Figure 2](image2.png)
μl each) were made at 2, 4.5, and 6 h after the start of the reaction. After the final addition, the reaction mixture was incubated at 30°C at 150 rpm overnight. The reaction was terminated by the addition of 90 ml of ethanol, shaken at 150 rpm at 30°C for 1 h, and centrifuged at 3000 rpm for 25 min, and the supernatant was used for the purification of metabolites.

**Purification of Metabolites.** The HPLC instrumentation consisted of either a Waters 600 model HPLC system equipped with a Waters Micromass 2M-D mass spectrometer (Waters) or a Shimadzu VP series (Shimadzu Scientific Instruments Inc.) equipped with a Thermo Electron LCQ spectrometer. Preparative separations were performed on a Supelco Discovery C18 column (10 × 250 mm; Supelco, Bellefonte, PA) with a flow rate of 4 ml/min. Analytical separations were performed on a Supelco Discovery C18 column (4.6 × 250 mm) with a flow rate of 1 ml/min. The mobile phase consisted of 10 mM aqueous ammonium acetate (mobile phase A) and acetonitrile (mobile phase B).

The supernatant from the 2D6 incubation was evaporated to approximately 100 μl and applied on two C18 preconditioned SPE columns. Each column was washed with 50 μl of water followed by 50 μl of methanol. The combined methanol extract was dissolved in 20 ml of 4:1 methanol/water, and divided into six portions, and each portion was chromatographed separately on a Supelco Discovery C18 column (10 × 250 mm) with a flow rate of 4 ml/min using a gradient profile: 0 min/10% B, 5 min/10% B, 50 min/60% B, 50.1 min/90% B, and 55 min/90% B. Column eluents were appropriately combined to yield fractions A (mostly containing metabolite 1), B, C (mostly containing metabolite 3), 1 and 90% of the dose was excreted in urine and feces, respectively, over 120 h. In mice, ~3 and 88% of the administered dose was recovered in urine and feces, respectively, over 72 h. In bile duct-cannulated rats, ~3, 34, and 59% of the dose was recovered in urine, feces, and bile, respectively, over 48 h. Thus, no significant difference was observed in the elimination routes of ruboxistaurin in any species.

**Metabolite Profiles of Ruboxistaurin.** The plasma and excreta samples from the dog study were analyzed in detail to profile the metabolites in these matrices, and the results from this study were extended to the other species. Representative radiochromatograms of 2-h plasma, 0- to 24-h feces, and 0- to 24-h urine after oral administration of a single dose of [14C]ruboxistaurin are shown in Table 1. In dogs, ~1 and 90% of the dose was excreted in urine and feces, respectively, over 120 h. In mice, ~3 and 88% of the administered dose was recovered in urine and feces, respectively, over 72 h. In bile duct-cannulated rats, ~3, 34, and 59% of the dose was recovered in urine, feces, and bile, respectively, over 48 h. Thus, no significant difference was observed in the elimination routes of ruboxistaurin in any species.

**Results**

**Excretion Profiles.** The recovery of radioactivity in urine and feces from female beagle dogs and male CD-1 mice, and in bile, urine, and feces from male bile duct-cannulated Fischer 344 rats following oral administration of a single dose of [14C]ruboxistaurin is summarized in Table 1. In dogs, ~1 and 90% of the dose was excreted in urine and feces, respectively, over 120 h. In mice, ~3 and 88% of the administered dose was recovered in urine and feces, respectively, over 72 h. In bile duct-cannulated rats, ~3, 34, and 59% of the dose was recovered in urine, feces, and bile, respectively, over 48 h. Thus, no significant difference was observed in the elimination routes of ruboxistaurin in any species.

**Table 2** Percentage radioactivity of ruboxistaurin and its metabolites in plasma at 2, 4, 8, 16, 24, and 48 h postdose following a single oral administration of 10 mg/kg [14C]ruboxistaurin in female beagle dogs

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>16 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruboxistaurin</td>
<td>20.0 ± 1.4</td>
<td>9.9 ± 1.9</td>
<td>10.5 ± 1.8</td>
<td>15.0 ± 4.8</td>
<td>6.4 ± 1.3</td>
<td>3.4 ± 1.4</td>
</tr>
<tr>
<td>1</td>
<td>59.4 ± 3.8</td>
<td>45.9 ± 14.3</td>
<td>20.2 ± 5.0</td>
<td>39.1 ± 2.3</td>
<td>40.6 ± 14.3</td>
<td>15.4 ± 8.7</td>
</tr>
<tr>
<td>2</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>2.1 ± 0.9</td>
<td>1.6 ± 0.7</td>
<td>0.6 ± 0.4</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>3</td>
<td>3.4 ± 0.3</td>
<td>2.3 ± 0.4</td>
<td>2.6 ± 0.6</td>
<td>1.6 ± 0.2</td>
<td>0.8 ± 0.5</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>5</td>
<td>0.9 ± 0.1</td>
<td>1.4 ± 0.7</td>
<td>2.1 ± 0.7</td>
<td>1.4 ± 0.3</td>
<td>0.5 ± 0.4</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>N.Q.</td>
<td>0.3 ± 0.3</td>
<td>2.4 ± 1.7</td>
<td>1.3 ± 1.1</td>
<td>4.2 ± 1.9</td>
<td>7.4 ± 4.6</td>
<td></td>
</tr>
</tbody>
</table>

N.Q., not quantifiable.

**Table 3** Percentage radioactivity of ruboxistaurin and its metabolites in plasma at 2, 4, and 8 h postdose following a single oral administration of [14C]ruboxistaurin in male CD-1 mice and male Fischer 344 rats

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>CD-1 Mice (4 h)</th>
<th>Fischer 344 Rats (8 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruboxistaurin</td>
<td>11.0</td>
<td>10.0</td>
</tr>
<tr>
<td>1</td>
<td>47.0</td>
<td>29.9</td>
</tr>
<tr>
<td>2</td>
<td>&lt;1.0</td>
<td>N.D.</td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>N.D.</td>
</tr>
<tr>
<td>19</td>
<td>9.0</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not detected.

a Plasma were pooled per time point.
matogram, metabolite 6 was not detected but was detected in all later samples.

In mice, parent (11% of the radioactive material in the sample), and metabolites 1 (47%), 2 (<1%), 5 (2%), and 19 (9%) were detected in 4-h pooled plasma samples. In 0- to 24-h urine, the radioactivity excreted was associated predominantly with metabolite 1 (41% of 25% of the dose excreted in this matrix), and in feces, the radioactivity excreted was associated with mostly parent (51.8% of 88% of the dose excreted in this matrix) and metabolite 1 (15.2%). In addition, 15 and 13 other metabolites were recovered from urine and feces, respectively (Table 5).

In rats, parent and only metabolite 1 were detected in the 2-h plasma sample. In bile duct-cannulated rats, over the 0- to 24-h collection period, approximately 63% of the radioactivity (data not shown) from 57% of the dose excreted in the bile was found to be hydroxy N-desmethyl metabolite 5. In addition, 12 other metabolites were detected in this matrix. In urine and feces, over the 0- to 24-h collection period, the predominant metabolite excreted was 1. In addition, four and nine other metabolites were excreted in urine and feces, respectively (Table 6).

**Biosynthesis and Isolation of Ruboxistaurin Metabolites.** Certain metabolites were of particular interest due to the fact that they were circulating in the plasma of dogs. To unambiguously determine the structures of these metabolites by NMR spectroscopy, recombinant human P450s and microbial cultures were screened for the production of sufficient amounts of these metabolites. Ruboxistaurin was incubated with recombinant human CYP2D6, which produced metabolites 3 and 5 in approximately 2 to 3% yields. Subsequent large-scale incubation with 55 mg of ruboxistaurin followed by chromatographic purification resulted in the isolation of approximately 0.2 and 0.1 mg of 3 and 5, respectively, suitable for structure determination by NMR. Similarly, microbial incubation with *M. zonata* produced metabolites 4 (3% yield) and 6 (1% yield). Metabolite 4 did not correlate with any in vivo dog metabolite but was found to be unique to microbial metabolism (similar direct comparison of 4 with in vivo metabolites of mice and rats was not carried out). After initial chromatographic purification, the mixture of 4 and 6 was subjected to LC/NMR. Although LC/NMR detected the two compounds cleanly,
the need to perform extensive two-dimensional NMR experiments, at least with 4, demanded drying down the sample for conventional NMR using a fully deuterated solvent.

**Structure Identification of Metabolites of Ruboxistaurin.** In general, structures of most of the metabolites detected in dog plasma and excreta were positively identified, and the results were then

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**FIG. 3.** Mass spectral fragmentation schemes. A, ruboxistaurin; B, metabolite 7; and C, metabolites 10 and 11.
Ruboxistaurin showed product ions at $m/z$ 424 and 289 (same as parent), 84, 70, and 44. The product ions observed at $m/z$ 440 (loss of dimethylamine plus a neutral moiety of formula C$_4$H$_9$NO$_2$), and $m/z$ 98, 84, and 58 supported hydroxylation of indole ring A. To define regiochemistry, metabolite 3 produced via biocatalysis of ruboxistaurin was subjected to NMR analysis. Detailed analysis of $^1$H and two-dimensional NMR (DQ COSY, HSQC, and HMBC) data of 3 led to the unambiguous assignment of all the protons and carbons (Tables 7 and 8) and structure assignment of 3, including the regiochemistry of oxidation. In contrast to ruboxistaurin, which showed two sets of aromatic four-proton spin systems due to two indole units, 3 showed one set of an aromatic four-proton spin system and one set of a three-proton aromatic spin system (Fig. 4), revealing hydroxylation of the phenyl part of one of the indole rings. Although ruboxistaurin possesses a near C2 symmetry, the two indole units can be distinguished due to the asymmetry of the macrocycle. Thus, the methylene groups alpha to the indole nitrogens can easily be distinguished based upon the spin connectivity.
on their attachment to the neighboring group (CH₂-CH versus CH₂-O). Long-range HMBC correlations from these groups will then facilitate identification of the proton and carbon resonances of the respective indole rings. However, due to the low concentration of 3, the HMBC experiment optimized for 8 Hz heteronuclear coupling did not provide the expected correlation from the methylene groups to the indole ring protons and carbons.

Fortuitously, the structure of another aromatic hydroxylated metabolite obtained from a microbial (M. zonata) biotransformation of ruboxistaurin, which was not correlated to any dog metabolite found in vivo and whose structure was unambiguously established by MS and NMR data as 4, was very helpful in the structure determination of 3. The NMR results of 4, reported in Tables 7 and 8, were obtained after a detailed analysis of ¹H, DQCOSY, HSQC, and HMBC. Unlike 3, the HMBC spectrum of 4 showed the anticipated correlations (Fig. 5) that established the location of the hydroxyl group. Thus, key multiple-bond heteronuclear correlations observed between H-10 and C-2, and H-21 and C-19, along with COSY cross-peaks observed between H-10 and H-11, and H-19 and H-18, first identified the pyrrolyl proton of the individual indole units with respect to the aliphatic portion of the macrocycle. Then, strong three-bond correlations observed in the HMBC experiment, optimized for 8 Hz heteronuclear coupling, from H-2 (δH 7.35, s) to C-4 (δC 127.1), which in turn showed correlation to H-8 (δH 7.28 d, J = 8.5 Hz), and from H-2 to C-9 (δC 129.9), which in turn showed correlation to H-5 (δH 7.17 d, J = 2 Hz), established the placement of the hydroxyl group at C-6. Furthermore, the up-field chemical shift (~6 ppm) of C-9 and the near constant chemical shift of C-4 observed in 4 when compared with ruboxistaurin (Table 8) are also consistent with the placement of the hydroxyl group at C-6. Accordingly, in the MS/MS spectrum of 4, a product ion at m/z 289 was observed as in 3, suggestive of hydroxylation of indole ring A. The fact that 3 and 4 showed the fragment ion at m/z 289, consistent with oxidation of indole ring A (see Fig. 3), and identical 4- and 3-proton spin systems (Fig. 4) for the indole rings, as evidenced by NMR, revealed that 3 and 4 are indeed regiosomers, and the possible location of the hydroxyl group in 3 was at C-7 instead of C-6 as in 4. The placement of the hydroxyl group at C-7 in 3 was further corroborated by the significant up-field shift (~6 ppm) observed for C-4 and a near constant chemical shift observed for C-9 in comparison with 4, when compared with ruboxistaurin.

Metabolite 5 revealed a protonated molecular ion at m/z 471, 14 Da less than 3, suggesting that it was an N-demethylated product of 3. Accordingly, in the MS/MS, significant product ions were observed at m/z 289 (defining the site of hydroxylation as indole ring A) and m/z 84 (defining the loss of N-methyl group compared with the parent), in addition to ions at m/z 453, 440, 428, 414, and 396. The structure of 5 was further confirmed by NMR. The notable feature in the ¹H NMR spectra of 5 and 3 was the close similarity, both in chemical shifts and coupling constants, of the aromatic resonances (Fig. 4), indicating that the hydroxyl group was located at C-7 in both compounds. Table 5 lists the entire proton chemical shifts of 5 in comparison with ruboxistaurin and 3. Due to limited sample availability, no carbon data were obtained for 5.

Metabolite 6 revealed a protonated molecular ion at m/z 485, 16 Da higher than the parent. The N-oxide structure was proposed for 6 based on the product ion observed at m/z 424 (loss of 61 Da from the parent), which was consistent with the loss of hydroxy-dimethylamine when compared with the parent, which showed the same product ion with the loss of dimethylamine. The structure of 6 was again confirmed by NMR as well as by direct comparison with a reference standard. The distinct feature in the ¹H NMR spectrum of 6 in contrast to the parent was the downfield shift (~0.25 ppm) exhibited by both the methyl groups attached to the nitrogen due to the oxidation of the amine to amine N-oxide.

Mouse and Rat. In addition to the metabolites 1, 2, and 5 identified in dog plasma, a glucuronide conjugate, 19, was also identified in mouse plasma. In rat plasma, however, only the N-desmethyl metabolite 1 was observed. Metabolite 19, detected only in the mouse plasma, showed a protonated molecular ion at m/z 647 and product ions at m/z 471, 440, 428, 414, and 289, suggesting hydroxylation of the N-desmethyl metabolite 1 followed by conjugation with glucuronic acid. The presence of the product ion at m/z 289 revealed hydroxylation of indole ring A; however, the exact position of hy-
FIG. 5. Selected $^1$H to $^{13}$C HMBC correlations of metabolites 3 and 4. The $J$-filter was optimized for $^1J_{C-H/11}$ = 140 Hz, and $N$ bond delay was set to 0.063 s corresponding to $^1J_{C-H/11}$ = 8 Hz.

TABLE 9

Product ion summary and interpretation of ruboxistaurin metabolites

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Product Ions</th>
<th>Identity</th>
<th>Dog</th>
<th>Mouse</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruboxistaurin</td>
<td>469 451, 424, 406, 398, 384, 289, 98, 84, 58</td>
<td>N-desmethyl</td>
<td>P</td>
<td>U</td>
<td>F</td>
</tr>
</tbody>
</table>

2 441 424, 423, 406, 398, 384, 289, 70   N,N-di-desmethyl  P U F  P U F  U B F
1 455 424, 412, 406, 398, 384, 289, 84  N-desmethyl        P U F  P U F  U B F
20 456 439, 413, 410, 385, 366, 354    Acid, oxidative deamination  F
17 471 454, 440, 428, 414, 289, 84     A-ring hydroxy-N-desmethyl  F
26 471 454, 453, 440, 428, 414, 390, 370, 327  hydroxy-N-desmethyl  F
10 471 453, 440, 428, 414, 197, 184, 156, 84  21-hydroxy-N-desmethyl  U F  U F  U B F
9 471 454, 428, 414, 271, 176, 169, 157, 145, 84  2-hydroxy-N-desmethyl  F
5 471 453, 440, 428, 414, 396, 289, 84  7-hydroxy-N-desmethyl  P U F  P U F  U B
15 471 453, 440, 428, 414, 305, 84  B ring-hydroxy-N-desmethyl  U F  U F  F
8 471 453, 440, 414, 271, 176, 169, 157, 145, 84, 70  2-hydroxy-N-desmethyl  U F  U F
11 471 453, 440, 197, 184, 156, 84  21-hydroxy-N-desmethyl  F
24 485 440, 414, 384, 271, 176, 169, 145, 98, 58  2-hydroxy  F
12 485 440, 422, 412, 400, 324, 184, 156, 98, 84, 58  21-hydroxy  U F  U F  B
3 485 467, 440, 242, 414, 289, 98, 84, 58  7-hydroxy  P U F  P U F  B U F
14 485 440, 305, 98, 84  B ring-hydroxy  U F  U F  F
7 485 467, 441, 440, 422, 414, 271, 176, 169, 145, 84, 58  2-hydroxy  U F  U F  F
13 485 440, 422, 412, 324, 184, 156, 98, 84, 58  21-hydroxy  U F  B
16 485 440, 422, 414, 289, 84, 58  A ring-hydroxy  F
6 485 468, 467, 424, 289, 98, 84, 58  N,N-dimethyl N-oxide  P  F
19 647 471, 440, 428, 414, 289  A ring-hydroxy-N-desmethyl + glucuronide  U  P  U  B
21 647 471, 440, 414  hydroxy-N-desmethyl + glucuronide  U  B
25 647 471  hydroxy-N-desmethyl + glucuronide  U  B
20 661 485, 440  hydroxy + glucuronide  U  B  F
22 661 485, 454, 440, 289, 58  A ring-hydroxy glucuronide  U  F  B
23 661 485, 440, 305  B ring-hydroxy glucuronide  U  B

P, plasma; U, urine; B, bile; F, feces.

$^3$ and $^{14}$ are not completely resolved in dog feces, and product ions listed for $^{14}$ may actually be product ions of $^3$. The major spectral difference is that $^3$ has an $m/z$ 289 ion and $^{14}$ has an $m/z$ 305 ion.

$^6$ was detected in a 4-h dog plasma sample.
droxylation and subsequent glucuronidation within the indole ring could not be determined from these data.

**Fecal Metabolites.** The metabolites 1, 2, 3, and 5 observed in plasma were also detected in feces. In addition, 10 other metabolites were detected, each less than 2% of the dose, and their structures were tentatively identified by MS with the exception of some metabolites whose structures could be confirmed by direct comparison with reference standards.

Metabolite 7 revealed a protonated molecular ion at \( m/z \) 485, 16 Da higher than the parent, and metabolites 8 and 9, isomeric in nature, showed identical protonated molecular ions at \( m/z \) 471, 14 Da less than 7. The product ion spectrum of 7 displayed an array of ions quite different from the ions observed in other oxidative metabolites such as 3, 5, and 6 (Table 9). Formulation of these ions at \( m/z \) 440, 271, 176, 169, 157, and 145, along with other ions, as shown in Fig. 3B, suggested oxidation of indole ring A at the C-2 position. The structure thus proposed by MS was further confirmed by direct comparison with a reference standard. The reference standard predominantly existed in the keto form as evidenced by NMR spectroscopy (data not shown). Metabolites 8 and 9 showed identical product ions at \( m/z \) 440, 271, 176, 169, 157, and 145, and ions 14 Da less in the segment bearing the dimethylamine functionality when compared with 7, suggesting oxidation of indole ring A at the C-2 position and N-demethylation, respectively. On this basis, the structures of the diastereoisomers 8 and 9, as a result of the keto-enol tautomerism, were proposed to be the N-desmethyl products of 7. The presence of both diastereoisomers of 7 was not detected in the fecal sample, and the stereochemistry of the additional chiral center in 7 could not be determined.

Metabolites 10 and 11 are a pair of diastereoisomers with protonated molecular ions at \( m/z \) 471. Their structures were proposed based on their mass spectral fragments derived from the observed product ions as depicted in Fig. 3C. It is apparent from the scheme that in 10 and 11, indole ring B was oxidized (C-21 position) instead of indole ring A (C-2 position) as in 8 and 9. The diastereoisomers 12 and 13 showed identical protonated molecular ions at \( m/z \) 485, 16 Da higher than the parent, and the same product ions (Table 9), consistent with the oxidation of parent at C-21 of indole ring B.

Metabolite 14 (protonated molecular ion at \( m/z \) 485) and its corresponding N-desmethyl metabolite 15 (protonated molecular ion at \( m/z \) 471) are distinct from the other hydroxy metabolites described above; in metabolites 14 and 15, the oxidation has occurred on the phenyl of indole ring B. This was borne out specifically by the presence of a fragment ion at \( m/z \) 305 (loss of trimethylamine plus a neutral fragment with formula C_8H_9NO) along with the ion at \( m/z \) 440 (loss of dimethylamine) in the product ion spectrum of both 14 and 15. Thus, the presence of a fragment ion at \( m/z \) 289 was used to characterize an unchanged indole ring B, and the presence of the ion at \( m/z \) 305 was used to characterize hydroxylated indole ring B. However, the exact position of hydroxylation on the phenyl ring could not be determined from these data.

Metabolite 16 (protonated molecular ion at \( m/z \) 485) and possibly its corresponding N-desmethyl metabolite 17 (protonated molecular ion at \( m/z \) 471) are yet two other hydroxy metabolites of the parent, the structures of which were proposed as shown in the formula on the basis of the fragment ions (Table 9), specifically the ion at \( m/z \) 289. Again, the exact position of hydroxylation could not be determined.
An additional hydroxy N-desmethyl metabolite, 26 (protonated molecular ion at m/z 471), was also observed; however, the site of hydroxylation could not be determined.

**Mouse and Rat.** The presence of the identified metabolites 1, 2, 3, 5, 6, 7, 10, 12, 14, 15, and 17 was observed in mouse feces. Additional metabolites tentatively identified in mouse were 20 (N,N-dimethyl-amino-methyl segment oxidized to a carboxylic acid), 22 (oxidation of the A ring with subsequent glucuronidation), and 24 (hydroxylation at the 2 position; diastereomer of 7). In rat, metabolites 1, 2, 3, 7, 8, 10, 12, 14, 15, and 17 were identified.

**Urinary Metabolites. Dog.** Plasma and fecal metabolites 1, 2, 3, 5 and 6 fecal metabolites 7, 8, 10, 12, 13, 14, and 15 were also detected in the urine. In addition, phase II glucuronide conjugates 18 and 19 (detected in mouse plasma) were observed in dog urine. The structure of 18 with a protonated molecular ion at m/z 661 was tentatively proposed to be the glucuronide conjugate of a hydroxylated parent. The location of hydroxylation and subsequent glucuronidation could not be determined.

**Mouse and Rat.** In mouse, previously identified metabolites 1, 2, 3, 5, 7, 8, 10, 12, 14, 15, 17, 19, and 22 were observed. In addition, three other oxidation products with subsequent conjugation with glucuronic acid (metabolites 21, 23, and 25) were observed. Metabolites 21 and 25 had protonated molecular ions at m/z 647, and their structures were proposed to be the glucuronides conjugates of hydroxylated N-desmethyl metabolites. The product ions observed in the MS/MS spectrum (Table 9) for metabolite 23 suggested oxidation of the B ring and subsequent glucuronidation (product ion at m/z 305). In rat, previously identified metabolites 1, 2, 3, 5, and 10 were observed.

**Rat Biliary Metabolites.** The major component observed in rat bile was identified as the hydroxy N-desmethyl metabolite 5. Other metabolites, each constituting approximately <1 to 5% of the dose, identified in this matrix were 1, 2, 3, 10, 13, 17, 18, 19, 21, 22, 23, and 25.

**Discussion**

The disposition of ruboxistaurin (LY333531), a potent and isoform-selective PKCβ inhibitor currently in development for the treatment of diabetic microvascular complications, has been studied previously in rats and dogs (Burkey et al., 2002). That study was limited to the detection and disposition of only the parent and its N-desmethyl metabolite 1 in plasma. The purpose of the present study was to investigate the metabolism of ruboxistaurin in dogs, mice, and rats and to identify distinct metabolites of ruboxistaurin in plasma and excreta since dog was chosen as the primary nonrodent species and mice and rats were chosen as the rodent species for the safety assessment of ruboxistaurin during development.

Following oral administration of a single dose of [14C]ruboxistaurin, the mean total recovery of radioactivity was approximately 92, 91, and 100% in dogs, mice, and rats, respectively. The radioactivity was primarily excreted through feces in dogs (90%) and mice...
The major circulating components in plasma were the N-desmethyl metabolite 1 and parent for all three species. The most notable exception was metabolite 19, a glucuronide conjugate of a hydroxy N-desmethyl ruboxistaurin, which was observed in significant amounts (9%) only in mice. In feces, parent and the N-desmethyl metabolite 1 were the major ruboxistaurin-related components observed in all species. Thus, overall, the major metabolic pathway of ruboxistaurin in all three species was N-demethylation.

Preliminary identification of metabolites was achieved by ion spray LC-MS/MS and, when necessary, by accurate mass measurements. The structures of significant metabolites were confirmed either by direct comparison with reference standards or by NMR spectroscopy. In an attempt to establish the exact sites of oxidation, a suite of two-dimensional NMR experiments was conducted. To support characterization by NMR, surrogates biocatalytic systems, including recombinant human P450s and microorganisms, were adopted to produce sufficient amounts of the desired metabolites. These efforts led to the unambiguous identification of all notable metabolites detected in dog plasma. Overall, in addition to parent, five metabolites (1, 2, 3, 5, and 6) were observed in dog plasma by radiochemical detection and identified by mass spectrometry. The structures of metabolites 1 and 2 were confirmed by comparison with reference standards, and those of 3, 5, and 6 were confirmed by NMR. Results from the metabolic profiling and identification experiments demonstrated that ruboxistaurin was primarily metabolized via N-demethylation to metabolite 1 (approximately 60% of the radioactive material observed in the 2-h dog plasma sample and comparable amounts in other species was the N-desmethyl metabolite 1). Minor routes of metabolism included hydroxylation of the indole ring (metabolite 3), oxidation of the tertiary amine to the N-oxide (metabolite 6), combined N-demethylation and hydroxylation of the indole ring (metabolite 5), combined N-demethylation and hydroxylation of the indole ring followed by subsequent glucuronidation (metabolite 19; the exact site of hydroxylation and subsequent glucuronidation could not be determined), and N,N-di-demethylation (metabolite 2). Based on the metabolites identified, the major metabolic pathways of ruboxistaurin are proposed as shown in Fig. 6.

With the exception of the N-oxide metabolite 6, which was observed only in dog plasma, the metabolites detected in plasma of all species were also detected in one or more of the excreta samples. In addition, several minor oxidative metabolites and oxidative metabolites with subsequent conjugation by glucuronic acid were also detected in these matrices. Figure 7 shows the minor metabolites of ruboxistaurin in excreta not shown in Fig. 6.

In rat, the fact that nearly 60% of the radioactivity was recovered from the bile after an oral dose of 5 mg/kg suggests that ruboxistaurin was well absorbed. Also, the fact that no parent was observed in this matrix indicates that ruboxistaurin was highly metabolized. Extensive metabolism of ruboxistaurin was also evident from the observation that the majority of the radioactivity detected in the circulating plasma was accounted for by the N-desmethyl metabolite 1 in all three species.

In summary, the excretion profiles of ruboxistaurin and its metabolites following oral administration of the compound were very similar in dogs, mice, and rats. Ruboxistaurin was well absorbed and highly metabolized, primarily to the N-desmethyl metabolite. The parent and its metabolites were predominantly eliminated via feces. The structures of most metabolites observed in plasma and significant metabolites observed in the excreta were positively identified by MS/MS, NMR, and by comparison with reference standards. Surrogate biocatalytic systems, including recombinant human P450s and microorganisms, were adopted to generate the desired metabolites in sufficient amounts for characterization by NMR. The positive identification of metabolites in plasma and excreta led to the proposal of metabolic pathways of ruboxistaurin in dogs, mice, and rats.

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References


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