DISPOSITION AND METABOLISM OF 2-[(1',3'-DIOXOLAN-2-YL)-2-METHYL-4-(2'-OXOPYRROLIDIN-1-YL)-6-NITRO-2H-1-BENZOPYRAN (SKP-450) IN RATS

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ABSTRACT:
The disposition and metabolism of the new antihypertensive agent 2-[(1',3'-dioxolan-2-yl)-2-methyl-4-(2'-oxopyrrolidin-1-yl)-6-nitro-2H-1-benzopyran (SKP-450) were investigated in male rats after single oral and i.v. doses of [14C]-labeled compound. After an oral 2.0 mg/kg dose, mean radioactive recovery was 98.2 ± 2.3% with 31.1 ± 7.3% in the feces and 67.1 ± 14.3% in the urine. Biliary excretion of radioactivity for the first 24-h period was approximately 40%, suggesting that SKP-450 is cleared either by hepatobiliary excretion or by renal excretion. SKP-450 was well absorbed; bioavailability calculated on the basis of radioactivity was 68 to 97%. Tissue distribution of the radioactivity was widespread with high concentrations in the liver and kidney but low central nervous system penetration. Radio-HPLC analysis of bile and urine from rats indicated the extensive metabolism of SKP-450 into oxidative metabolites. Oxidative metabolism of the dioxolanyl ring resulted in an aldehyde intermediate, subsequently confirmed in vitro, which was further oxidized to the corresponding carboxylic acid (M1) or reduced to the corresponding alcohol (M3). No parent drug was detected in the urine or bile. Glucuronide conjugate of M3 was also detected in urine and bile, accounting for 5.8 ± 2.1 and 8.9 ± 3.7% of the excreted radioactivity, respectively. Quantitative data obtained from plasma samples suggest that the majority of circulating radioactivity was associated with metabolites. Our results suggest that the long duration of pharmacological activity of SKP-450 (>10 h) is largely attributable to its metabolites.

Potassium channel modulation has been known to be an important mechanism for the regulation of vascular smooth muscle function and blood pressure (Robertson and Steinberg, 1990; Pinheiro and Malik, 1992). As part of an effort to control high blood pressure, a number of structurally diverse compounds with potassium channel-opening activity have been developed and their pharmacological properties were characterized (Lawson et al., 1992; Nakajima et al., 1992; Damiano et al., 1994; Keiji et al., 1994).

2-[(1',3'-dioxolan-2-yl)-2-methyl-4-(2'-oxopyrrolidin-1-yl)-6-nitro-2H-1-benzopyran (SKP-450; Fig. 1), a novel benzopyran derivative closely related to lemakalim, has been shown to be 10 times more potent than lemakalim in lowering blood pressure in rats with less incidence of tachycardia and in vitro coronary smooth muscle contraction activities (Kwak et al., 1995; Lee et al., 1998a). Cardioprotective activity of SKP-450 was also reported in the coronary occluded rat model of ischemia (Lee et al., 1997). In in vitro metabolism studies, SKP-450 was extensively metabolized to two major metabolites; these metabolites were found to possess pharmacological activity (Shin et al., 1998). The present report describes the metabolism and disposition of [14C]-SKP-450 after single oral and i.v. administration in rats, the species used in pharmacological and toxicological evaluation of this drug candidate.

Materials and Methods

Chemicals. [14C]-SKP-450 (specific activity: 36.2 mCi/mmol) was synthesized at Life Science Center, Sunkyung Industries Co. (Suwon, Korea) with a radiochemical purity ≥98% as judged by HPLC-radiochromatography. Unlabeled SKP-450 and potential metabolites M1, M2, and M3 were synthesized at Korea Research Institute of Chemical Technology (Taeduk, Korea) with a chemical purity of 99%.

Animals. Male Sprague-Dawley rats weighing 200 to 250 g were purchased from Daehan Laboratory Animal Research Center Co. (Taejeon, Korea). They were housed in a temperature- (23 ± 2°C) and moisture- (55 ± 10°C) controlled room and were exposed to a controlled 12-h light/dark cycle and allowed free access to food and water.

Preparation of Dosing Solution. Appropriate quantities of [14C]-labeled SKP-450 were diluted with cold SKP-450 to adjust the specific activity required for dose preparation. For the i.v. dosing, SKP-450 was dissolved in normal saline to a final concentration of 0.25 mg/ml. For oral dosing, SKP-450 was suspended in 0.5% carboxymethylcellulose solution with continuous stirring.

Study Design and Sample Collection. Pharmacokinetics. Two days before the experiments, the femoral artery and vein (i.v. only) were cannulated using PE-50 and PE-10 tubing (Becton Dickinson & Co., Lincoln Park, NJ) and the cannula was fixed to head neck. The rats were fasted overnight before use and until 6 h after dosing. For oral experiments, the rats in groups of four were given a single dose of [14C]-SKP-450. Heparinized samples of blood (0.4 ml) were collected at 0, 5, 15, 30 min, and 1, 1.5, 2, 3, 4, 6, 8, and 10 h postdose. For the i.v. experiment, the rats were given a single dose of 0.5 mg/kg bolus of [14C]-SKP-450 and blood samples were collected at 0, 1, 5, 10, 20, 30, and 45 min, and 1, 1.5, 2, 3, 5, and 8 h postdose. Plasma was harvested after centrifugation and stored frozen at −20°C until analyzed.

Biliary excretion. The bile duct was cannulated with PE10 tubing 1 h before...
dosing under pentobarbital anesthesia (40 mg/kg i.p.). The rats (four animals) were dosed with \(^{14}C\)SKP-450 orally at 2.0 mg/kg. Bile was collected at 1-h intervals for the first 10 h and then from 10 to 24 h. To demonstrate enterohepatic circulation, rats were implanted with double cannula in the proximal and distal duodenum. Pooled bile collected in the aforementioned experiment was infused into the duodenum and bile was collected at 1-h intervals for 10 h.

**Tissue distribution.** Rats (four animals per group) were dosed with \(^{14}C\)SKP-450 orally at 2.0 mg/kg. At 0.5, 4, and 24 h after dosing, each animal was lightly anesthetized with ether, blood was collected by heart puncture, and then the animal was sacrificed by cervical dislocation. The representative tissues or organs were rapidly dissected, weighed, and patted dry on a combustion pad in preparation for sample oxidation.

**Plasma protein binding.** Plasma protein binding was determined by ultrafiltration using Amicon centrifuge micropartition devices (Amicon Inc., Beverly, MA; molecular weight cutoff, 30,000). \(^{14}C\)SKP-450 was added to plasma from untreated rats (n = 5, in triplicate) to yield final concentrations of 10, 100, and 1000 ng/ml and incubated for 30 min at 37°C. After incubation, 1.0-ml aliquots were added to the ultrafiltration units and centrifuged at 37°C for 10 min at 3000 rpm. The concentrations of \(^{14}C\)SKP-450 in the plasma and ultrafiltrate were determined by liquid scintillation spectrometry.

For the ex vivo determination of plasma protein binding, a 2.0 mg/kg dose of \(^{14}C\)SKP-450 was orally administered and blood samples were drawn at 0.5, 1, and 5 h after dosing. Plasma samples were then prepared and protein binding was analyzed as described above.

**Excretion.** After an overnight fast, four rats were dosed with \(^{14}C\)SKP-450 orally at 2.0 mg/kg and housed individually in metabolic cages equipped with urine and feces separators. Urine, feces, and washing samples were collected and weighed at the following intervals: predose, 0 to 6, 6 to 12, 12 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, and 120 to 144 h. Urine and fecal samples were stored separately at 4°C until analysis. Aliquots of urine from the first 10 h and then from 10 to 24 h, were stored separately at 4°C until analysis.

**Analytical Methods.** Radioactivity measurements. Plasma, urine, and bile were mixed with 10% of scintillation fluid (Insta Gel XF, Packard Instrument Co., Inc., Meriden, CT) and counted directly for radioactivity. Aliquots of solid samples (blood, organs, and feces) were weighed and combusted in a sample oxidizer (Tri-Carb model 307, Packard). The resulting \(^{14}C\)CO\(_2\) was adsorbed on Carbosorb and then mixed with Permafluor V scintillation fluid. Radioactivity of samples was counted using a liquid scintillation counter (Tri-Carb, Packard).

**Analysis of plasma SKP-450 and metabolites.** Analysis of SKP-450 and metabolites in plasma was performed by thin layer chromatography (TLC) after precipitation of proteins with 2 volumes of acetonitrile containing 0.1 N HCl. Concentrated samples were applied on a TLC plate (LKC Si60) and developed using a mixture of ethyl acetate/chloroform/methanol (10:1:1, v/v/v). R\(_f\) values of SKP-450, M\(_1\), and M\(_3\) under these conditions were 0.78, 0.32, and 0.55, respectively. Radioactive spots were identified by exposing TLC plates to an imaging plate (IP, 20 cm × 25 cm, Fuji Film Co., Kanagawa, Japan) for 24 h and processed by a BAS 2000 Image analyzer (Fuji Film Co.). Quantification of radioactivity was made by comparison with intensities of external standard radioactivity exposed under the same condition.

**HPLC analysis of urinary and biliary compounds.** One milliliter of urine and bile was passed through activated Sep-pak C\(_18\) cartridges and the final methanol eluate was dried under nitrogen evaporation and the residue was dissolved in HPLC buffer. To hydrolyze glucuronide conjugates, 1 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 5 U of \(\beta\)-glucuronidase was added to the same volume of urine and bile, and the samples were heated at 50°C for 1 h. After the incubation, the samples were passed through activated Sep-pak C\(_18\) cartridges and the final methanol eluate was dried under nitrogen evaporation and the residue was reconstituted in HPLC buffer. Chromatographic separation of metabolites was achieved on a Partisil ODS column (4.6 mm × 15 cm, 5 \(\mu\)m, Beckman) using a linear gradient from 10% acetonitrile in 20 mM ammonium acetate buffer (pH 5.0) to 30% acetonitrile over 25 min followed by 10 min isocratic elution at a flow rate of 1.0 ml/min. Radioactivity was monitored using a \(\beta\)-RAM radioactivity flow detector (IN/US Corporation, Tampa, FL).

**Pharmacokinetic analysis.** Pharmacokinetic parameters were determined using standard noncompartmental methods. Plasma area under the curve (AUC) was calculated using PCNONLIN software (Scientific Consulting, Inc., Lexington, KY) with a log-linear trapezoidal method. Oral bioavailability was calculated as:

\[
\text{Bioavailability} = \frac{\text{AUC}_{\text{oral}}}{\text{Dose}_{\text{oral}}} \times \frac{\text{Dose}_{\text{i.v.}}}{\text{AUC}_{\text{i.v.}}} \times 100
\]

**Results**

**Plasma Concentration of Total Radioactivity, SKP-450, and its Metabolites.** After i.v. administration of \((^{14}C\)SKP-450 (0.5 mg/kg), the compound was rapidly cleared with a half-life of 0.05 h, whereas the radioactive equivalents in plasma declined more slowly (Fig. 2). SKP-450 was extensively metabolized to M\(_1\) and M\(_3\) after injection and the parent SKP-450 was no longer detected after 15 min. AUC of the parent compound was only 2.1% compared with AUC of the total radioactivity (Table 1). Total radioactivity was eliminated in a biphasic fashion with a half-life of 1.21 ± 0.21 h (Table 2). \(V_d\) was lower than body weight as 357 ± 40 ml/kg and clearance occurred rapidly (250 ± 30 ml/min/kg). The plasma concentration of M\(_3\) peaked at 5 min after i.v. injection whereas M\(_1\) showed its maximum level at 1 min after injection.
circulating radioactivity in plasma was associated with plasma protein.

After oral administration of $[^{14}C]$SKP-450, the drug was absorbed rapidly with a peak concentration of total radioactivity occurring in 0.18 to 0.24 h (Fig. 2). The parent SKP-450 peaked at 0.19 to 0.21 h with a peak concentration of total radioactivity occurring in plasma protein.

Plasma Protein Binding. In vitro incubation of $[^{14}C]$SKP-450 with rat liver microsomes in the presence of NADPH-generating system resulted in three metabolites with a retention time of 17.0 (M1), 22.4 (M2), and 24.7 min (M3), respectively (Fig. 4A). HPLC radiochromatograms of 0- to 24-h urine and 0- to 10-h bile collected after oral administration of $[^{14}C]$SKP-450 shown in Fig. 4 demonstrated three to four radioactive peaks. Urinary and biliary distribution of metabolites is described in Table 5. M1 was a major metabolite, making up greater than 50% of the total radioactivity in urine and bile. Parent drug was not detected in the urine and bile. Each of the radioactive peaks was isolated either from urine or from in vitro incubations by semipreparative HPLC and characterized by mass spectral analysis. Thermospray chemical ionization and electron impact mass spectroscopy were used to analyze each metabolite sample. The identities of metabolites of $[^{14}C]$SKP-450 were established by coinjection of metabolites with synthetic standards.

Metabolism Study. HPLC analysis of organic extracts after in vitro incubation of SKP-450 with rat liver microsomes in the presence of NADPH-generating system resulted in three metabolites with a retention time of 17.0 (M1), 22.4 (M2), and 24.7 min (M3), respectively (Fig. 4A). HPLC radiochromatograms of 0- to 24-h urine and 0- to 10-h bile collected after oral administration of $[^{14}C]$SKP-450 shown in Fig. 4 demonstrated three to four radioactive peaks. Urinary and biliary distribution of metabolites is described in Table 5. M1 was a major metabolite, making up greater than 50% of the total radioactivity in the urine and bile. Parent drug was not detected in the urine and bile. Each of the radioactive peaks was isolated either from urine or from in vitro incubations by semipreparative HPLC and characterized by mass spectral analysis. Thermospray chemical ionization and electron impact mass spectroscopy were used to analyze each metabolite sample. The identities of metabolites of $[^{14}C]$SKP-450 were established by coinjection of metabolites with synthetic standards.

M1. The peak eluting at 17.0 min yielded a protonated molecular ion at $m/z$ 319 and ammonium adducted ion at $m/z$ 336 (Fig. 5A). Positive thermospray ionization/mass spectrometry analysis also showed a protonated molecular ion at $m/z$ 319. Isolated M1 was not eluted through capillary GC column. El mass of this metabolite obtained after methyl derivatization showed a molecular ion at $m/z$ 332 and fragment ion at $m/z$ 273 [M-59 (COOCH$_3$)]$^+$, 244, and 227 [M-59-46 (NO$_3$)]$^+$ (Fig. 6B). The base fragment ion at $m/z$ 273 as with that of parent SKP-450 (Fig. 5A) indicated that the benzopyran moiety of this compound was not transformed and was a carboxyl derivative of SKP-450. M1 was further confirmed by cochromatography with the authentic standard and by comparison to mass fragmentation of derivatized authentic compound.

**Table 1**

<table>
<thead>
<tr>
<th>Route</th>
<th>AUC</th>
<th>$V_{ss}$</th>
<th>$t_{1/2}$</th>
<th>$C_{max}$</th>
<th>$T_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>oral</td>
<td>63 ± 3</td>
<td>7880 ± 380</td>
<td>1183 ± 198</td>
<td>67 ± 11</td>
<td>147 ± 21</td>
</tr>
<tr>
<td>oral</td>
<td>20 ± 3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>147 ± 21</td>
</tr>
<tr>
<td>oral</td>
<td>2.0</td>
<td>49 ± 10</td>
<td>—</td>
<td>147 ± 21</td>
<td>0.19 ± 0.09</td>
</tr>
</tbody>
</table>

**Tissue Distribution.** The tissue distribution of total radioactivity after single oral administration of $[^{14}C]$SKP-450 is described in Table 3. Radioactivity was widely distributed into all tissues, except brain, fat, and testes. Concentrations of radioactivity in kidney and liver at 30 min after dosing were higher than in plasma. This result was mainly due to the accumulation of radioactivity in the excretory organs. Concentrations of radioactivity in other tissues were lower than in plasma, ranging from 200 to 600 ng equivalents/ml. The radioactivity in all tissues appeared to decline by first order kinetics up to 24 h after dosing.

**Excretion of Radioactivity.** Excretion of total radioactivity in the urine and feces after single oral administration of $[^{14}C]$SKP-450 is shown in Table 4. Urinary and fecal recoveries of total radioactivity for the first 24-h period were 64.2 ± 6.3% and 23.9 ± 2.8% of administered dose, respectively. Total radioactivity recovered within 7 days was 98.2 ± 2.3%, with 31.1 ± 7.3% appearing in the feces and 67.1 ± 14.3% excreted in the urine. Biliary excretion of radioactivity and metabolites after single oral administration of $[^{14}C]$SKP-450 is shown in Fig. 3. Radioactivity excreted in bile over 24 h was 41.1 ± 2.6% of administered dose and approximately 60% of the radioactivity was associated with M1. The amount of radioactivity excreted into bile was higher than that excreted in the feces for the first 24 h, suggesting that enterohepatic circulation of the radioactivity occurred in the rat.
with NaBH₄ resulted in the complete disappearance of the peak and appearance of a new peak at the retention time of M3. This metabolite was considered to be an aldehyde intermediate, which was confirmed by cochromatography with authentic standard.

**M3.** This metabolite eluting at the retention time of 24.7 min yielded a protonated molecular ion at m/z 305 and ammonium adducted ion at m/z 322 in thermospray ionization mass spectrum (Fig. 5C). El mass of this metabolite after trimethyl silylation derivatization showed a molecular ion at m/z 376 and fragment ions at m/z 361 [loss of methyl], 273 [loss of CH₃OTMS], 257 [273-(O)], 227 [257-(NO₂)] (Fig. 6C). The same base ion at m/z 273 and two higher mass unit compared to M2 suggested that the dioxolane moiety was transformed to a hydroxymethyl group. Authentic standard has the same mass fragment pattern and showed the same HPLC retention time.

**M4.** This metabolite was tentatively identified as the glucuronide adduct of hydroxymethyl SKP-450 (M3). Enzymatic hydrolysis with β-glucuronidase or acid hydrolysis with 6 N HCl resulted in the complete disappearance of the peak and appearance of a new peak at the retention time of M3.

**Discussion**

After oral administration of [¹⁴C]SKP-450, maximal plasma concentration of radioactivity was achieved within 30 min, indicating that absorption of the radiolabeled dose from the gastrointestinal tract was rapid. The maximum plasma concentration (Cₘₚₓ) of total radioactivity and the AUC value increased almost proportionally to the dose and other kinetic parameters (T₁/₂, Vₘₚₓ, and Tₘₚₓ) were not different between doses of 0.5 and 2 mg/kg. These results demonstrate that SKP-450 has linear kinetics over a dose range of 0.5 to 2.0 mg/kg. The bioavailability of total radioactivity (F) was calculated by comparison of AUC between oral administration and i.v. injection at a dose of 0.5 mg/kg.
**mg/kg.** $F$ values were comparatively high as 97.3% and 68.8% at the dose of 0.5 mg/kg and 2 mg/kg, respectively. The AUC values of SKP-450 at oral doses of 0.5 and 2 mg/kg were less than 1% compared with those of total radioactivity, and the ratio of these two values was similar between 0.5 and 2.0 mg/kg. These results indicated that metabolism of SKP-450 was not saturated up to the dose of 2.0 mg/kg and SKP-450 was extensively metabolized once absorbed. Hepatic blood flow has been reported to be 60 to 70 ml/min/kg for the rat (Lin et al., 1982). Total blood clearance of SKP-450 was much greater than hepatic blood flow in rats. $T_{\text{max}}$ of M1 and M3 was 1 and 5 min, respectively, when the compound was i.v. injected. These results suggest that the metabolism of SKP-450 to M1 proceeded faster than to M3. After oral administration of $[^{14}\text{C}]$SKP-450 to rats, the major route of elimination of radioactivity was via the urine (67%) and a substantial amount of radioactivity was also detected in the feces (31%). Part of the radioactivity present in the feces after oral administration seems to be attributed to biliary excretion of metabolites because a substantial amount of the radioactivity administered was detected in the bile. Approximately 40% of the radioactivity was excreted in bile for 24 h in bile duct-cannulated rats, larger than the amount of radioactivity detected in the feces, suggesting that enterohepatic circulation might occur in the rat. This phenomenon has been confirmed by observation of biliary excretion of the radioactivity after injection of pooled bile into duodenum (data not shown).

$[^{14}\text{C}]$SKP-450 was well distributed into all tissues examined, except brain, fat, and testes. Lower levels in the brain tissue indicate that penetration of SKP-450 or its metabolites across blood-brain barrier may be limited. The concentration of radioactivity in liver and kidney was higher than in plasma and tissue/plasma concentration ratios in other tissues were in the range of 0.3 to 0.9, suggesting that SKP-450 or its metabolites may have relatively low tissue affinity in the rat.

Incubation of $[^{14}\text{C}]$SKP-450 with rat liver microsomes produced three distinct metabolite peaks with M2 as a major metabolite, which was not found in in vivo studies. HPLC/radiochromatogram analysis of urine and bile extracts revealed similar metabolic profiles between the urine and bile. The proposed metabolic pathway of $[^{14}\text{C}]$SKP-450 in the rat is described in Fig. 7. Intact parent drug was not detected in the urine and bile. The compound was primarily metabolized by initial oxidation of the dioxolanyl ring. Hydroxylation at the carbon of the 1-dioxolanyl ring may result in ring cleavage followed by rearrangement to an aldehyde (M2). The structure of M2 was characterized by thermospray CI mass and by its reduction to the corresponding alcohol (M3) by sodium borohydride. The reaction was dependent on NADPH, indicating that cytochrome P-450 could be involved in this biotransformation. The enzymatic transformation of acetal group to aldehyde is not well documented. Grosta et al. (1986) reported the transformation of the acetal group in doxophylline to an ester by rat liver microsomes and complete loss of the 16a,17a acetal group from budesonide was demonstrated after incubation with human liver 9000 g supernatant (Edsbacker et al., 1983). The metabolic product of the acetal group might be governed by the structure of parent. Once M2 is generated, it is presumed to be further metabolized either to the corresponding carboxylic acid (M1) by oxidation or to the corresponding alcohol (M3) by reduction. Reductive conversion of aldehyde intermediates to the corresponding alcohols has been proposed in the metabolism of naftifine (Schatz et al., 1986) and pholcodine (Maurer and Fritz, 1990). M1 was a major radioactive component of urine and bile collected after administration of $[^{14}\text{C}]$SKP-450, suggesting that oxidation of M2 to the corresponding carboxylic acid seemed to be the preferable metabolic pathway in rat. M1 was detected only as a free acid, whereas the glucuronide conjugate of M3 was found both in the urine and bile. Given the rapid metabolism of $[^{14}\text{C}]$SKP-450 and the major circulating radioactivity associated with M1 and M3, pharmacological activities of these metabolites may be an important factor in overall pharmacodynamics of the drug. Pharmacological activities of the metabolites have been described elsewhere (Shin et al., 1998).

In conclusion, the present study demonstrated that administered SKP-450 was absorbed in the GI-tract very quickly and showed linear pharmacokinetics. Passing through the GI-tract and the liver, most of
FIG. 5. Thermospray CI mass spectra of M1 (A), M2 (B), and M3 (C).

FIG. 6. EI mass spectra of SKP-450 (A), methylated M1 (B), and trimethylsilylated M3 (C).
this compound was metabolized to M1 and M3, because the majority of circulating radioactivity in plasma was associated with these metabolites. Thus the pharmacological activity of these metabolites is presumed to give a great contribution to the blood pressure lowering in rats.

Fig. 7. Proposed biotransformation pathway of SKP-450 in rats.

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References


