Class III antiarrhythmic agents prolong the duration of the cardiac action potential by inhibition of repolarizing potassium currents and are effective in the treatment of malignant ventricular tachyarrhythmias (Claremont et al., 1993; Elliot et al., 1992; Lynch et al., 1992, 1993, 1994; Morgan and Sullivan, 1992; Sanguinetti, 1992). Patients with previous myocardial infarctions are susceptible to electrical instability in their heart membranes, and class III compounds may be useful prophylactic agents for these patients by preventing sudden death.

The synthesis of a series of 4-oxospirobenzopyran 2’,4’-piperidine class III agents was described by Elliot et al. (1992). Metabolic disposition studies on one member of the series, L-691,121, indicated that in the dog the pharmacological activity was curtailed because of a metabolic reduction of the benzopyran ketone to an alcohol metabolite (Vickers et al., 1993) that had significantly less class III activity (Elliot et al., 1992). Metabolic studies showed that MK-499 was useful prophylactic agents for these patients by preventing sudden death.

The purpose of the present study was to study the metabolic disposition and pharmacokinetics of MK-499 in rats and dogs, as these were the species used in toxicology studies. A preliminary report on the in vivo metabolism of MK-499 was presented earlier (Vickers et al., 1994).

Materials and Methods

Chemicals. MK-499 was labeled either with 3H on the carbon number 3 of the benzopyran ring or with 14C at carbon number 4 of the benzopyran ring (fig. 1). The radioactive preparations were at least 98–99% pure based on HPLC1 and were diluted with carrier drug when necessary. D-Mannitol, citric acid, sodium citrate, and phosphoric acid (HPLC grade) were obtained from Fisher Scientific (Pittsburgh, PA), and triethylamine was obtained from Aldrich. Ammonium acetate was reagent grade (Merck).

Animals and Treatments. Male Sprague-Dawley rats and male pure-bred beagle dogs were used. Doses were calculated on the basis of the free base. Food was provided to dogs at the end of each day during the studies. Rats were fed ad libitum, and water was available to the animals during the course of all studies.

Rats. For iv dosing, MK-499 was dissolved in aqueous solutions containing in each ml mannitol (27.3 mg), citric acid (2.88 mg), and sodium citrate (10.3 mg). Oral doses of MK-499 were dissolved in aqueous 0.5% methylcellulose. The oral and iv doses were given by gavage and tail vein, respectively.

Plasma concentrations. Rats received oral and iv doses of MK-499 at 6.25 and 0.5 mg/kg, respectively. Blood was collected, and plasma was harvested and stored at −20°C prior to analysis by radioimmunoassay.

1 Abbreviations used are: HPLC, high pressure liquid chromatography; AUC, area under the curve.
Mass balance. Urines and feces were collected (0–72 hr) from two groups of four rats housed in stainless steel metabolism cages. One group received 6.25 mg/kg [3H]MK-499 po and the other 0.5 mg/kg [3H]MK-499 iv. In similar experiments, rats received [14C]MK-499. Urines were cooled by dry ice. To support toxicokinetic studies on MK-499, additional experiments were performed in which rats received 50 mg/kg [14C]MK-499.

Biliary excretion. Rats were anesthetized with a short-acting anesthetic before undergoing aseptic surgery for bile duct cannulation. The bile duct was cannulated with polyethylene tubing (PE-10). A group of three rats received 10 mg/kg [3H]MK-499 po, and another group of four rats received 6.25 mg/kg [14C]MK-499 po. Bile was collected (0–24 hr) in containers cooled by dry ice and subsequently analyzed for radioactivity prior to metabolite isolation.

Tissue distribution of radioactivity. Plasma and tissue concentrations of radioactivity were determined in rats that were sacrificed in groups of three at 5 min, 4 hr, 24 hr, and 72 hr after they were dosed with [14C]MK-499 (0.5 mg/kg) iv. Blood samples were collected by cardiac puncture in the presence of heparin and centrifuged; the plasma was then harvested. Brain, heart, lung, liver, kidney, testes, muscle, fat, stomach, small intestine, large intestine, pancreas, spleen, mesenteric lymph nodes, adrenals, and eyes were also collected. The tissues were rinsed, blotted, weighed, and (except for lymph nodes, adrenals, and eyes) homogenized with water. Tissue homogenates, including the contents of the stomach and small and large intestine, were combusted and assayed by radiometric technique.

Dogs. Dosing solutions were formulated as described above for rats.

Plasma concentrations. In a cross-over study, dogs received oral and iv doses of MK-499 at 3 and 2.5 mg/kg, respectively. Blood was collected from the femoral vein, and plasma was harvested and stored at −20°C prior to analysis of parent MK-499 by radioimmunoassay.

Mass balance. A crossover study was performed in which dogs were given either a 0.1 mg/kg iv dose or a 1 mg/kg oral dose of [3H]MK-499. Urine and feces were collected (0–96 hr) from four dogs that were individually housed in stainless steel metabolism cages.

Biliary excretion. Dogs were anesthetized before undergoing aseptic surgery for bile duct cannulation. Upon recovery, the dogs received [3H]MK-499 (5 mg/kg) by gavage, and bile (0–24 hr) was collected, frozen, and stored at −20°C before analysis and isolation of metabolites.

Instrumental Methods. Radioactivity was measured in a Packard Tricarb 2500 TR liquid scintillation counter. Samples of urine and HPLC eluates were added directly to polyethylene vials containing 5 ml of Ready Solv (Beckman Instruments, Fullerton, CA). Fecal and tissue homogenates were combusted to H2O or 14CO2 in a Packard Sample Oxidizer 306, and the radioactivity was measured with Monophase or a combination of PermaFluor and Carbo-Sox. The combustion efficiency of the sample oxidizer was determined by comparing the radioactivity recovered from the combustion of samples spiked with a radioactive standard to that obtained by spiking the trapping solution with the same amount of standard. Radioactivity counting time was usually 10 min. An external standard (133Ba) was used to determine efficiency. Combined liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed on a Sciex API III triple quadruple mass spectrometer interfaced via a Sciex-heated nebulizer probe to a Hewlett Packard Series 1050 pump. Mass spectra were recorded in the positive-ion mode. 1H-NMR spectra were obtained at frequencies of 400 MHz from samples dissolved in CD3OD by using a Varian model Unity-400 spectrometer. Tetramethylsilane was used as an internal standard.

Chromatography of Urinary and Biliary Radioactivity. Chromatography of rat and dog urine and bile was performed on an Applied Biosystems HPLC system (model 400 pumps, model 783 programmable absorbance detector) with a Waters WISP710B Autosampler and a Foxy II fraction collector. The HPLC fractions were assayed for radioactivity. A Flow 1/Beta radioactivity detector was used to analyze either concentrated acetone extracts of bile or untreated bile. Acetone extracts were prepared by mixing bile (0.4 ml) and

FIG. 1. Biotransformation of MK-499 in rats and dogs.

Values in parentheses represent per cent of excreted label.
acetic acid and acetonitrile (B). Gradient elution was used in which the mobile phase was a mixture of 0.01 M ammonium acetate (A) and acetonitrile (B). One-milliliter aliquots from each of the reconstituted urines were added to tubes containing β-glucuronidase (1–2.5 mg, 1000 units/mg of solid from abalone entrails, type VIII Sigma) was added. The sample was incubated overnight at 37°C. A control experiment was performed to confirm the absence of nonspecific binding of 

\[
\text{\textsuperscript{3}H} \text{MK-499} (0.1–1 \muCi, 108–126 \muCi/iv).\]

Results

Excretion. Rats. Recoveries of excreted label were similar for 

\[\text{\textsuperscript{3}H}\text{MK-499}\] and \[\text{\textsuperscript{14}C}\text{MK-499}\]. Fecal recoveries exceeded urinary recoveries and were indicative of extensive biliary excretion of label (table 1). Most of the label was excreted within 24 hr after dose. Mean recoveries of radioactivity in 0–24 hr bile after dosing rats orally with either \[\text{\textsuperscript{3}H}\text{MK-499}\] or \[\text{\textsuperscript{14}C}\text{MK-499}\] were 38.6 and 41.0%, respectively.

Dogs. Recoveries of \[\text{\textsuperscript{3}H}\] label in (0–96 hr) urines and feces were similar after oral or iv administration (table 2). Consistent with the high fecal recoveries of label, a significant amount of label was excreted in bile; approximately 34% of the labeled dose was recovered in 0–24 hr bile.

Chromatography of Radioactivity in Rat and Dog Urine. There were three major radioactive fractions in HPLC chromatograms of urine from rats that received oral doses of \[\text{\textsuperscript{3}H}\text{MK-499}\]. Each represented approximately 30% of the rat urinary label. They were identified (vide infra) as I, II, and MK-499 (figs. 1 and 2). Fractionation of dog urinary \[\text{\textsuperscript{3}H}\] radioactivity yielded two labeled species: the more polar one was identified as I and the other was MK-499. Metabolite I and MK-499 represented approximately 20 and 70% of the dog urinary label, respectively. Recoveries of radioactivity from the fractionation columns were approximately 90% of the amounts applied.

Chromatography of Radioactivity in Rat and Dog Bile. HPLC chromatography of bile from rats that received either \[\text{\textsuperscript{3}H}\text{MK-499}\] or \[\text{\textsuperscript{14}C}\text{MK-499}\] indicated that the metabolism of MK-499 was complex. Metabolites II, IV, V, and VI (fig. 1) were identified (vide infra) and represented 20, 10, 5, and 5%, respectively, of the biliary label. MK-499 represented another 10%.

An HPLC chromatogram of dog bile (fig. 3) showed the presence of MK-499 and VII (fig. 1), a more polar radioactive metabolite,

TABLE 1

<table>
<thead>
<tr>
<th>Label</th>
<th>PO</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>Feces</td>
</tr>
<tr>
<td>\textsuperscript{3}H</td>
<td>(10.5 \pm 2.5) (N = 4)</td>
<td>(78.0 \pm 7.4) (N = 4)</td>
</tr>
<tr>
<td>\textsuperscript{14}C</td>
<td>(9.1 \pm 2.7) (N = 4)</td>
<td>(82.8 \pm 9.0) (N = 4)</td>
</tr>
</tbody>
</table>

\(\text{\textsuperscript{3}H}\) values (mean ± SD) were from rats that weighed 265–315 g and received 1–2 μCi. \(\text{\textsuperscript{14}C}\) values (mean ± SD) were from rats that weighed 305–338 g and received 0.5–2 μCi.
which had a UV spectrum that was almost identical to that of MK-499 (fig. 4). This indicated that metabolism had not affected the UV properties of the chromophore. Dog bile samples that had been incubated with either glucuronidase or sulfatase were fractionated by HPLC. The fractions were analyzed for label content. After glucuronidase treatment, the relative amount of label associated with VII was reduced, whereas that which was associated with MK-499 was increased. It was concluded that the polar compound VII was the ether glucuronide of MK-499.

During the first 3 hr, the majority of the biliary label was excreted as unchanged MK-499. Subsequently, the excreted biliary radioactivity was proportioned between unchanged [3H]MK-499 and VII in a relatively constant fashion. There was no evidence for any other major labeled species in dog bile during the 0–24-hr collection period.

Identification of Metabolites. The structures of the metabolites are shown in fig. 1. There were species differences in the excretion of metabolites, with rats showing a more extensive and complex metabolism. Dogs excreted a glucuronide conjugate of MK-499 in bile; in contrast, rats did not excrete glucuronides but did excrete a glutathione conjugate (and the mercapturic acid) of an MK-499 metabolite. Other rat metabolites were formed by excision of the methanesulfonanilide moiety and by aliphatic hydroxylation of the cyanotetrahydronaphthalene ring. Metabolic loss of the cyanotetrahydronaphthalene ring occurred in both species.

Urinary metabolites. A polar urinary radioactive metabolite with a retention time of approximately 7 min was isolated from rat and dog urine. When the metabolite was analyzed by LC-MS/MS, the product ion spectrum was identical to synthetic I (fig. 5). Another polar metabolite (II) in rat urine was eluted at 12.5 min. The NMR spectrum of II indicated that metabolic excision of the methanesulfonanilide moiety and by aliphatic hydroxylation of the cyanotetrahydronaphthalene ring. Metabolic loss of the cyanotetrahydronaphthalene ring occurred in both species.
MK-499. The NMR spectra of III and the reference were identical (fig. 7).

**Biliary metabolites.** II was also present in rat bile. Another rat biliary metabolite was IV. Evidence for hydroxylation of the cyanotetrahydronaphthalene ring in IV included the presence of a pseudomolecular ion at \( m/z \) 484 and ions at \( m/z \) 269 and \( m/z \) 154 (fig. 8). The UV spectra of metabolites V and VI were distinct from those of the other metabolites in that there were additional absorption maxima at 286 and 298 nm. Metabolites IV, V, and VI were generated under appropriate *in vitro* conditions (V and VI required the presence of glutathione and N-acetylcysteine, respectively). Chemical and structural assignments were made from NMR and MS data: IV, V, and VI were identified, respectively, as an isomer of III, a glutathione conjugate, and the corresponding mercapturic acid of a metabolite in which ring fission had occurred (Slaughter et al., 1994). The glucuronide VII was found in dog bile. This metabolite was characterized as a glucuronide of MK-499 on the basis of HPLC-UV diode array spectroscopy and its lability in the presence of glucuronidase. The purified metabolite was subjected to HPLC-MS/MS; Q1 spectra showed the anticipated molecular ion at \( m/z \) 644, and daughter ion spectra showed ions at \( m/z \)
468 (consistent with the loss of the glucuronide moiety) and at m/z 253, 186, 156, and 98, all characteristic of MK-499 itself (fig. 9).

Tissue Distribution and Protein Binding. A study of the tissue distribution of radioactivity in rats 5 min after they were dosed with [14C]MK-499 (0.5 mg/kg) iv showed that radioactivity was distributed throughout most of the tissues of the body. Liver, kidney, and small intestine contained a relatively large percentage of the dose, whereas the brain and the eyes contained insignificant amounts of label. Highest concentrations of radioactivity were in liver, kidney, adrenal, heart, and lung (table 3). At 24 hr, the liver showed the highest concentration of labeled compounds. At 72 hr post-dosing, the tissue radioactivity levels were universally low (table 3), with liver, kidney, and testes having the highest concentration. MK-499 was highly bound to rat plasma proteins but less so to those of dog (table 4).

Pharmacokinetics. Concentrations of MK-499 in rat and dog plasma are shown in fig. 10. Pharmacokinetic parameters for MK-499 in rats and dogs are shown in table 5. There was a species difference in that the values for \( C_{\text{max}} \) plasma half-life and bioavailability were markedly greater in dogs.

Discussion

Biliary elimination was the principal mode of excretion for MK-499 and its metabolites in rats and dogs. Glucuronides of xenobiotics are frequently excreted in bile (Abou-El-Makaren et al., 1967) as are glutathione conjugates (Awasthi, 1990; Morganstern et al., 1982, 1984; Vore, 1993). In dog bile, the major metabolite was MK-499 glucuronide (VII). In rat bile, VII was not evident, but there was a glutathione conjugate (V) and a mercapturic acid (VI) of a MK-499 metabolite that resulted from a ring cleavage. The formation of mercapturic acids is usually accomplished by an interorgan pathway; glutathione conjugates formed by the liver enter the systemic circulation to be delivered to the kidneys where they are converted first to cysteine conjugates (Guder and Ross, 1984; Hughey et al., 1978; Jones et al., 1979a, 1979b) and then to the corresponding mercapturic acids that are excreted in urine (Heuner et al., 1991). However, hepatocytes can form mercapturic acids (Inoue et al., 1984), and when isolated perfused livers from rats were infused with 1-chloro-2,4-

### TABLE 3

| Tissue distribution of radioactivity in Sprague-Dawley rats dosed intravenously with [benzopyran-14C]MK-499 (0.5 mg/kg) |
|---|---|---|---|
| 5 min | 4 hr | 24 hr | 72 hr |
| Plasma | 0.128 ± 0.008 | 0.018 ± 0.002 | — | — |
| RBC | 0.118 ± 0.002 | — | — | — |
| Brain | 0.024 ± 0.016 | — | — | — |
| Heart | 1.226 ± 0.307 | 0.141 ± 0.011 | 0.012 ± 0.001 | — |
| Lung | 1.683 ± 2.50 | 0.077 ± 0.006 | — | — |
| Liver | 2.144 ± 0.601 | 2.069 ± 0.181 | 0.435 ± 0.066 | 0.100 ± 0.002 |
| Kidneys | 4.736 ± 1.290 | 1.077 ± 0.080 | 0.110 ± 0.018 | 0.017 ± 0.003 |
| Testes | 0.061 ± 0.044 | 0.032 ± 0.002 | 0.031 ± 0.005 | 0.018 ± 0.004 |
| Muscle | 0.249 ± 0.054 | — | — | — |
| Fat | 0.101 ± 0.009 | — | — | — |
| Stomach | 0.734 ± 0.108 | 0.035 ± 0.012 | — | — |
| Small intestine | 0.703 ± 0.108 | 0.057 ± 0.021 | — | — |
| Large intestine | 0.557 ± 0.330 | 0.114 ± 0.012 | — | — |
| Pancreas | 0.961 ± 0.145 | 0.038 ± 0.009 | — | — |
| Spleen | 0.744 ± 0.178 | 0.063 ± 0.017 | — | — |
| Mesenteric lymph node | 0.247 ± 0.021 | 0.025 ± 0.013 | — | — |
| Adrenals | 4.754 ± 2.313 | 0.113 ± 0.012 | — | — |
| Eyes | 0.276 ± 0.059 | — | — | — |

*Mean ± SD (N = 3).

Average of two rats.

*Background radioactivity; specific activity, 28,880 dpm/μg.
dinitrobenzene, a glutathione conjugate and a mercapturic acid metabolite were excreted in bile (Hinchman et al., 1991). The hepatic conversion of the glutathione-conjugated metabolite of 1-chloro-2,4-dinitrobenzene to the corresponding mercapturic acid was inhibited by the retrograde biliary infusion of acivicin, an irreversible inhibitor of γ-glutamyltransferase (Hinchman et al., 1991). These findings provided direct evidence for the intrahepatic biosynthesis of mercapturic acids. Thus, the presence of VI in rat bile was possibly a consequence of the hepatic metabolism of V.

Flavones and flavonones contain benzopyrone-substituted rings. Both classes of compounds are metabolized by scission of the heterocyclic rings, and the position of scission varies with species (Parke, 1968). An example from the early literature is that of hesperetin (a flavonone and the aglycone of hesperidin, which occurs in citrus fruits). The heterocyclic ring of hesperetin underwent metabolic cleavage by rats and rabbits to yield a substituted phenylpropionic acid. However, humans who ingested hesperetin excreted a substituted phenylhydracrylic acid (Booth et al., 1958). Because metabolite II also contains a hydrated acrylic acid moiety, the sites of bond cleavages in the benzopyrone ring of hesperetin and the benzopyran ring of MK-499 are probably the same. It has been proposed that for MK-499 an NADPH-dependent hydroxylation occurs followed by ring cleavage and iminoquinone formation (Slaughter et al., 1994). The iminoquinone is postulated to be a precursor of both II and V (fig. 11).

During toxicokinetic studies, rats received subchronic high doses of MK-499. HPLC analysis of the urines indicated that in addition to unchanged MK-499, there was a major metabolite of MK-499. Consequently, a study was done in which urine was collected from rats that received 50 mg/kg [14C]MK-499 po. The major urinary 14C metabolite was isolated and identified as III, a positional isomer of IV. However, there was no evidence for IV, which had been previously identified as a rat bile metabolite. Apparently, formation and excretion of metabolite III was associated with high doses of MK-499, as it was not evident in urines from rats that received lower doses of MK-499.

Metabolite I was a urinary metabolite in both dogs and rats and was previously identified as a metabolite of L-691,121, a class III antiarhythmic agent that contained a benzofurazan moiety rather than the cyanotetrahydronaphthalene group of MK-499 (Vickers et al., 1993). In vitro experiments indicated that I was formed by a P-450-catalyzed loss of N-substitution (Slaughter et al., 1994).

MK-499 was highly bound to plasma proteins of rats, dogs, and humans. Five minutes after an iv dose of [14C]MK-499, radioactivity was concentrated in rat tissues, including the heart (the target organ). An exception was the brain, where radioactivity concentrations were lower than those of plasma. Drug-plasma protein complexes do not usually prevent extravascular drug distribution (Tillement et al., 1986; Barre et al., 1990), and it is possible that penetration of MK-499 into the CNS was limited by the blood-brain barrier.

Overall, the oxidative metabolism of MK-499 was less pronounced in dogs than in rats. This may have resulted in the relatively low clearance and high bioavailability of MK-499 in dogs. MK-499 disposition data in humans indicated that, relative to the rat, the dog was...
a better model with respect to plasma half-life and clearance values (Goldberg et al., 1994).

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References


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