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IMPLICATION OF P450-METABOLITE COMPLEX FORMATION IN THE NONLINEAR PHARMACOKINETICS AND METABOLIC FATE OF (±)-(1′*R**,3*R**)-3-PHENYL-1-[(1′,2′,3′,4′-TETRAHYDRO-5′,6′-METHYLENE-DIOXY-1′-NAPHTHALENYL) METHYL] PYRROLIDINE METHANESULFONATE (ABT-200) IN DOGS

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ABSTRACT:

Following a single oral or intravenous administration of the *R*,*R*(+) and *S*,*S*(−) ^{14}C-pseudoracemate of (±)-(1′*R,3*R**)-3-phenyl-1-[(1′,2′,3′,4′-tetrahydro-5′,6′-methylene-dioxy-1′-naphthalenyl) methyl] pyrrolidine methanesulfonate (ABT-200/I) to dogs, a total of six (*R*,*R*[+]) and eight (*S*,*S*[−]) metabolites were identified by high-pressure liquid chromatography/mass spectral techniques. Greater than 99% of the dose was eliminated as metabolites indicating that the clearance of I was predominantly metabolic. The catechol was the major excreted metabolite (fecal), whereas the urine and bile predominantly contained metabolites resulting from secondary biotransformation of the catechol via *O*-methylation, glucuronidation, and sulfation. After a single 12 mg/kg oral dose of racemic I to dogs, the mean area under the plasma curve (AUC_{0-24h}) averaged 4.55 μg · h/ml, with an apparent plasma clearance value of 2.70 l/h · kg. After 14 daily doses, the apparent plasma clearance was 3.5-fold lower (0.78 l/h · kg) and the AUC_{0-24h} about 4-fold higher (18.58 μg · h/ml). Isolation of liver microsomes from these animals indicated that a cytochrome P450 (P450)-metabolite complex (MI complex) was formed in the liver after both single and multiple dosing. The mean concentration of the MI complex 24 h after a single dose averaged 31 pmol/mg of microsomal protein, whereas the amount in the animals given multiple doses of I averaged 163 pmol/mg. There was a positive correlation (R^2 = 0.993) between the plasma AUC for I and the amount of the MI complex found in the liver of each animal. Formation of the MI complex was demonstrated in vitro in control dog liver microsomes, was NADPH-dependent, and was dissociated from P450 with 2-methylbenzimidazole. In vitro preincubation studies indicated that I was a mechanism-based inhibitor and that formation of the complex inhibited catechol formation. These results demonstrate that the liver P450s that metabolize I form an inhibitory MI complex after in vivo administration in dogs. Formation of the complex increases during multiple dosing and inhibits the enzymes from further metabolism of I resulting in nonlinear pharmacokinetics.**

ABT-200[1] (**I**; Fig. 1) is a novel pyrrolidine derivative that exists as a racemic mixture of the *S*,*S*(−)- and *R*,*R*(+)-enantiomers. Preliminary studies demonstrated that the compound exhibited nonlinear pharmacokinetics during oral administration in the dog. Whereas initial studies with dog and human liver microsomes generated only the NADPH-dependent catechol metabolite of **I**, liver slices from these species formed extensive amounts of secondary metabolites resulting from further biotransformation of the catechol derivative via *O*-methylation, sulfation, and glucuronidation (Ferrero et al., 1997). These results suggested that oxidation of the methylenedioxy bridge carbon to form the catechol was a major initial metabolic step in the metabolism of **I**.

Certain methylenedioxyphenyl compounds interact with the mixed-function oxidases to form metabolite complexes with cytochrome P450 (P450) (Franklin, 1971; Philpot and Hodgson, 1972; Hodgson and Philpot, 1974; Fennell et al., 1980; Murray et al., 1985). Treatment of rats with isosafrole followed by isolation of hepatic microsomes results in the formation of a P450-metabolite complex, which exhibits absorption maxima at 455 nm in the dithionite-reduced difference spectrum (type III spectra) (Franklin, 1971). Formation of these complexes is believed to occur by NADPH-dependent oxidation of the methylenedioxy ring to yield a carbene and is accompanied by the inhibition of monooxygenase activity (Ullrich, 1977; Dickins et al., 1979; Mansuy, 1980).

The objective of these studies was to evaluate the metabolic fate of **I** in the dog and the potential relationship between metabolism and the nonlinear pharmacokinetic behavior. This report summarizes the re-

[1] Abbreviations used are: ABT-200/**I**, (±)-(1′*R**,3*R**)-3-phenyl-1-[(1′,2′,3′,4′-tetrahydro-5′,6′-methylene-dioxy-1′-naphthalenyl) methyl] pyrrolidine methanesulfonate; P450, cytochrome P450; HPLC, high-performance liquid chromatography; AUC, area under the curve; ESI-MS, electrospray ionization-mass spectrometry; MS/MS tandem mass spectometry.

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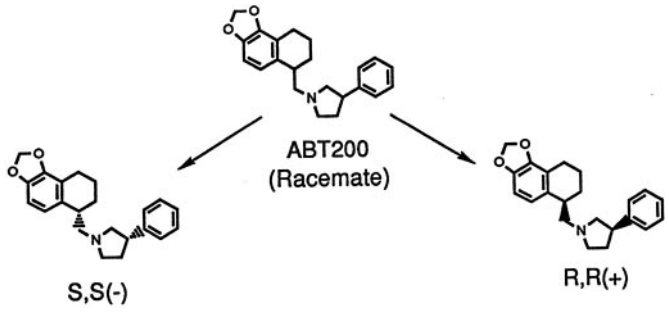

FIG. 1. *Chemical structure of racemic* **I**.

sults of a series of in vivo and in vitro studies, which examined the metabolism of each enantiomer of **I** (as the respective ^{14}C-pseudoracemate), the putative formation of a P450-metabolite complex, and the potential involvement of a P450-metabolite complex in the nonlinear pharmacokinetics in the dog.

Methods and Materials

Chemicals. The carbon-14 labeled and unlabeled *R,R*(+)- and *S,S*(−)-enantiomers of **I** were synthesized by Abbott Laboratories (Abbott Park, IL). The structures of the enantiomers of **I** and the position of the carbon-14 label are depicted in Fig. 1. The authentic catechol reference standard and the two *O*-methyl reference standards, in which the methyl moiety is attached at the hydroxy position of the catechol either *meta* or *ortho* to the six-membered saturated ring, were synthesized by Abbott Laboratories. Sulfatase, β-glucuronidase and Krebs-Henseleit buffer were purchased from Sigma-Aldrich (St. Louis, MO).

Animals. Fifteen male beagle dogs were used in these studies (Marshall Research Animals, North Rose, NY). The dogs weighed 6 to 10 kg and were 1 to 3 years of age. The animals were fed 12 h after drug administration and daily thereafter. During the study, the dogs were housed individually in stainless steel metabolism cages.

For the bile cannulation studies, the dogs were fasted overnight before the study but allowed water ad libitum. On the day of the study, the dogs were anesthetized with an initial sodium pentobarbital intravenous dose and maintained under anesthesia with a slow intravenous drip infusion of sodium pentobarbital. This was delivered through the cephalic vein using a butterfly infusion set. A midline incision was made in the abdomen, the gall bladder was clamped off, and the bile duct cannulated with polyethylene tubing.

In Vivo Metabolism Studies. *Metabolism and disposition of the ^{14}C-pseudoracemates of **I**.* This was a multicrossover study in which the same animals were used for both the oral and intravenous legs with the ^{14}C-pseudoracemates of **I**.

*Dosing of the ^{14}C-pseudoracemates of **I*** and *sample collection.* The carbon-l4 labeled *R,R*(+)-enantiomer of **I** was mixed with an equal amount of unlabeled *S,S*(−)-enantiomer (to form the ^{14}C-*R,R*(+)-pseudoracemate). Likewise, to form the ^{14}C-*S,S*(−)-pseudoracemate, carbon-14 labeled *S,S*(−)-enantiomer of **I** was mixed with an equal amount of unlabeled *R,R*(+)-enantiomer. Both pseudoracemates were dissolved in 5% dextrose in sterile water at a concentration of 2.0 mg/ml (2.6 mg mesylate salt/ml). The ^{14}C-*R,R*(+)-pseudoracemate of **I** was administered to three dogs orally by gavage at a dose of 2 mg/kg, corresponding to 1 ml/kg, and about 50 μCi/animal. Two weeks later, these dogs were given a 2 mg/kg dose of the ^{14}C-*R,R*(+)-pseudoracemate (corresponding to 1 ml/kg and about 50 μCi/animal) intravenously through the cephalic vein. Approximately three weeks after the intravenous segment of the ^{14}C-*R,R*(+)-pseudoracemate study was completed, the same three dogs were given a 2 mg/kg oral dose of the ^{14}C-*S,S*(−)-pseudoracemate, corresponding to 1 ml/kg, and about 50 μCi/animal. Two weeks later, these dogs were given a 2 mg/kg dose of the ^{14}C-*S,S*(−)-pseudoracemate (corresponding to 1 ml/kg and about 50 μCi/animal) intravenously through the cephalic vein. After dosing was completed, the radiochemical purity of the dose solution was determined to be at least 98%. After each dosing segment was completed, the radiochemical purity of the dose solutions were determined to be at least 98%.

All urine and feces excreted by the dogs were collected for 5 days after drug administration. During the first 24 h after dosing, the urine was collected in containers surrounded by dry ice. The cages of these animals were also washed daily with a small amount of water that was saved for radioassay. A small amount of ascorbic acid was added to all sample containers to retard the oxidation of possible catechol metabolites.

For the bile cannulation study, the ^{14}C-*S,S*(−)- or ^{14}C-*R,R*(+)-pseudoracemate of **I** was administered to previously untreated dogs intravenously or intraduodenally at a dose of 2 mg/kg, corresponding to 1 ml/kg, and about 25 μCi/animal. Bile was collected from 0 to 2, 2 to 4, and 4 to 6 h after drug administration. A small amount of ascorbic acid (ca. 3 mg) was added to each collection tube to retard the oxidation of possible catechol metabolites.

*Metabolism and disposition of the ^{14}C-enantiomers of **I***. Since only one of the enantiomers was radiolabeled in each pseudoracemate formulation, only the metabolites from the respective radiolabeled enantiomer were detected by HPLC/radioassay. The samples taken from these animals, however, necessarily contained a mixture of both *S,S*(−) and *R,R*(+) metabolites. Consequently, the resolution of metabolites from these preparations by the present nonchiral HPLC method would result in the isolation of a single metabolite peak, which would likely contain the metabolites of both enantiomers. Therefore, to more accurately characterize the metabolites of each enantiomer of **I**, separate dogs were dosed orally (by gavage) with either the single ^{14}C-*S,S*(−)- or ^{14}C-*R,R*(+)-enantiomer orally by gavage at a dose of 2 mg/kg (corresponding to 1 ml/kg, and about 50 μCi/animal). The urine of these animals was collected for metabolite isolation and mass spectral characterization.

Pharmacokinetic Study and In Vivo P450-Metabolite Complex Formation of Racemic I. Dogs were given a single or multiple doses of racemic **I**, and the pharmacokinetic parameters were examined after the first and last dose. The animals were sacrificed after the last dose, and the livers were excised. Microsomes were prepared from the liver of each animal and the amount of P450-metabolite complex was determined as described below.

Dose and sample collection. In the multiple dose group, racemic **I** was administered to two dogs orally in capsules at a dose of 12.0 mg base/kg/day for 14 days. The corresponding two single dose animals were given empty gelatin capsules on days 1 through 13 followed by a single 12.0 mg base/kg oral dose of **I** on day 14. The control animal received empty gelatin capsules for 14 days.

Samples of venous blood were collected in heparinized tubes from the dogs given **I** daily for 14 days at 0, 0.5, 1.5, 3, 6, 10, and 24 h after treatment on day 1 and at the same times after the final dose on day 14. Samples of venous blood were also collected from the dogs given a single oral dose of **I** on day 14 at the same time points. Twenty-four hours after the last dose, all animals on study were euthanized and the livers were removed.

Measurement of P450-metabolite complex formed in vivo. Livers were homogenized and microsomes prepared according to standard procedures (Omura and Sato, 1964). Measurement of the P450-metabolite complex was determined using a Shimadzu model UV-2101PC UV/visible scanning spectrophotometer (Scientific Instruments Inc., Hawthorne, NY). The microsomes were diluted in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and divided equally into sample and reference tubes. Formation of the P450-metabolite complex was monitored by recording the reduced minus oxidized spectrum between 400 and 500 nm. Reduction was effected by addition of sodium dithionite to the sample cuvette. Quantitation of the P450-methylenedioxyaryl complex was determined by $\Delta A_{455-490\ nm}$ using the extinction coefficient of 75 $mM^{-1}cm^{-1}$ (Elcombe et al., 1975; Fennell et al., 1980; Murray et al., 1985). The concentration of protein in the liver microsomes was determined by the method of Smith et al. (1985).

Plasma analytical method. The concentration of racemic **I** in each sample was determined by reverse-phase HPLC with electrochemical detection following liquid-liquid extraction (ethyl acetate/hexane) of the plasma matrix. The assay was linear (correlation coefficient >0.99) over the concentration range 0 to 220 ng/ml with a pooled standard deviation of <5%. Standards were analyzed in triplicate at six different concentrations with an estimated detection limit of 0.1 ng/ml. Samples with **I** concentrations in excess of 30 ng/ml were subjected to a second analysis to determine the enantiomeric distribution. The chiral analysis used liquid-liquid extraction with ethyl acetate/hexane to separate the compounds of interest from the plasma contaminants. Chiral separation was achieved on a 10 cm × 4.0 mm 5 μm α1-acid glycoprotein column

with an acetonitrile/0.01 M KH_2PO_4 pH 5.5 (12:88, by volume) mobile phase at a flow rate of 0.7 ml/min and with detection at 205 nm. Column temperature was maintained at 35°C.

Pharmacokinetic analysis. The area under the plasma concentration-time curve from 0 to 24 h (AUC_{0-24h}) after dosing was calculated using the linear trapezoidal rule. Since the bioavailability of racemic **I** was not known for the animals in this study, the apparent plasma clearance of **I** was determined using the equation of Cl = dose/AUC.

Radioassay. In the radiolabeled metabolism studies, duplicate aliquots of the bile, urine, and cagewash samples were radioassayed directly in Insta-Gel (PerkinElmer Life Sciences, Boston, MA) scintillation cocktail. The feces were homogenized in water using a motor-driven high frequency homogenizer. Duplicate aliquots of the whole blood samples and fecal homogenates were placed in Combusto-Cones (PerkinElmer Life Sciences) containing cellulose powder (Whatman, Clifton, NJ) and burned in a Tri-Carb (PerkinElmer Life Sciences) model 307 sample oxidizer. The resulting $^{14}CO_2$ was trapped in Carbosorb and radioassayed in Permafluor V (PerkinElmer Life Sciences) liquid scintillation fluid and counted in a Tri-Carb (PerkinElmer Life Sciences) model 2500TR liquid scintillation spectrometer. All samples were corrected for quenching with an automatic external standardization technique.

HPLC/Radioassay. Analyses of **I** and metabolites in biological samples, as well as incubations from the in vitro studies with dog liver microsomes, were accomplished using an IBM Instrument, Inc., LC 9560 liquid chromatograph and a diode array detector (Applied Biosystems, Inc., Foster City, CA) operated at 215 nm. Separations were achieved in a reversed phase mode using a C_{18} Altima 5-μ column (4.6 × 250 mm; Alltech Associates, Deerfield, IL) at ambient temperature. **I** and its metabolites were resolved by gradient system using two mobile phases. Mobile phase (A) was composed of 0.01 M triethylammonium formate, whereas mobile phase (B) contained 75% acetonitrile in 0.01 M triethylammonium formate. The gradient was run from 80% A/20% B at 0 min, 65% A/35% B at 15 min, 60% A/40% B at 30 min, 100% B at 40 min and then isocratically with 100% B for 10 min thereafter. The flow rate was 0.9 ml/min. The column effluent was passed through a Flo-One Beta radioactive flow detector (Radiomatic Instruments, Inc., PerkinElmer Life Sciences) where it was combined with scintillation cocktail (Flo-Scint III, Radiomatic Instruments, Inc., PerkinElmer Life Sciences) at a ratio of 1:3.6. The radioactivity eluting from the column was monitored at 6-s intervals to obtain the metabolic pattern.

Urine samples were filtered through Acrodisc 0.45-μ filters (Pall Corp. Port Washington, NY) prior to HPLC analysis. Samples were pooled to provide a representative 0- to 5-day urinary metabolite patterns. Fecal homogenates were treated with three volumes of acetonitrile and concentrated as described above. Samples were pooled to provide a representative 0- to 5-day fecal metabolite patterns. Recovery of fecal radioactivity averaged about 85 to 90%. Bile samples were vortexed prior to HPLC analysis and pooled to provide representative 0- to 6-h metabolic patterns.

For the enzymatic hydrolysis of possible conjugated metabolites, one volume of urine or bile (one-tenth volume) was combined with either one volume of a β-glucuronidase preparation or one volume of a sulfatase preparation. Control incubations contained only 0.1 M potassium phospate buffer (pH 7.4). Samples were incubated in a Dubnoff metabolic water bath at 37°C for 16 h. Prior to HPLC analysis, samples were extracted with acetonitrile, as described above. Urine or bile samples were also treated with 1.0 N HCl or 1.0 N NaOH at 70°C for 1 h. The samples were neutralized and extracted, as described above, prior to HPLC analysis.

Isolation of Metabolites. Urine samples from the radiolabeled enantiomer metabolism study were carefully applied to an ion-exchange column and washed with several column volumes of distilled water to remove endogenous urinary components yet retain metabolites. Metabolites of **I** were eluted with methanol, and the methanol was evaporated under vacuum at room temperature. The metabolites were dissolved in HPLC mobile phase prior to chromatography. Samples containing metabolites of the individual *S,S*(−)- and *R,R*(+)-enantiomers were chromatographed using the system previously described. Fractions were collected at 0.5-min intervals, and a small aliquot of each fraction was counted to obtain the overall metabolic pattern. Fractions containing a radioactive peak were evaporated to a small volume (<100 μl) and analyzed by electrospray ionization and MS/MS techniques.

Characterization of Metabolites by ESI MS/MS. Electrospray-ionization mass spectral analysis (ESI-MS) was done using a Finnigan-MAT TS700 (Thermo Finnigan, San Jose, CA) with 2-methoxy ethanol as a sheath liquid. The ESI MS/MS analysis was done on the TS700 using argon gas for ESI analysis and MS/MS to verify the metabolite structure.

In Vitro Studies. *Metabolism of **I** by control dog liver microsomes.* Dog livers were homogenized and microsomes prepared according to standard procedures (Omura and Sato, 1964). For generation of oxidative metabolites of **I**, the microsomes were diluted to a concentration of about 1 mg/ml in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and radiolabeled **I** (100 μM). Reactions were initiated with NADPH (1 mM). In some experiments, unlabeled racemic (final concentration 100 μM) was incubated with control dog liver microsomes in the presence or absence of NADPH for 20 min followed by the addition of a trace amount of the radiolabeled compound (and NADPH was added to incubations not yet containing this cofactor) to initiate radiolabeled metabolism and the reactions were stopped at various times with acetonitrile/methanol (9:1, v/v). Extracts were analyzed by HPLC/radioassay. Radiolabeled metabolite conversion over the time course averaged less than 12%.

*Formation of the P450-metabolite complex of **I** by control dog liver microsomes.* For in vitro formation of P450-metabolite complex, liver microsomes from control dogs were diluted with 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and divided into two tubes (sample and reference). Racemic **I**, or the respective *S,S*- and *R,R*-enantiomers of **I**, were dissolved in dimethyl sulfoxide and added to the sample cuvette while dimethyl sulfoxide alone was added to the reference cuvette. The final concentration of drug was 100 μM. Preliminary experiments indicated that P450-metabolite complex formation was linear from 50 to 100 μM. Measurement of complex formation at concentrations of **I** below 50 μM, however, resulted in poor signal-to-noise. Therefore, 100 μM was used. Complex formation was initiated with NADPH (1 mM, added to both sample and reference cuvette), and the spectrum was scanned from 400 to 500 nm at zero time and approximately every 5 min thereafter until complex formation was maximal (20 min). Incubations were run at 37°C. Quantitation of the P450-metabolite complex was determined by $\Delta A_{455-490nm}$ using the extinction coefficient of 75 $mM^{-1}cm^{-1}$.

Results

Characterization of Metabolites of I by ESI MS/MS. Identification of the metabolites of **I** by mass spectral analysis of the urinary metabolites isolated from dogs given the ^{14}C-*R,R*(+)- or ^{14}C-*S,S*(−)-enantiomer is presented in the metabolic pathway in Fig. 2. In many cases, the metabolites were present as triethylammonium adducts resulting from the paired ion buffer contained in the mobile phase HPLC used during metabolite isolation. An estimate of the relative positions of the mono-conjugates or the *O*-methyl sulfate and *O*-methyl glucuronide moieties on the respective hydroxyl groups of the catechol derivative of **I** were determined by the HLPC characteristics of each aglycone after hydrolysis compared with that of the authentic *O*-methyl reference standards, which were methylated at either the *meta* or *ortho* hydroxyl positions of the catechol.

Metabolite Profiles in Urine After Administration of the ^{14}C-Enantiomers of I. The urinary metabolite profiles from dogs given the ^{14}C-enantiomers of **I** were virtually identical to those observed in dogs given the ^{14}C-pseudoracemates of **I**. In addition, the effect of various hydrolysis treatments on the urinary metabolite profiles were similar whether the animals were dosed with the ^{14}C-enantiomers or the ^{14}C-pseudoracemates of **I**. These results indicated that the presence of either enantiomer did not affect the metabolite product specificity of the antipole.

Metabolism and Disposition of the ^{14}C-Pseudoracemates of I. *Excretion of Radioactivity.* Within 5 days after oral and intravenous administration, respectively, 28.6 and 30.2% of the ^{14}C-*S,S*(−)-pseudoracemate dose and 32.1 and 31.6% of the ^{14}C-*R,R*(+)-pseudoracemate dose was eliminated in the urine. Fecal elimination after oral and intravenous administration, respectively, comprised 60.0 and 60.9%

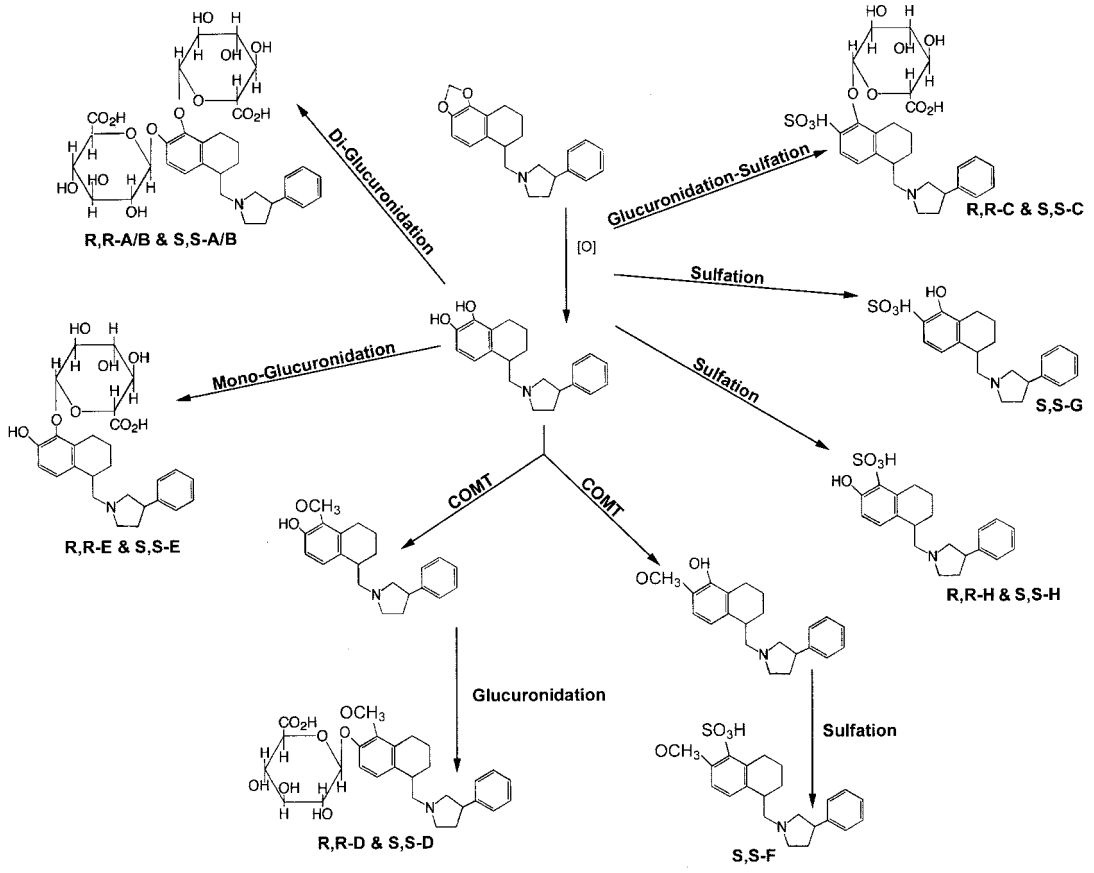

FIG. 2. *Proposed metabolic pathways of* **I** *in dog.*

of the ^{14}C-*S*,*S*(−)-pseudoracemate dose and 63.9 and 62.5% of the ^{14}C-*R*,*R*(+)-pseudoracemate dose. The similar urinary excretion of radioactivity after oral and intravenous administration indicated that radioactivity was well absorbed after oral administration of both ^{14}C-pseudoracemates. Within 6 h after administration of the ^{14}C-*S*,*S*(−)- and ^{14}C-*R*,*R*(+)-pseudoracemate, respectively, to dogs with surgically implanted biliary catheters, about 45.2 and 56% of the intravenous dose and 56.9 and 35.5% of the intraduodenal dose was recovered in the bile.

Metabolite Profiles in Urine, Feces, and Bile. A summary of the amounts of parent drug and metabolites excreted in the urine and feces is presented in Table 1.

The metabolite patterns in the urine samples were qualitatively similar after administration of either pseudoracemate. Unchanged parent drug was not detected in the urine of any animals after either route of administration. Hydrolysis of urine with β-glucuronidase indicated that metabolite peaks **A**, **B**, **C**, **D**, **E**, and **F** contained glucuronic acid conjugates (i.e., the size of these peaks decreased significantly after β-glucuronidase treatment with an increase in the amount of the catechol and the *ortho*-*O*-methyl metabolite). Mass spectral characterization of these peaks isolated from the urine of dogs given only the ^{14}C-*S*,*S*(−)- or ^{14}C-*R*,*R*(+)-enantiomer of **I** confirmed that peaks **A** and **B** were di-glucuronic acid conjugates of the catechol, peak **C** was a glucuronic acid/sulfate conjugate of the catechol, peak **D** was the glucuronic acid conjugate of the *ortho*-*O*-methyl metabolite, and peak **E** was the mono-glucuronic acid conjugate of the catechol derivative of **I**. Sulfatase or acid hydrolysis of the urine indicated that peaks **F**, **G**, and **H** contained sulfate conjugates; as these three peaks decreased while the *ortho*-*O*-methyl and catechol metabolite peaks increased. Isolation of these peaks indicated that peak **F** was the sulfate conjugate of the *ortho*-*O*-methyl metabolite, and peaks **G** and **H** were identified as mono-sulfate conjugates of the catechol derivative of **I**.

After administration of either ^{14}C-pseudoracemate to dogs, the major component in the feces was the catechol metabolite of **I**. The catechol derivative represented about 50% of the administered dose, whereas the sulfate conjugates comprised about 10%. No unchanged parent drug was detected in the feces after administration of either pseudoracemate.

The biliary metabolite patterns were qualitatively similar to those observed in the urine. The major biliary metabolite was the di-glucuronic acid conjugate of **I**. Unchanged parent drug was a minor biliary component comprising only about 1% of the administered dose (data not shown).

Pharmacokinetic Study of Racemic I After Single and Multiple Dosing. A comparison of the pharmacokinetic parameters in dogs after single and multiple doses of **I** are presented in Table 2 and Figs. 3, A and B. After 14 daily doses of **I**, the mean plasma C_{max} and AUC_{0-24h} were 2.024 μg/ml and 18.576 μg · h/ml, respectively, compared with mean values of 0.590 μg/ml and 3.283 μg · h/ml after a single dose. In addition, the plasma clearance values averaged 6.06 l/h · kg after a single dose of **I** decreasing approximately 8-fold to 0.78 l/h · kg after the multiple dosing regimen. These results indicate that the (metabolic) clearance of **I** decreases substantially during chronic dosing.

Formation of a Liver P450-Metabolite Complex of I. *In vivo.* A redox difference spectra of the liver microsomes isolated from dogs given a single or multiple doses of racemic **I** are presented in Fig. 4. The amount of the P450-metabolite complex in the liver microsomes of dogs treated with **I** is presented graphically in Fig. 5. In addition, the correlation between the amount of liver P450-metabolite complex

TABLE 1

*Summary of parent drug and metabolites eliminated by dogs after oral and intravenous administration of the ^{14}C-S,S(−)- and ^{14}C-R,R(+)-pseudoracemates of **I***

See Figure 2 for metabolite identification.

Metabolites	% of Administered Dose					
	14-*S*,*S*(−)			14-*R*,*R*(+)		
	Urine	Feces	Total	Urine	Feces	Total
Oral						
A	5.29	< 0.5	5.29	6.57	<0.5	6.57
B	1.67	< 0.5	1.67	3.22	< 0.5	3.22
C	11.40	< 0.5	11.40	0.79	< 0.5	0.79
D	0.66	1.06	1.72	1.38	< 0.5	1.38
E	4.46	10.52	14.98	< 0.5	9.83	9.83
F	0.77	< 0.5	0.77	< 0.5	< 0.5	< 0.5
G	1.12	< 0.5	1.12	18.03	3.89	21.92
Catechol	< 0.5	47.06	47.06	< 0.5	50.24	50.24
ABT-200(**I**)	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Total	25.36	58.64	84.01	29.85	63.96	93.95
Intravenous						
A	6.00	< 0.5	6.00	6.05	< 0.5	6.05
B	1.23	< 0.5	1.23	2.67	< 0.5	2.67
C	12.87	< 0.5	12.87	1.41	< 0.5	1.41
D	1.53	< 0.5	1.53	1.92	< 0.5	1.92
E	4.75	4.69	9.44	< 0.5	11.13	11.13
F	0.78	< 0.5	0.78	< 0.5	< 0.5	< 0.5
G	0.81	< 0.5	0.81	17.1	7.6	24.70
Catechol	< 0.5	54.68	54.68	< 0.5	38.62	38.62
ABT-200(**I**)	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Total	27.92	59.37	87.29	29.01	57.35	86.36

TABLE 2

*Comparison of the single and multiple dose pharmacokinetics of racemic **I** in the dog*

Dog No.	C_{max}	T_{max}	AUC	app. CL
	mcg/ml	*hr*	*mcg · hr/ml*	*l/hr · kg*
Single Dose				
D-2	0.499	3	3.230	3.72
D-3	0.214	0.5	0.794	15.11
D-4 Day-1	1.058	0.5	5.267	2.28
D-5 Day-1	0.589	3	3.842	3.12
Mean (all dogs)	0.590	1.8	3.283	6.06
Mean (D-4 & D-5)	0.824	1.8	4.555	2.70
Multiple Dose				
D-4 Day-14	1.195	0.5	10.996	1.09
D-5 Day-14	2.853	0.5	26.156	0.46
Mean	2.024		18.576	0.78
Ratio MD/SD (Dogs 4 & 5) Dose: 12.0 mg/kg	2.5		4.1	0.29

formed during in vivo drug administration and the plasma clearance of **I** is depicted in Fig. 6.

Both dogs given a single oral dose of **I** demonstrated the formation of a methylenedioxyaryl complex with P450 24 h after receiving drug. The amount of the P450-metabolite complex in the single dose animals averaged 31 pmol/mg of microsomal protein (range 17–45 pmol/mg of protein) representing 5.0% of total liver P450. Considerably greater amounts of the P450-metabolite complex were found in the liver of dogs receiving **I** chronically for 14 days. The amount of the P450-metabolite complex in liver microsomes isolated from the multiple dose animals averaged 163 pmol/mg of protein (range 112–215 pmol/mg of protein) representing 19.6% of total liver P450. The liver of dogs given multiple doses of **I** contained, on average, 5.3-fold more of the P450-metabolite complex than did the single dose animals. No evidence of a liver P450-metabolite complex was apparent in the control dogs. Figure 6 demonstrates that despite the considerable amount of variability between animals in both the amount of the P450-metabolite complex formed in the liver and the plasma clearance after single and multiple dosing of **I**, there was a significant correlation ($R^2 = 0.99$) between these parameters.

In vitro. Incubation of **I** with control dog liver microsomes in the presence of NADPH demonstrated the formation of a P450-metabolite complex (155–262 pmol/mg). No P450 complex was formed in the absence of NADPH indicating that **I** does not form a complex with P450 directly but requires metabolism. Formation of the P450-metabolite complex was maximal by 20 to 25 min. Similar amounts of the P450-metabolite complex were formed after incubation of the individual *R*,*R*- and *S*,*S*-enantiomers (196 and 204 pmol/mg, respectively), indicating no stereoselectivity regarding formation of the P450-metabolite complex. Addition of 10 mM 2-methylbenzimidazole to the cuvette following complex formation resulted in the displacement of 70 to 80% of the metabolite from P450 (data not shown). These results indicated that **I** can form a metabolite complex with liver P450

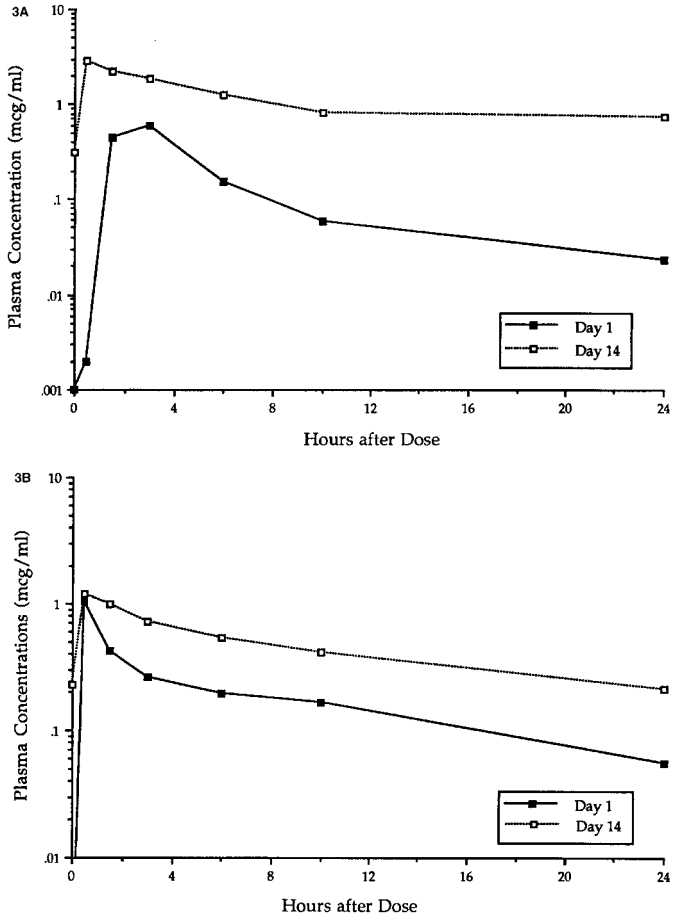

FIG. 3. *Plasma concentration-time curves after single or multiple doses of **I** in dogs; curves A and B correspond to dogs 5 and 4, respectively.*

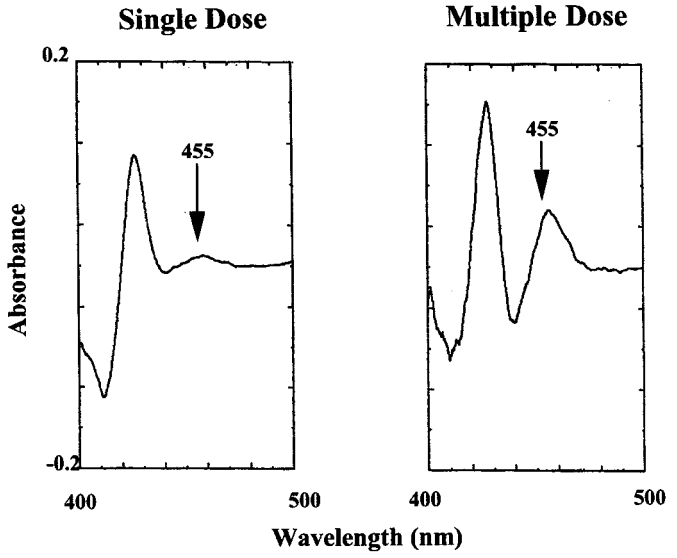

FIG. 4. *In vivo formation of a P450-metabolite complex in dog liver after single or multiple doses of **I**.*

Livers were removed from dogs after a single or 14 daily doses of I. Microsomes were prepared and diluted in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and divided equally into sample and reference tubes. Formation of the P450-metabolite complex was monitored by recording the reduced minus-oxidized spectrum between 400 and 500 nm.

both in vivo and in vitro and that the binding of the metabolite to P450 is reversible and not stereoselective.

Metabolism of ABT-200 by Dog Liver Microsomes. Incubation of **I** with dog liver microsomes in the presence of NADPH generated only the catechol metabolite. There was a significant decrease in the rate of catechol formation during the 30-min incubation period, decreasing by an average of 62%. The rate of catechol formation was also compared in dog liver microsomes preincubated with unlabeled **I** in the presence or absence of NADPH for 20 min prior to the addition of a trace amount of radiolabeled **I** (to initiate radiolabeled metabolism) and NADPH (to incubations not yet containing the cofactor). Preincubation of **I** with NADPH and microsomes decreased the initial rate of catechol formation by 48 to 60% compared with preincubation without NADPH.

Discussion

This study was undertaken to test the hypothesis that the considerable decrease in the clearance of **I** during chronic dosing was due to the formation of an inhibitory methylenedioxyaryl metabolite complex with the P450s that metabolize the compound. In support of this, a small amount of the P450-metabolite complex was formed in dogs within 24 h after a single dose of **I**, whereas considerably more of the complex was found after 14 days of treatment. Since the study design enabled the determination of the plasma pharmacokinetics of **I** and the formation of a P450-metabolite complex from the same animals, it was possible to evaluate the correlation between these two parameters. Although there was a 30-fold variation in the plasma AUC or clearance of **I** between animals and a 12-fold variation in the amount of the liver P450-metabolite complex formed between dogs, there was a positive correlation between these pharmacokinetic parameters and the amount of the P450-metabolite complex formed in the livers of each animal (Fig. 6). Studies with control dog liver microsomes indicated that the in vitro formation of the P450 complex with **I** was NADPH-dependent and reversible with 2-methylbenzimidazole. This indicated that **I** did not form a complex with P450 directly but required oxidative metabolism and that the binding was not covalent but reversible. The rate of conversion of **I** to the catechol decreased with time in control dog liver microsomes. In a separate set of experiments, preincubation of unlabeled **I** with control dog liver microsomes and NADPH for 20 min followed by the addition of a tracer amount of radiolabeled **I** resulted in a significant decrease in the rate of catechol formation compared with the rate without preincubation. These results indicated that **I** appears to be a mechanism-based inhibitor and that formation of the complex inhibits the rate of catechol formation. Currently, however, it is not known whether the mechanism of catechol formation is tightly coupled to P450-metabolite complex formation, but the data suggest that other mechanisms may be involved. Preliminary in vitro experiments indicated that even when formation of the P450-metabolite complex is maximal, the rate of catechol formation is still about 40 to 50% of the initial rate. The fact that both the *R*,*R*- and *S*,*S*-enantiomers formed similar amounts of the P450-metabolite complex in vitro was in agreement with the nonstereoselective accumulation of **I** during multiple dosing in dogs. Whereas the *S*,*S*(−)-enantiomer predominated in the plasma after administration of racemic **I**, the nonlinear accumulation was observed with both enantiomers. Preliminary studies in human liver microsomes suggest that CYP3A4 is the major isozyme involved in metabolite complex formation of **I**.

The pharmacokinetic results in this study were consistent with previous preliminary investigations of **I** in dogs, which demonstrated considerably higher plasma levels of **I** after multiple dosing compared with a single dose. In the present study, the plasma area under the

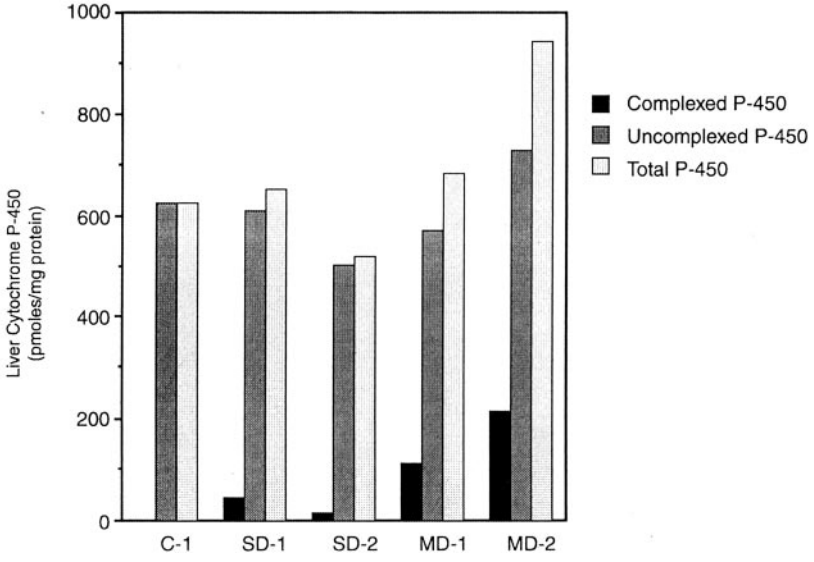

FIG. 5. *Amount of complexed, uncomplexed, and total P450 in the liver of dogs given single or multiple doses of* ***I****.*

Dose, 12.0 Mg/Kg. Animal treatment abbreviations are C-1 (control, dog-1), SD-1 (single dose, dog-1), SD-2 (single dose, dog-2), MD-1 (multiple dose, dog-1), and MD-2 (multiple dose, dog-2).

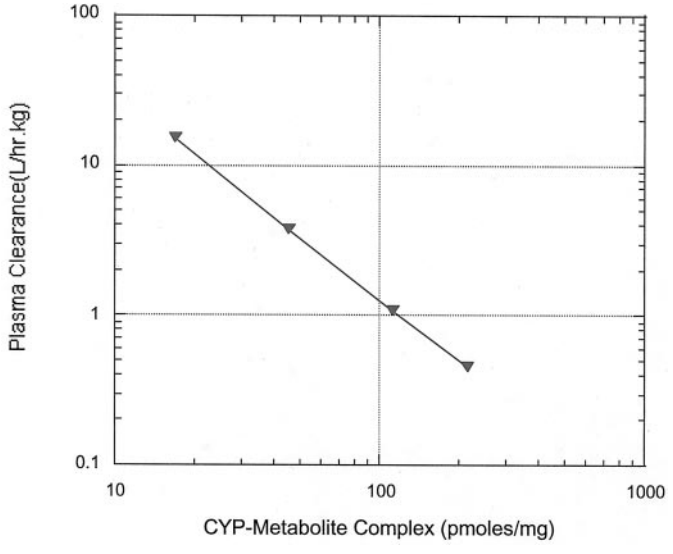

FIG. 6. *Correlation between the amount of liver P450-metabolite complex and the plasma clearance of* ***I*** *in dogs given single or multiple doses of* ***I****.*

curve values in dogs given multiple doses of **I** averaged 4-fold greater than in the same animals given a single dose. Correspondingly, there was a 9-fold decrease in the apparent plasma clearance after multiple dosing compared with a single dose of **I**. Since the clearance of **I** in the dog was due entirely to metabolism, the large decrease in drug clearance during chronic dosing was predominantly due to a decline in the rate of metabolism.

Figure 2 depicts the proposed metabolic pathways of **I**. The first metabolic step involving the metabolism of **I** was oxidation at the methylenedioxy region to form the catechol derivative. The catechol then underwent *O*-methylation, sulfation and/or glucuronidation. Some stereoselectivity in the metabolic patterns was also apparent, with the *O*-methyl/sulfate metabolite formed only by the *S,S*(−)-pseudoracemate of **I**. In addition to the formation of mono-glucuronide and sulfate conjugates, di-conjugates (glu/glu and glu/sulfate) were also confirmed by mass spectral analysis. The in vivo formation of di-conjugated metabolites has been demonstrated with steroids and bile acids, including glucuronide/glucuronide, sulfate/sulfate and sulfate/glucuronide conjugates (Billing et. al., 1957). The di-glucuronic acid conjugate of *p*-hydroxybenzoic acid has also been reported in the urine of dogs after administration of the aglycone (Quick, 1932).

The mass spectral data demonstrating the formation of two di-glucuronic acid conjugates of the catechol derivative of **I** was somewhat perplexing. These two conjugates, designated as metabolites "A" and "B", exhibited different chromatographic retention times. Thus, these metabolites were clearly distinct entities with no apparent cross contamination prior to mass spectral analysis. Metabolite B was hydrolyzable by β-glucuronidase, whereas metabolite A was resistant to hydrolysis by β-glucuronidase, acid or base. A possible explanation for these results may be that metabolite A is a intramolecular rearrangement of the glucuronic acid sugar moieties. This has been shown to occur with some ester glucuronides in which the glucuronic acid attached to the aglycone at the control dog-1 position can rearrange or migrate and attach to the 2-, 3-, or 4-position of the sugar (Dickinson and King, 1991). These positional isomers generally have shorter retention times on reverse-phase HPLC and are resistant to β-glucuronidase hydrolysis, as was metabolite A for both pseudoracemates in the present study. Although the intramolecular rearrangement of ester glucuronides is well documented, a similar occurrence for ether glucuronides has not been reported.

The effects of methylenedioxyaryl compounds that form metabolite complexes with P450 are frequently biphasic when administered to animals in vivo. Their inhibitory effects on drug metabolism are often followed by an increase in mixed function oxidase activity due to the induction of liver P450 (Karmienski et al., 1971; Kumagai et al., 1994). In the present study, one of the two dogs dosed chronically with **I** demonstrated P450 levels that were significantly above those in the control animal. Although this was not investigated, there may exist the potential for induction of P450 by **I**.

Several, but not all, methylenedioxyaryl-containing compounds have been shown to form metabolite complexes with P450 (Franklin, 1971). Structural requirements appear to include both a methylenedioxyaryl configuration and compound lipophilicity. **I** fits these criteria. The NADPH-dependence of complex formation indicated that oxidative metabolism of **I** is required. Based on previous mech-

anistic studies of P450 complex formation with isosafrole, another lipophilic methylenedioxyphenyl compound, the formation of a P450-metabolite complex with **I** may involve oxidation of the methylenedioxy bridge carbon of ABT-200, possibly through a carbene intermediate, which then binds to the heme iron of P450 forming a complex as proposed by Dickins et al. (1979).

Collectively, these results demonstrate that some of the liver P450s that are responsible for the metabolism of **I** form a metabolite complex after administration in dogs. Formation of these complexes increases during chronic dosing and inhibits the enzyme(s) from further metabolism of **I**, resulting in a marked decrease in drug clearance and nonlinear accumulation in the plasma.

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