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HEPATOBILIARY EXCRETION OF BERBERINE

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ABSTRACT:

Berberine is a bioactive herbal ingredient isolated from the roots and bark of *Berberis aristata* or *Coptis chinensis*. To investigate the detailed pharmacokinetics of berberine and its mechanisms of hepatobiliary excretion, an in vivo microdialysis coupled with high-performance liquid chromatography was performed. In the control group, rats received berberine alone; in the drug-treated group, 10 min before berberine administration, the rats were injected with cyclosporin A (CsA), a P-glycoprotein (P-gp) inhibitor; quinidine, both organic cation transport (OCT) and P-gp inhibitors; SKF-525A (proadifen), a cytochrome P450 inhibitor; and probenecid to inhibit the glucuronidation. The results indicate that berberine displays a linear pharmacokinetic phenomenon in the dosage range from 10 to 20 mg kg^{-1}, since a proportional increase in the area under the concentration-time curve (AUC) of berberine was observed in this dosage range. Moreover, berberine was processed through hepatobiliary excretion against a concentration gradient based on the bile-to-blood distribution ratio (AUC_{bile}/AUC_{blood}); the active berberine efflux might be affected by P-gp and OCT since coadministration of berberine and CsA or quinidine at the same dosage of 10 mg kg^{-1} significantly decreased the berberine amount in bile. In addition, berberine was metabolized in the liver with phase I demethylation and phase II glucuronidation, as identified by liquid chromatography/tandem mass spectrometry. Also, the phase I metabolism of berberine was partially reduced by SKF-525A treatment, but the phase II glucuronidation of berberine was not obviously affected by probenecid under the present study design.

The plant alkaloid, berberine (Fig. 1), is derived from the roots and bark of *Berberis aristata* and *Coptis chinensis*, extracts of which have been used in traditional oriental medicine for the treatment of gastroenteritis and secretory diarrhea. Its chemical structure has a quaternary base, and it is commercially prepared for clinical application as various salts such as berberine chloride or sulfate. Multiple pharmacological effects have been attributed to berberine and its relative derivatives, such as antidiarrheic (Taylor and Baird, 1995), antimicrobial (Kaneda et al., 1991), anticancer (Iizuka et al., 2000), anti-inflammatory (Ckless et al., 1995), and antiarrhythmic (Sanchez-Chapula, 1996).

Phytomedicine, including natural products from traditional herbal medicines for medical and health-fortifying purposes, is gaining international popularity. However, little is known about the mechanisms of action of their active principles and even less about their pharmacokinetic mechanisms. Recently, three sulfate-conjugated metabolites of berberine in healthy volunteers' urine were identified after oral administration of berberine at 0.9 g per day for 3 days. In this case, little berberine was recovered in urine compared with its metabolites (Pan et al., 2002). At present, there is little information available on the protein-unbound berberine levels in biological fluids of blood and bile following administration. Therefore, studying its detailed pharmacokinetic mechanism is warranted.

The elimination process through the primary active transport mechanisms is now designated as "phase III" in the detoxification for xenobiotics, in addition to phase I by cytochrome P450 and phase II by conjugation (Ishikawa, 1992). Transport by ATP-dependent efflux pumps, such as P-glycoprotein (P-gp[1]), is an increasingly recognized determinant of drug disposition. For example, P-gp encoded by the human *MDR1* and mouse *mdr1a/1b* genes functions as a drug efflux transporter expressed in normal tissues such as brain, liver, kidney, and intestine (Matheny et al., 2001). P-gp-mediated transport in the liver has been shown to be responsible for the excretion of xenobiotics via the canalicular membrane of hepatocytes into bile, and this physiological function may be a control mechanism to accelerate the processes of hepatobiliary excretion (Schinkel, 1997).

It has been reported that berberine might up-regulate the multidrug resistance transporter expression and function in human and murine hepatoma cells (Lin et al., 1999a). Moreover, berberine could antagonize the action of paclitaxel, a well known P-gp substrate in tumor cells mediated by *MDR1* expression and thereby reduce its uptake and resistance (Lin et al., 1999b). Furthermore, there have been reports indicating that plant amphipathic cations, like berberine alkaloids, are good MDR substrates (Hsieh et al., 1998; Lewis, 1999). Since berberine interacts with the P-gp, clarification of the transport mechanism

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[1] Abbreviations used are: P-gp, P-glycoprotein; HPLC, high-performance liquid chromatography; SKF-525A, proadifen; CsA, cyclosporin A; LC/MS-MS, liquid chromatography/tandem mass spectrometry; ESI, electrospray ionization; AUC, area under the concentration-time curve; CL, clearance; PSC 833, valspodar; MRP2, multidrug resistance-associated protein 2.

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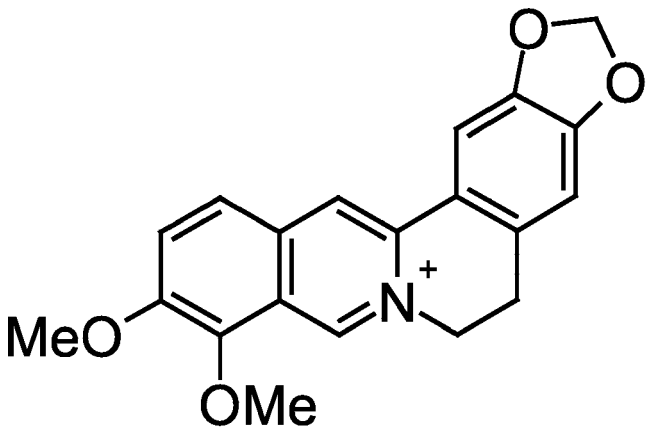

FIG. 1. *Chemical structure of berberine.*

may provide important information for studying the pharmacokinetics of berberine.

Thus, to obtain more detailed information about the disposition of berberine in vivo, this study investigates the pharmacokinetics of unbound berberine in rat blood, liver, and bile using a microdialysis sampling technique coupled with HPLC. In addition, further exploration of its mechanism concerning the hepatobiliary excretion and liver metabolism is also observed by comparing the pharmacokinetics of berberine present both with and without a P-gp inhibitor, an organic cation transport inhibitor, a cytochrome P450 inhibitor, or a glucuronidation inhibitor, separately.

Materials and Methods

Chemicals and Reagents. Berberine injection (5 mg ml^{-1} per vial) was purchased from Kyorin (Taoyuan, Taiwan). Cyclosporin A (Sandimmun) was purchased from Novartis (Basel, Switzerland). Quinidine was purchased from Aldrich Chemical Co. (Milwaukee, WI). SKF-525A (proadifen) and probenecid were purchased from Sigma-Aldrich (St. Louis, MO). Liquid chromatographic grade solvents and reagents were obtained from Merck (Darmstadt, Germany). Triply deionized water (Millipore Corporation, Bedford, MA) was used for all preparations.

Animals. All experimental protocols involving animals were reviewed and approved by the institutional animal experimentation committee of the National Research Institute of Chinese Medicine, Taiwan. Male specific pathogen-free Sprague-Dawley rats (300 ± 50 g) were obtained from the Laboratory Animal Center of the National Yang-Ming University, Taipei, Taiwan. Following arrival, the animals were kept in our animal facilities to acclimatize for about 7 days in the animal quarters with air conditioning and an automatically controlled photoperiod of 12 h of light daily. Animals had free access to food (Laboratory Rodent Diet 5P14, PMI Feeds Inc., Richmond, IN) and water until 18 h before being used in experiments, at which time only food was removed. The rats were initially anesthetized with 1 g ml^{-1} urethane and 0.1 g ml^{-1} (1 ml kg^{-1} i.p.) α-chloralose, and remained anesthetized throughout the experimental period as needed. The femoral vein was exposed for further drug administration. During the experiment, the rats' body temperature was maintained at 37°C with a heating pad.

Microdialysis Technique. Blood, liver, and bile microdialysis systems consisted of a CMA/100 microinjection pump (CMA/Microdialysis, Solna, Sweden), multiple microdialysis probes, and a CMA/140 fraction collector, as described previously (Tsai, 2001; Tsai et al., 2001). The dialysis probes for blood and liver (1 cm in length) were made of silica capillary in a concentric design with their tips covered by dialysis membrane (150-μm outer diameter with a nominal molecular weight cutoff of 13,000; Spectrum Laboratories, Inc., Rancho Dominguez, CA). All tubing unions and the end of the dialysis membrane were cemented with epoxy, and at least 24 h was allowed for the epoxy to dry. The blood and liver microdialysis probes were separately positioned within the jugular vein/right atrium and the hepatic median lobe, and then perfused with anticoagulant citrate dextrose solution (3.5 mM citric acid, 7.5 mM sodium citrate, 13.6 mM dextrose) at a flow rate of 3 μl min^{-1}. The microdialysis probes for bile sampling were constructed in our own laboratory according to the design originally described by Scott and Lunte (1993). A 7-cm dialysis membrane was inserted into a polyethylene tube (PE-60; 0.76-mm i.d., 1.22-mm o.d.; Clay Adams, Parsippany, NJ). The ends of the dialysis membrane and PE-60 were inserted into silica tubing (40-μm i.d, 140-μm o.d.; SGE Australia, Ringwood, Australia) and PE-10 (0.28-mm i.d.; 0.61-mm o.d.), respectively. Both ends of the tubing and the unions were cemented with epoxy, and at least 24 h was allowed for the epoxy to dry (Tsai, 2001). After bile duct cannulation into a rat, the microdialysis probe was perfused with Ringer's solution at a flow rate of 3 μl min^{-1}. The sampling interval was 10 min for each microdialysis probe.

Microdialysis Recovery. An in vivo retrograde calibration technique was utilized to obtain the microdialysate recovery. The blood, liver, and bile microdialysis probes were individually inserted into the jugular vein, hepatic median lobe, and bile duct under anesthesia. Anticoagulant citrate dextrose solution (for blood and liver microdialysis) or Ringer's solution (for bile microdialysis) containing berberine was perfused through the probe at a constant flow rate of 3 μl min^{-1} using the infusion pump CMA/100. Then, 2 h after probe implantation, the perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of berberine were measured by HPLC. The in vivo relative recoveries (R_{dial}) of berberine across the microdialysis probe were calculated according to the following equation: $R_{dial} = (C_{perf} - C_{dial})/C_{perf}$ (Evrard et al., 1996).

Drug Administration. After a 2-h postsurgical stabilization period, berberine (10 or 20 mg kg^{-1}) was administered via femoral vein by i.v. bolus injection for the control group. For the drug-treated groups, CsA (20 mg kg^{-1}), quinidine (10 mg kg^{-1}), SKF-525A (10 mg kg^{-1}), or probenecid (100 mg kg^{-1}) was injected i.v. via the femoral vein 10 min before berberine administration. Six animals were used in each group. All blood, liver, and bile dialysates were collected every 10 min and then measured by a validated HPLC system.

HPLC System for Dialysates. The HPLC system consisted of a chromatographic pump (BAS PM-80; BAS Bioanalytical Systems, West Lafayette, IN), an injector (CMA 160; CMA/Microdialysis) equipped with a 20-μl sample loop, and an ultraviolet detector (Varian, Inc., Palo Alto, CA). Berberine was separated from the dialysates using a Zorbax SB-phenyl column (150 × 4.6 mm i.d.; particle size 5 μm) maintained at ambient temperature. The mobile phase was composed of 350 ml of acetonitrile, 200 ml of methanol, 20 mM monosodium phosphate (pH 3.0), and 0.1 mM 1-octanesulfonic acid in 1 liter. The buffer was filtered through a Millipore 0.45-μm filter and degassed before use. The flow rate of the mobile phase was 1 ml min^{-1}. The UV detector for berberine was set at a wavelength of 346 nm. Output data from the detector were integrated via an EZChrom chromatographic data system (Scientific Software, San Ramon, CA). The chromatographic method has previously been reported (Tsai and Tsai, 2002).

Liquid Chromatography/Tandem Mass Spectrometry (LC/MS-MS) for Metabolite Identification. LC/MS-MS analysis was performed using a Waters 2690 with a 996 photodiode assay detector, together with an automatic liquid chromatographic sampler and an autoinjection system hyphenated to a Micromass Quattro Ultima tandem quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization (ESI) source. The separation was achieved using a reversed-phase C18 column (150 × 4.6 mm i.d.) (Agilent Technologies, Palo Alto, CA). The mobile phases A, B, and C were water, methanol, and 2% acetic acid, respectively. The solvent system was kept constant at 1 ml min^{-1} and it obeyed a linear gradient elution according to the following profile: 0 to 15 min, 60 to 20% A, 20 to 60% B, 20 to 20% C. The volume of injection was 10 μl. For operation in MS-MS mode, a mass spectrometer with an orthogonal Z-spray ESI interface was used. The infusion experiment was performed using a Mode 22 multiple syringe pump (Harvard Apparatus Inc., Holliston, MA). During the analyses, the ESI parameters were set as follows: capillary voltage, 1.6 kV for positive mode; source temperature, 80°C; desolvation temperature, 250°C; cone gas flow, 69 l h^{-1}; and desolvation gas flow, 666 l h^{-1}. The cone voltages of *m/z* 336, 322, and 498 were 20, 25, and 25 V, and the collision voltages were 25, 22, and 22 eV, respectively. All LC/MS-MS data were processed by the MassLynx version 4.0 NT Quattro data acquisition software.

Bile Sample Preparation for LC/MS-MS. To investigate the berberine metabolism in bile, an i.v. bolus of 10 mg kg^{-1} berberine was administered

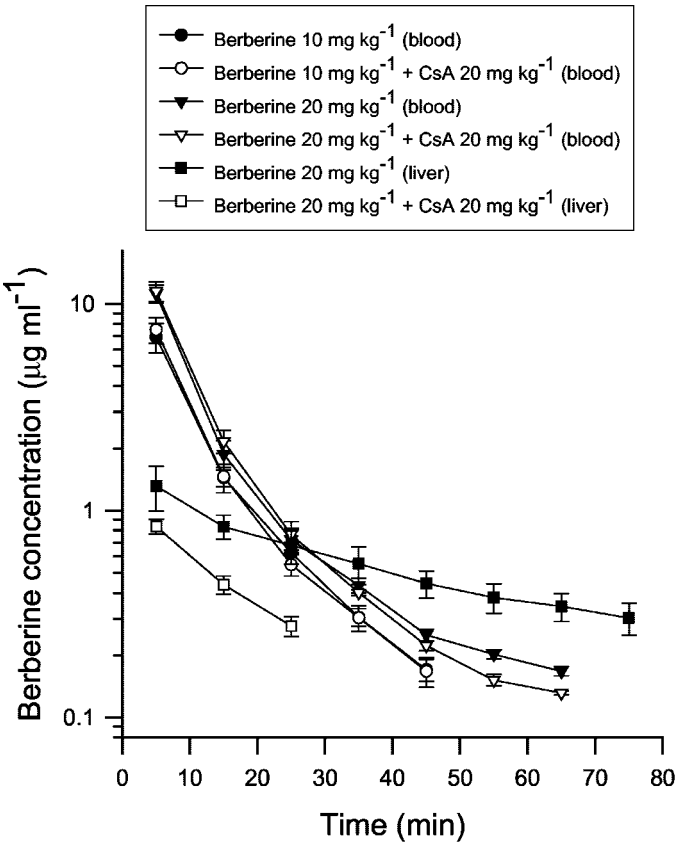

FIG. 2. *Concentration-time profiles for berberine in blood and liver dialysate after berberine i.v. administration at doses of 10 and 20 mg kg^{-1} with and without CsA 20 mg kg^{-1}.*

The data are means ± S.E.M. from six individual experiments for each group.

through the femoral vein after bile duct cannulation for bile sampling. The bile juice was collected between 30 and 60 min after berberine administration to analyze its metabolites using LC/MS-MS. The sampling time interval was according to our pilot study because the content of the metabolites was sufficient for the measurements. To remove interfering components in bile, the bile sample was diluted and proteins were precipitated with acetonitrile at the volume ratio of 1:2. After vortex mixing and centrifugation at 8000*g* for 10 min, an aliquot of the supernatant was injected into the LC/MS-MS system.

Pharmacokinetics and Statistics. Dialysates of berberine unbound concentrations (C_u) converted from berberine microdialysate concentrations (C_m) were corrected by the estimated in vivo recoveries (R_{dial}) from the respective microdialysis probes ($C_u = C_m/R_{dial}$). The midpoint of the 10-min period was used as the sample time for blood, liver, and bile berberine concentration-time profiles. Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic program, WinNonlin Standard Edition Version 1.1 (Pharsight, Mountain View, CA) by the noncompartmental method (Gabrielsson and Weiner, 1994). The area under the concentration-time curve (AUC) was calculated according to the log linear trapezoidal method. The clearance (CL) and half-life ($t_{1/2}$) were calculated as follows: CL = dose/AUC; $t_{1/2} = 0.693/k$ (k is the elimination rate constant). The liver-to-blood or bile-to-blood distribution was calculated as follows: $AUC_{liver\ or\ bile}/AUC_{blood}$ (Lin et al., 1982). All data were presented as mean ± S.E.M. Comparisons of pharmacokinetic data were performed by one-way analysis of variance or *t* test, and the statistically significant difference was set at $p < 0.05$.

Results

Dose Dependence of Berberine in Blood. The validation method of chromatographic analysis for berberine in a microdialysis system has been reported previously (Tsai and Tsai, 2002). Mean unbound berberine blood concentrations-time profiles in dose dependence (10 and 20 mg kg^{-1} i.v.) are shown in Fig. 2. After a 20 mg kg^{-1} administration, berberine could be detected in extracellular blood for up to 70 min. However, for profiles of the 10 mg kg^{-1} dose, the later concentration values were omitted because they were below the detection limit of the present HPLC system. The pharmacokinetic parameters, as derived from these data and calculated by the WinNonlin program, are shown in Table 1. The AUC and $t_{1/2}$ were significantly increased, but CL was constant when compared with 10 mg kg^{-1}. Since the increase in the AUC was proportional to that in berberine dose through 10 to 20 mg kg^{-1}, it appears in a linear pharmacokinetic manner during these dosage ranges.

Pharmacokinetics of Berberine in Liver and Bile. Mean unbound berberine concentration-time profiles at the doses of 10 and 20 mg kg^{-1} in the rat liver and bile are presented in Figs. 2, 3, and 4, respectively. The disposition of berberine in liver has a slower elimination phase as compared with that in blood, and berberine could be detected in liver for up to 80 min with berberine at 20 mg kg^{-1}. The concentration of berberine in bile reached peak concentration at about 10 to 20 min and could be detected for up to 6 h. All the berberine concentrations in bile were significantly higher than those in blood and liver at each dose. The hepatobiliary excretion of berberine was defined as the liver-to-blood or bile-to-blood distribution ratio ($k = AUC_{liver\ or\ bile}/AUC_{blood}$). The liver-to-blood distribution ratios were the same (0.18 ± 0.03) after berberine administration at doses of 10 and 20 mg kg^{-1}. The bile-to-blood distribution ratios were 6.6 ± 1.6 and 7.4 ± 0.9 after berberine administration at doses of 10 and 20 mg kg^{-1}, respectively. The pharmacokinetic data of berberine in liver and bile are shown in Tables 1 and 2.

CsA Interaction with Berberine. After 20 mg kg^{-1} CsA was coadministered, the berberine concentration in blood was not significantly altered (Fig. 2). The AUCs of berberine alone and with CsA added in blood were 265.4 ± 44.0 and 506.6 ± 39.3 min μg ml^{-1} and 306.4 ± 55.5 and 471.6 ± 55.9 min μg ml^{-1}, respectively, at the berberine doses of 10 and 20 mg kg^{-1}. The CL and $t_{1/2}$ were almost constant when compared with berberine alone at each dose. Since there were no significant differences between these two groups, the results suggest that CsA could not alter the pharmacokinetics of berberine in blood. However, the berberine level in liver and bile decreased dramatically after it was coadministered with CsA (Fig. 3). Above the limit of detection, berberine could be detected in the liver for only 30 min and in bile for only 50 min after CsA was coadministered with berberine at 20 and 10 mg kg^{-1}, respectively. The AUCs of berberine alone and with CsA coadministered in liver became 84.6 ± 6.4 and 30.7 ± 4.0 min μg ml^{-1}, respectively, at the berberine dosage of 20 mg kg^{-1}. The AUCs of berberine alone and with CsA coadministered in bile became 1470.0 ± 134.0 and 3575.0 ± 165.0 min μg ml^{-1}, and 14.9 ± 3.9 and 109.1 ± 27.8 min μg ml^{-1}, respectively, at the berberine dosage of 10 and 20 mg kg^{-1}. These pharmacokinetic data are shown in Table 1. Since the liver-to-blood and bile-to-blood distribution ratios were significantly reduced at each dose after coadministration with CsA, it can be seen that the bile efflux transport system of berberine may be markedly affected by the treatment of CsA, a P-gp inhibitor.

Quinidine Interaction with Berberine. After coadministration of quinidine and berberine at the dose of 10 mg kg^{-1} each, the berberine concentration in blood was not significantly altered (Fig. 4). The AUC of berberine alone and with quinidine added in blood were 265.4 ± 44.0 and 342.6 ± 29.6 min μg ml^{-1}, respectively, which was a slight increase, but there was no statistical difference between these two groups. The CL and $t_{1/2}$ were also slightly changed, but there was also

TABLE 1

Pharmacokinetic data of berberine in rat blood, liver, and bile dialysate after i.v. administration of berberine (10 or 20 mg kg^{-1}) alone and with CsA (20 mg kg^{-1})

Data are expressed as means ± S.E.M. from six individual experiments for each treatment group.

Treatment	AUC	CL	$t_{1/2}$	$AUC_{liver\ or\ bile}/AUC_{blood}$
	min μg ml^{-1}	*ml kg^{-1} min^{-1}*	*min*	
Blood				
Berberine, 10 mg kg^{-1}	265.4 ± 44.0	43.7 ± 7.6	15.5 ± 3.2	
+CsA, 20 mg kg^{-1}	306.4 ± 55.5	37.7 ± 5.8	16.8 ± 5.2	
Berberine, 20 mg kg^{-1}	506.6 ± 39.3[a]	40.9 ± 3.7	33.6 ± 4.3[a]	
+CsA, 20 mg kg^{-1}	471.6 ± 55.9	45.5 ± 5.3	29.0 ± 4.9	
Liver				
Berberine, 20 mg kg^{-1}	84.6 ± 6.4			0.18 ± 0.03
+CsA, 20 mg kg^{-1}	30.7 ± 4.0[b]			0.07 ± 0.002[b]
Bile				
Berberine, 10 mg kg^{-1}	1470 ± 134			6.6 ± 1.6
+CsA, 20 mg kg^{-1}	14.9 ± 3.9[a]			0.05 ± 0.01[a]
Berberine, 20 mg kg^{-1}	3575 ± 165[a]			7.4 ± 0.9
+CsA, 20 mg kg^{-1}	109.1 ± 27.8[b]			0.3 ± 0.1[b]

[a] $p < 0.05$ compared with the 10 mg kg^{-1} berberine-alone group.
[b] $p < 0.05$ compared with the 20 mg kg^{-1} berberine-alone group.

TABLE 2

Pharmacokinetic data of berberine in rat blood, liver, and bile dialysate after i.v. administration of berberine (10 mg kg^{-1}) alone and with quinidine (10 mg kg^{-1}), SKF-525A (10 mg kg^{-1}), or probenecid (100 mg kg^{-1})

Data are expressed as means ± S.E.M. from six individual experiments for each treatment group.

Treatment	AUC	CL	$t_{1/2}$	$AUC_{liver\ or\ bile}/AUC_{blood}$
	min μg ml^{-1}	*ml kg^{-1} min^{-1}*	*min*	
Blood				
Berberine, 10 mg kg^{-1}	265.4 ± 44.0	43.7 ± 7.6	15.5 ± 3.2	
+Quinidine, 10 mg kg^{-1}	342.6 ± 29.6	30.8 ± 3.8	23.2 ± 3.6	
+SKF-525A, 10 mg kg^{-1}	322.6 ± 42.6	32.5 ± 3.9	18.9 ± 3.8	
+Probenecid, 100 mg kg^{-1}	167.8 ± 18.3	56.6 ± 4.9[a]	21.6 ± 3.7	
Liver				
Berberine, 10 mg kg^{-1}	47.4 ± 4.5			0.18 ± 0.03
+SKF-525A, 10 mg kg^{-1}	126.8 ± 14.9[a]			0.56 ± 0.05[a]
Bile				
Berberine, 10 mg kg^{-1}	1470 ± 134			6.6 ± 1.6
+Quinidine, 10 mg kg^{-1}	n.c			n.c.
+SKF-525A, 10 mg kg^{-1}	3526 ± 163[a]			11.6 ± 1.5[a]
+Probenecid, 100 mg kg^{-1}	1376 ± 147			8.8 ± 1.3

n.c., not calculable.
[a] $p < 0.05$ compared with the 10 mg kg^{-1} berberine-alone group.

no statistical difference between these two groups. The pharmacokinetic data in blood are summarized in Table 2. However, the berberine concentration in bile gradually increased and was maintained at a constant level 20 min after drug coadministration (Fig. 5). Especially during the first hour after drug treatment, the berberine level in bile was relatively reduced when quinidine was coadministered. After that, the steady-state berberine concentrations were higher than those of the berberine-alone group. Since no tendency of decay was seen during the berberine elimination in bile, the AUC computed from time 0 to infinity could not be calculated by the noncompartmental approach. However, AUC_{last} computed to the last observation could be acquired and compared with other groups if needed. Consequently, for up to 60 min, the values of AUC_{last} for berberine alone and with quinidine added in bile were 682.2 ± 78.0 and 218.4 ± 13.6 min μg ml^{-1}, respectively. These results reveal that biliary excretion of berberine might be significantly affected by quinidine with the properties of either P-gp or organic cation transport inhibitor during the period of the first hour. As for the later interval, between 1 and 6 h, further illustration is discussed below.

Metabolism of Berberine Identified by LC/MS-MS. Figure 6A shows the chromatogram of the bile blank. Figure 6B shows the chromatogram of a bile sample after berberine administration (10 mg kg^{-1} i.v.), illustrating three major peaks (I–III) at retention times (t_R) of 3.65/3.97, 7.51, and 8.34 min, although peak I overlapped with the endogenous compounds in bile. After identification by the full scan and daughter scan, peak III was consistent with berberine (mol. wt. 336; t_R 8.34 min) and peaks I and II belonged to berberine metabolites with molecular weights of 498 (t_R 3.65/3.97 min) and 322 (t_R 7.51 min), respectively. As for the full scan of the metabolites, Fig. 7A shows the fragment ion *m/z* 322 (336 − 14), which might be derived from a demethylation (*m/z* 14) at the methoxyl group at C_9 or C_{10} of berberine, and was assumed to be $[336 - CH_2]^+$. Figure 7, B and C, shows a similar fragment ion *m/z* 498 at the different t_R values of 3.65 and 3.97 min, which were suspected to be structural isomers. The fragment ion *m/z* 498 (322 + 176) might be caused by a glucuronidation (*m/z* 176) at the hydroxyl group at C_9 or C_{10} of *m/z* 322 and was assumed to be $[322 + C_6H_8O_6]^+$. To further verify that metabolites *m/z* 322 and 498 were originally from berberine *m/z* 336, subsequent LC/MS-MS analyses were performed to fingerprint the daughter ion spectra between them. The daughters of berberine 336 included the peaks of *m/z* 336, 322, and 307 (Fig. 8A). The daughters of metabolite *m/z* 322, 498 separately had the peaks of *m/z* 322, 307 and 498, 322, 307 (Fig. 8, B and C). Hence, based on the above confirmation, berberine could be metabolized

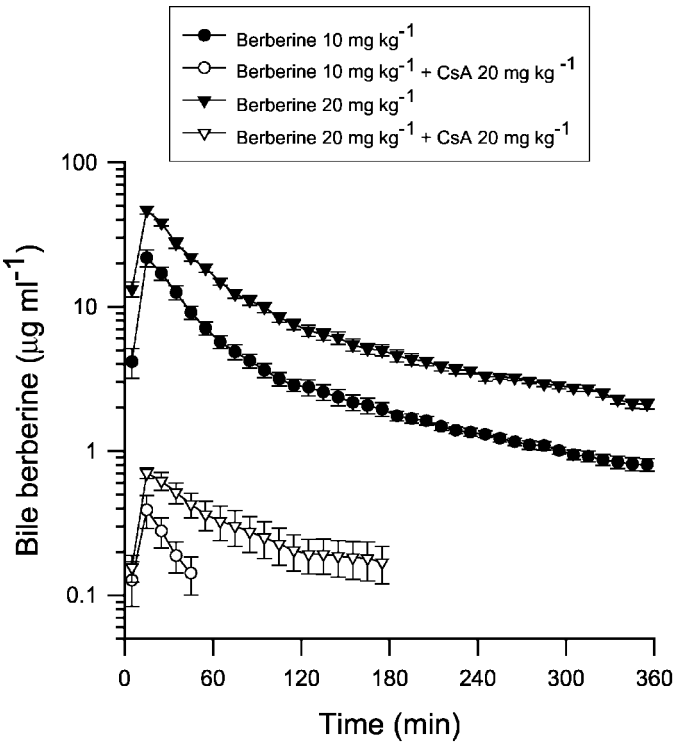

FIG. 3. *Concentration-time profiles for berberine in bile dialysate after berberine i.v. administration at doses of 10 and 20 mg kg^{-1} with and without 20 mg kg^{-1} CsA.*

The data are means ± S.E.M. from six individual experiments for each group.

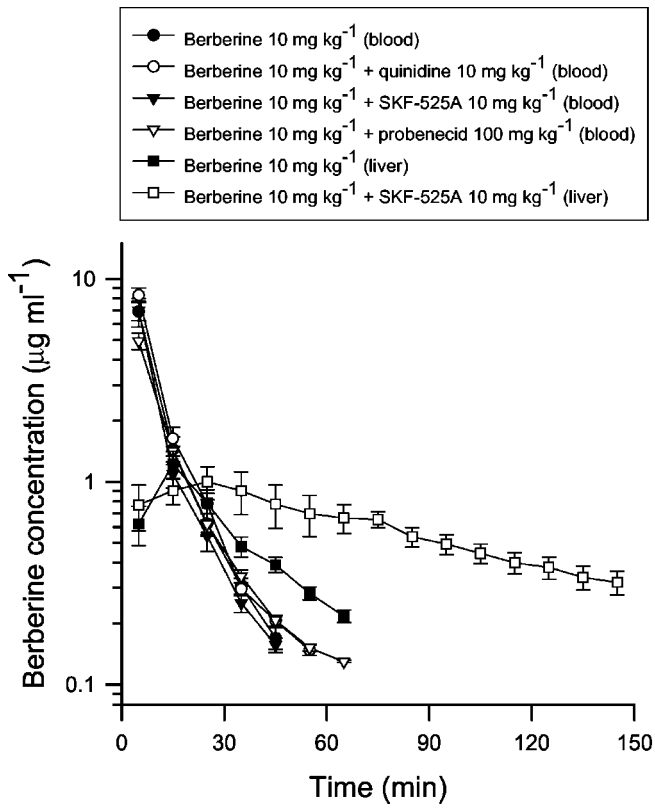

FIG. 4. *Concentration-time profiles for berberine in blood and liver dialysate after administration of 10 mg kg^{-1} berberine, alone, and with 10 mg kg^{-1} quinidine, 10 mg kg^{-1} SKF-525A, or 100 mg kg^{-1} probenecid.*

The data are means ± S.E.M. from six individual experiments for each group.

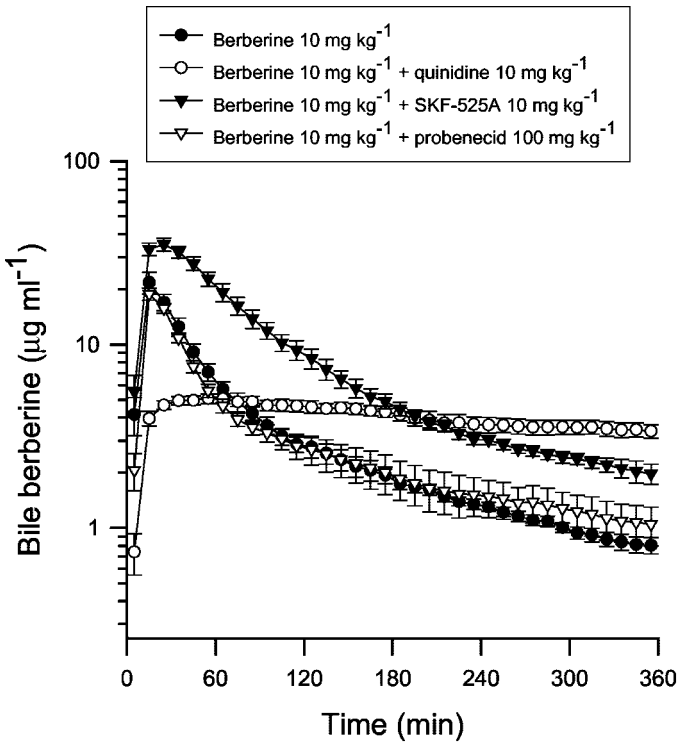

FIG. 5. *Concentration-time profiles for berberine in bile dialysate after administration of 10 mg kg^{-1} berberine, alone, and with 10 mg kg^{-1} quinidine, 10 mg kg^{-1} SKF-525A, or 100 mg kg^{-1} probenecid.*

The data are means ± S.E.M. from six individual experiments for each group.

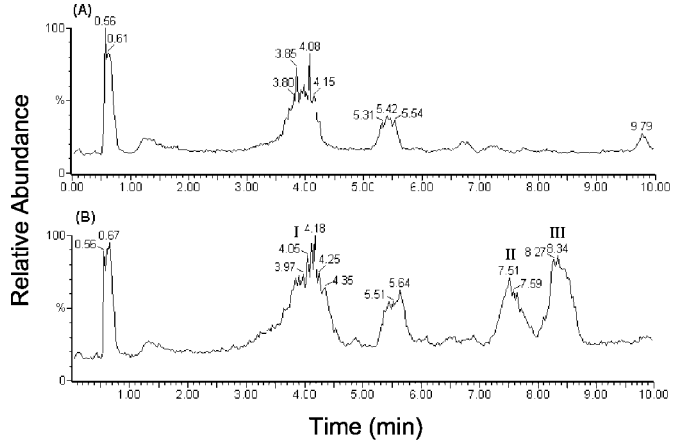

FIG. 6. *LC/MS chromatograms of (A) bile blank (A) and bile sample after berberine administration (B) (10 mg kg^{-1}) illustrating three major peaks, I to III (peak I: metabolite m/z 498, t_R 3.65/3.97 min; peak II: metabolite m/z 322, t_R 7.51 min; peak III: berberine m/z 336, 8.34 min).*

by cytochrome P450 with phase I demethylation and then followed by phase II glucuronidation.

SKF-525A Interaction with Berberine. After coadministration of berberine and SKF-525A at the dose of 10 mg kg^{-1} each, the berberine concentration and pharmacokinetic parameters in blood were also not significantly altered. However, the berberine level in bile was increased and the duration of berberine in the liver was prolonged (Figs. 4 and 5). Comparing the liver-to-blood and bile-to-blood ratios of berberine distribution, both values were increased from 0.18 ± 0.03 and 6.6 ± 1.6 to 0.56 ± 0.05 and 11.6 ± 1.5 when SKF-525A was coadministered (Table 2). The results indicated that metabolism of berberine in the liver was clearly affected by the

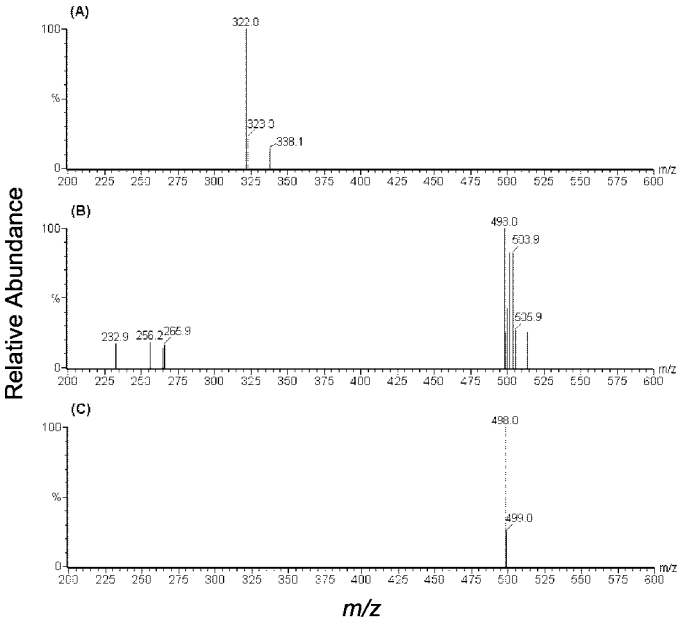

FIG. 7. *Full scan of berberine metabolites m/z 322, t_R 7.51 min (A); m/z 498, t_R 3.65 min (B), and m/z 498, t_R 3.97 min (C).*

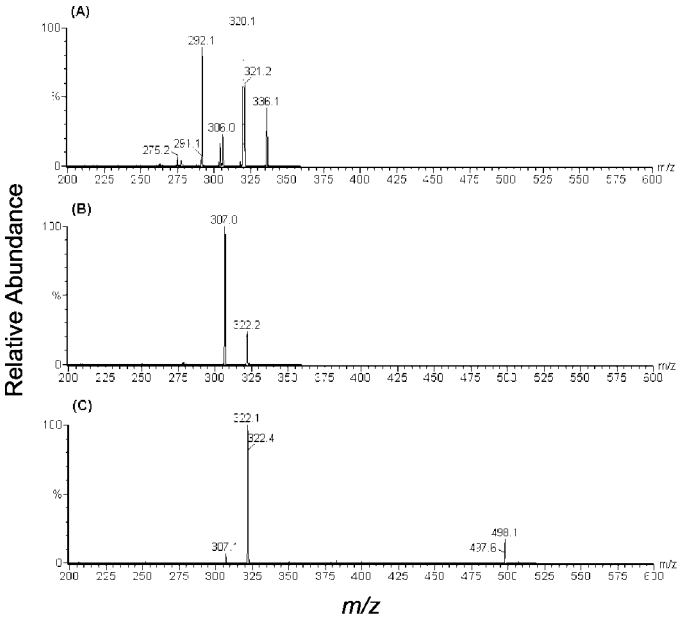

FIG. 8. *Daughter ion scan of authentic berberine (A), metabolite m/z 322 (B), and metabolite m/z 498 (C).*

cytochrome P450 inhibitor, SKF-525A, thus promoting the biliary excretion of berberine.

Probenecid Interaction with Berberine. A slightly decreased but not significantly different berberine concentration in blood was noted after 100 mg kg^{-1} probenecid was coadministered (Fig. 4). Similarly, the berberine level in bile was also not significantly altered between these two groups (Fig. 5). The pharmacokinetic data are shown in Table 2, which shows that the AUC, $t_{1/2}$, and bile-to-blood distribution ratio of berberine were still unchanged after coadministration with probenecid. Hence, probenecid, a glucuronidation inhibitor, seems to have no effect on the berberine disposition in rats since no backflux of berberine into blood was observed, or else more berberine should be rapidly excreted into bile.

Discussion

Although the unbound drug concentration is undoubtedly more closely related to the activity of the drug than the total drug concentration, conventional pharmacokinetic techniques that determine the total concentrations are routinely done since the methods to measure unbound concentrations are tedious, costly, and lack accuracy. Microdialysis sampling, as an alternative method, offers the unique opportunity for in vivo determination of protein-free substances in the extracellular fluid of virtually any tissue, organ, or biological fluid. Several characteristics of microdialysis make it appropriate for pharmacokinetic studies. For example, microdialysis allows the continuous monitoring of the investigated analytes, either endogenous or exogenous compounds at multiple sites in the same animal. In addition, because no fluid is actually removed during sampling, there is minimal disruption of the homoeostasis and physiological processes (Davies et al., 2000).

In recent years, noncompartmental approaches to pharmacokinetic data analysis have been increasingly utilized since they are not required to describe the data by a specific compartmental model. We therefore use a noncompartmental model to calculate the pharmacokinetic parameters for the following reasons. First, according to the minimum Akaike's information criterion as the best representation of the blood concentration-time course data (Yamaoka et al., 1978), fitting a specific one- or multicompartmental model was difficult for all the data in each group. Second, the acquired data in peripheral tissues, like liver or bile, were often not adaptable to a compartmental model, so that it was more convenient to use a noncompartmental approach for the pharmacokinetic evaluation model for comparison between different tissues.

Chen and Chang (1995) found that hepatobiliary excretion of berberine in rabbits was one of the elimination routes following i.v. bolus injection. Our results are in agreement with this report, indicating that berberine is rapidly transferred from blood into liver and bile through the active transportation in rats. Previously, several in vitro studies have shown that berberine is correlated to a certain degree with the multidrug resistance transporter, P-gp (Lin et al., 1999a; Lewis, 1999). In addition, several studies have also indicated that P-gp may play a transportation role in excreting some drugs from the liver into bile (Meijer et al., 1997; Kusuhara et al., 1998). Some P-gp-related transport inhibitors, including verapamil (Watanabe et al., 1992), cyclosporin A (Chu et al., 1999), quinidine (Fromm et al., 1999), and PSC 833 (valspodar) (Song et al., 1999) have been reported to decrease the hepatobiliary excretion of substrates from the liver into bile.

Considering the minimal acute peripheral cardiovascular side effects attributable to CsA, it was used in this study. At a dose of 20 mg kg^{-1} CsA, the hepatobiliary excretion of berberine in rats was sufficient to be inhibited, indicating that P-gp may act as a high-affinity component in the berberine excretion into bile. Although the AUC for unbound berberine in blood was not altered in the CsA-treated group, this might be because of the large blood volume in the body or because the process of redistribution compensated the berberine regulation of hepatobiliary excretion. In the study by Chen and Chang (1995), only 4.93% and 0.5% of the dose was unchangeably eliminated from the urine and bile after i.v. bolus administration of 2 mg kg^{-1} berberine. Similarly, a very small amount of unchanged berberine was secreted into urine, so that berberine thereby seems to be extensively metabolized in the body, which could probably explain the absence of a significant change in unbound systemic berberine concentration, whereas CsA gave the immediate inhibitory effect at the biliary excretion of berberine. However, the liver-to-blood distri-

bution ratio of berberine was decreased rather than increased after apparent inhibition of CsA on the hepatic P-gp-mediated berberine efflux activity. Generally, the drugs with a high hepatic extraction rate are mainly sensitive to change in hepatic blood flow, which may cause the extensive vascularization of liver. For microdialysis experiments performed in liver, capillary exchange and the rate of hepatic blood flow appear to be the dominant processes that affect net transport from a microdialysis probe (Stenken et al., 1997). Sandberg et al. (1994) found that the hepatic blood flow was not influenced by CsA (9.5 mg kg^{-1} i.v.) in an anesthetized Sprague-Dawley rat model. Whether CsA changes hepatic blood flow, accompanied by a decreased recovery, or reduces the uptake of berberine from the liver needs more evidence.

The well known digoxin-quinidine interaction has described a range of changes in digoxin disposition when quinidine is administered concomitantly, including enhanced digoxin absorption, and reduced renal secretion and biliary excretion. However, it is now recognized that inhibition of P-gp-mediated digoxin disposition at these sites may be a common mechanism leading to elevated digoxin concentrations (Fromm et al., 1999). Quinidine has been shown to be an inhibitor of P-gp and organic cation transporter. Smit et al. (1998) proposed that MDR substrates/reversal agents, such as quinidine, strongly reduced both small (type I) and bulky (type II) organic cation excretion into bile. In addition, quinidine has been reported to inhibit the proton gradient-stimulated tetraethylammonium uptake and the tetraethylammonium counter-transport, which are two potential mechanisms in the hepatocyte basolateral membrane for transport of organic cations (McKinney and Hosford, 1992). All the above might be able to explain the decreased biliary excretion of berberine in the initial stages and the increased, but not significantly so, plasma berberine concentration when quinidine was coadministered in this study. However, our results indicate that berberine concentration was maintained as almost constant 20 min after the drug was administered and lasted for a long time, almost 6 h, which might be attributed to the following reasons. It has been reported that quinidine inhibited the renal excretion of digoxin, not by competition between the two on a mutual transport, but rather by the pharmacological effect of quinidine in decreasing renal blood flow; in addition, a parallel decrease in biliary clearance of digoxin has been documented, which might suggest a similar mechanism (Koren et al., 1988). The results indicate that biliary excretion of berberine was significantly affected by quinidine. The long-lasting effect of quinidine prolongs the biliary excretion rate of berberine, which may cause the saturation of the capacity-limited metabolism.

Matsubara et al. (1982) demonstrated that despite uniform protein concentration throughout rat liver, P450 monooxygenase activity was higher in homogenates prepared from the median and the right lobe relative to homogenate from the left lobe. Among these three lobes of the liver, the median lobe is the largest, with the thickest part in the center and positioned above the other two. Thus, we chose the median lobe as the sampling site for microdialysis in the liver. Sampling of the extracellular fluids surrounding hepatocytes in the median lobe of the liver was performed by microdialysis probe insertion through the hole made larger by the needle than the probe outer diameter. A microdialysis probe implanted in liver tissue will reflect the extracellular fluid composition over a wider area since the arrangement and size of the structural/functional units of the liver are not uniform and the probe is too large to sample only periportal or only perivenous regions (Davies and Lunte, 1996).

According to the chromatograms of berberine in liver and bile (Tsai and Tsai, 2002), the peak areas of berberine and metabolites (suspected temporarily) in the liver were much smaller than those in bile. Also, the fact that the AUC of berberine in the liver was less than that in blood, based on the liver-to-blood distribution ratio, was a response to the downhill substrate concentration gradient from blood to liver during the hepatic uptake, which was maintained by intracellular metabolism and export from the hepatocyte by active pumps (Faber et al., 2003). Thus, rapid excretion of berberine or metabolites from the liver into bile is supposed.

Characterization and structural elucidation of the berberine metabolites were achieved by LC/MS-MS. It has been verified that demethylation followed by sulfate conjugation at position 2, 3, or 10 was a major metabolite route of berberine in male humans (Pan et al., 2002). The berberine derivative, 7-(4-chlorbenzyl)-7,8,13,13*a*- tetrahydroberberine, was also metabolized in rats by demethylation at position 10 to produce a new entity (Feng et al., 1998). Here, the finding that berberine underwent demethylation followed by glucuronidation was first proposed with confirmation by the tandem mass spectrometry. This indicates that berberine was metabolized in rats by P450, which was sequentially verified by treatment with a nonspecific P450 inhibitor SKF-525A. Until now, almost no study has reported which P450 isoform is more likely to be involved in berberine metabolism, and SKF-525A was chosen to inhibit berberine metabolism without P450 form selectivity, because it can block metabolic activities of all P450 forms tested to various degrees, depending on the P450 form (Ono et al., 1996). Pretreatment with SKF-525A has been reported to cause higher plasma concentration, greater plasma AUC and slower total body CL for some drugs (Bu et al., 2000; Hurh et al., 2000), indicating the drugs were metabolized by P450 isozymes. In this study, the berberine concentration and AUC in plasma were not statistically different from those in the control rats, which could be due to rapid redistribution of berberine into bile, since the resultant AUC and CL of bile were significantly greater and slower, respectively.

Probenecid has been reported to impair biliary excretion of some glucuronide conjugates, possibly due to the inhibition of glucuronidation, such as acetaminophen (Savina and Brouwer, 1992), zidovudine (Mays et al., 1991), and indomethacin (Baber et al., 1978). Unfortunately, no pharmacokinetic data of berberine were altered in blood and bile by treatment with probenecid; thus, it is difficult to determine whether the formation of the new isomeric metabolites following glucuronide conjugation was obstructed. Berberine may still be able to go through other metabolic pathways beyond glucuronidation, or the rapid bolus dosing of probenecid cannot be targeted to a concentration that has been shown to inhibit glucuronidation (Savina and Brouwer, 1992). Hence, further investigation on the eliminated proportion for berberine metabolites should be carried out. Moreover, probenecid is also a well known inhibitor for organic anion transporters such as MRP2 (multidrug resistance-associated protein 2) (Horikawa et al., 2002; Zamek-Gliszczynski et al., 2003). In general, transport across the bile canalicular membrane of neutral and cationic compounds is mediated by P-gp, whereas that of anionic compounds is mediated by MRP2 and its superfamily (Kusuhara et al., 1998). However, berberine is indeed a quaternary base with a positive charge and is almost not a MRP2 substrate. Therefore, the possible effect of probenecid on MRP2 is excluded here. Whether berberine glucuronide metabolites are also secreted into bile by MRP2 needs further confirmation.

In conclusion, the studies reveal that CsA, quinidine, and SKF-525A all interact with berberine and, additionally, that P-gp and P450 may play important roles in the regulation of hepatobiliary excretion and liver metabolism for berberine in rats. Future studies should focus on the pharmacokinetics of berberine by oral administration since the P-gp is expressed in intestinal cells and the significant first-pass extraction by P450-dependent processes may severely limit its oral bioavailability.

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