Hepatobiliary Excretion of Silibinin in Normal and Liver Cirrhotic Rats

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Received June 1, 2007; accepted November 27, 2007

ABSTRACT:

Silibinin is the major active flavonolignan extracted from the seeds and fruits of milk thistle and has potential efficacy in the treatment of liver disease. The aim of the present study was to examine the hepatobiliary excretion of silibinin and its effect on dimethylnitrosamine (DMN)-induced liver cirrhosis. The experiments were divided into five groups: 10, 30, and 50 mg/kg silibinin alone, 30 mg/kg silibinin coadministered with cyclosporin A (CsA), and 50 mg/kg silibinin with liver cirrhosis induced by DMN. The data indicated that silibinin had dose-related pharmacokinetics in the dose ranges of 10 to 50 mg/kg. All of the unconjugated or total (unconjugated + conjugated) silibinin concentrations in the bile were significantly higher than those in plasma at the sampling time points at each dose, suggesting active hepatobiliary excretion. When coadministered with CsA, the area under the concentration versus time curve (AUC) in bile was significantly decreased. This result suggested that the active silibinin efflux might be partially inhibited by P-glycoprotein. In the DMN-induced liver cirrhotic rats, the AUC of plasma unconjugated silibinin was reduced by 53%; however, total silibinin was increased by 182%. These results together suggest that the phase II conjugative reaction of silibinin was blocked by treatment with DMN.

Silymarin is the biological extract of milk thistle (Silybum marianum L.), and silibinin is the major active constituent of silymarin, which is widely used in traditional European medicine (Morazzoni and Bombardelli, 1996). Silymarin has been evaluated clinically in the treatment of hepatitis and liver damage caused by alcohol (Wellington and Jarvis, 2001) or by antituberculosis drugs (Tasduq et al., 2005). Silibinin (Fig. 1) is a very strong antioxidant compound capable of scavenging both free radicals and reactive oxygen species. A recent report in rodents suggested that silibinin may be useful in the chemoprevention of malignancies in the skin, bladder, liver, cervix, and colon (Gazék et al., 2007).

P-gp acts as a biological barrier by extruding xenobiotics out of cells. The modulator of P-gp has been reported to be the cause of drug-drug interactions when potent P-gp enhancers/inhibitors and P-gp substrates are administered together (Lin, 2003). 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). Silymarin inhibits P-gp-mediated efflux in Caco-2 cells; this effect was concentration-dependent, and increased daunomycin accumulation in P-gp-positive cells but not in P-gp-negative cells has been reported (Gazék et al., 2007).

Silibinin is eliminated predominantly by glucuronic acid conjugation with extensive biliary excretion in the conjugated form and its primary therapeutic application is treatment of liver disorders (Wellington and Jarvis, 2001). Chronic liver disease, in particular liver cirrhosis, can modulate many factors, determining the behavior of drugs in the body (Delcò et al., 2005). Although the pharmacokinetics of silibinin in patients treated with CsA and in patients with liver cirrhosis and healthy volunteers has already been investigated (Barzaghi et al., 1990; Orlando et al., 1990), no information on the hepatobiliary excretion of silibinin in the presence of cirrhosis is available.

DMN is a potent hepatotoxic, carcinogenic, and mutagen. DMN-induced liver injury in rats seems to be a good animal model for early liver cirrhosis (George et al., 2001). A model of cirrhosis induced by chronic, discontinuous treatment with a low dose of DMN in the rat has been reported to reproduce a number of characteristics of this liver disease (Jézéquel et al., 1987).

It is important to clarify the possible mechanisms of biliary excre-

ABBREVIATIONS: P-gp, P-glycoprotein; CsA, cyclosporin A; DMN, dimethylnitrosamine; HPLC, high-performance liquid chromatography; AUC, area under the concentration versus time curve.
tion for the pharmacokinetic study of silibinin. Until now, the mechanism responsible for the biliary excretion of silibinin has not been fully characterized, and no information is available on the hepatobiliary excretion of silibinin in the presence of cirrhosis. Hence, our hypothesis is that silibinin is actively transported across the sinusoidal membrane into hepatocytes and secreted into biliary canaliculi via P-gp modulation, and biliary excretion appears to be affected by the status of liver cirrhosis. In this study we characterize the pharmacokinetics of silibinin and its interaction with CsA in rats with cirrhosis. In addition, the pharmacokinetic mechanism of hepatobiliary excretion of silibinin was investigated. Our results demonstrate that silibinin was excreted into the bile via P-gp for hepatobiliary excretion.

**Materials and Methods**

**Chemicals and Reagents.** Silibinin (molecular mass 482.44 Da), naringenin (internal standard), dimethylnitrosamine, and β-glucuronidase (type H-1 from *Helix pomatia*, 338,000 units/g) were purchased from Sigma-Aldrich (St. Louis, MO). Liquid chromatographic grade solvents and reagents were obtained from E. Merck (Darmstadt, Germany). Triply deionized water from Millipore Corporation (Bedford, MA) was used for all preparations. Sodium acetate buffer solution (pH 5) was prepared using sodium acetate (9.45 g) and glacial acetic acid (1.725 ml) in 500 ml of deionized water according to a previous report (Taylor et al., 2005). The β-glucuronidase enzyme solution (3000 units/ml) was freshly prepared using sodium acetate buffer (pH 5).

**Experimental Animals.** All experimental protocols involving animals were reviewed and approved by the institutional animal experimentation committee of National Yang-Ming University. Male specific-pathogen Sprague-Dawley from *Helix pomatia*, 338,000 units/g) were purchased from Sigma-Aldrich (St. Louis, MO). Liquid chromatographic grade solvents and reagents were obtained from E. Merck (Darmstadt, Germany). Triply deionized water from Millipore Corporation (Bedford, MA) was used for all preparations. Sodium acetate buffer solution (pH 5) was prepared using sodium acetate (9.45 g) and glacial acetic acid (1.725 ml) in 500 ml of deionized water according to a previous report (Taylor et al., 2005). The β-glucuronidase enzyme solution (3000 units/ml) was freshly prepared using sodium acetate buffer (pH 5).

**Experimental Animals.** All experimental protocols involving animals were reviewed and approved by the institutional animal experimentation committee of National Yang-Ming University. Male specific-pathogen Sprague-Dawley rats weighing 280 to 320 g were from the Laboratory Animal Center of the National Yang-Ming University. The animals had access to food (Laboratory Rodent Diet 5001; PMI Feeds Inc., Richmond, IN) and water until 18 h before being used in experiments, and after that only food was removed.

The rats were initially anesthetized with pentobarbital (50 mg/kg i.p.), and remained anesthetized throughout the experimental period. During the experiments, the body temperature of each rat was maintained at 37°C with a heating pad. The right atrium (through a catheter (cannula) implanted into the right external jugular vein) was used for blood samples, and a catheter (PE-10) was inserted into the proximal portion of the bile duct toward the liver for bile juice collection (Wu and Tsai, 2007). Phenobarbital stimulates varied pathways of metabolism by liver microsomes. With daily administration of phenobarbital to rats, the maximal increase of enzyme activity (3- to 10-fold) is not reached for at least 3 days (Conney, 1967). In this study, animals were anesthetized by pentobarbital, and samples were obtained within 5 h, which may not affect the metabolism of silibinin.

**Induction of Liver Cirrhosis by DMN.** DMN (dissolved in 0.9% NaCl-injectable solution to produce 0.01 mg/ml) was injected i.p. on 3 consecutive days per week for 4 weeks (Jézéquel et al., 1987). For control rats, the same volume of 0.9% NaCl-injectable solution (pH 5) was injected. During the pretreatment, rats had free access to food and water. Five days after the last DMN injection (or 9 days after NaCl-injectable solution), the experiment was performed. Liver cirrhosis induced by DMN was observed on each individual set of data using the pharmacokinetic software WinNonlin Standard Edition (version 1.1; Pharsight, Mountain View, CA) and the noncompartmental method. The AUC was calculated according to the linear trapezoidal method.

**Statistics.** The results are presented as means ± S.E.M. Statistical analysis was performed with SPSS (version 10.0; SPSS Inc. Chicago, IL). Comparisons of pharmacokinetic data between un conjugated silibinin and total silibinin at doses of 10 and 30 mg/kg alone and 30 mg/kg coadministered with CsA and 50 mg/kg alone and 50 mg/kg in the rat group with cirrhosis were performed by Student’s t test, and the significant difference was set at p < 0.05.
Results

Chromatography in Plasma and Bile. Typical chromatograms of silibinin in rat plasma and bile are shown in Figs. 2 and 3, respectively. Separation of silibinin from endogenous substances in the plasma and bile was achieved in an optimal mobile phase containing acetonitrile-10 mM monosodium phosphate (pH 5.45 adjusted with orthophosphoric acid) (50:50, v/v), at a flow rate of 1 ml/min.

Figure 2A shows the chromatogram of a blank plasma sample before drug administration, and Fig. 2B shows a calibration sample of silibinin (10 μg/ml). None of the observed peaks interfered with the analyte within the retention times of the analytes. Figure 2C shows the chromatogram of a plasma sample containing silibinin (7.91 μg/ml) collected from rat plasma at 15 min after silibinin administration (30 mg/kg i.v.).

Figure 3A shows the chromatogram of a blank bile sample obtained from the bile duct before the drug administration. Figure 3B shows a calibration sample of standard silibinin (50 μg/ml). Figure 3C shows the chromatogram of bile sample containing silibinin (97.25 μg/ml) collected from the rat bile 10 min after silibinin administration (30 mg/kg i.v.). The unretained matrix components on the chromatogram in Fig. 3C slightly affect the first bile sample, which may be the cause of the unidentified endogenous silibinin or metabolites of silibinin after drug administration.
Because the unretained matrix was gradually reduced in the later samples, the potential error of the chromatogram shoulder for the biliary excretion of silibinin may be <1%.

Method Validation. The calibration curve of silibinin was obtained before HPLC analysis of plasma and bile over a concentration range of 0.5 to 100 μg/ml. The limit of detection and the limit of quantitation of silibinin in rat plasma and bile were determined to be 0.1 μg/ml at a signal-to-noise ratio of 3 and 0.5 μg/ml, respectively. The limit of quantitation was defined as the lowest concentration of silibinin in plasma and bile samples that could be quantified with acceptable criteria (interassay coefficient of variation and accuracy within ±15%). Our analytical data were within the criteria of 15%.

Pharmacokinetics of Silibinin in Plasma and Bile. Figures 4 and 5 show unconjugated silibinin and total silibinin in plasma and bile for the 10 and 30 mg/kg silibinin groups, respectively. The pharmacokinetic data for unconjugated silibinin and total silibinin are presented in Tables 1 through 3. After silibinin administration at 10, 30, and 50 mg/kg, the plasma unconjugated silibinin level declined rapidly in the rat plasma up to 60 to 180 min. The plasma AUCs of unconjugated and total silibinin did not increase proportionally for the doses of 10, 30, and 50 mg/kg. Silibinin seems to show dose-related pharmacokinetics in this dose range, which may be due to the saturation of the metabolic or excretion pathway.

The pharmacokinetic data for the silibinin-alone group reflect the fact that disposition of unconjugated silibinin in rat bile exhibited a
peak concentration at 5 min after silibinin administration. The hepatobiliary excretions of unconjugated silibinin, which were defined as blood to bile excretion (k value) calculated by the AUC ratio (k = AUC_{bile}/AUC_{blood}), were 1.3, 2.3, and 4.9 at doses of 10, 30, and 50 mg/kg, respectively (Tables 1–3) (de Lange et al., 1997).

After silibinin administration at doses of 10 or 30 mg/kg, the total plasma silibinin level decreased slowly and could be detected in rat plasma for up to 300 min (Fig. 5). The pharmacokinetic data for the silibinin-alone group reflect the fact that disposition of total silibinin in rat bile exhibited a peak concentration at 10 min after silibinin administration. The hepatobiliary excretions of silibinin in total and unconjugated forms were 41 ± 7.3 and 1.3 ± 0.3, 31 ± 5.4 and 2.3 ± 0.6, and 45 ± 4.4 and 4.9 ± 1.4, respectively, for doses of 10, 30, and 50 mg/kg (Tables 1–3). These data indicate that most of silibinin went through hepatobiliary excretion and enterohepatic recirculation in the conjugated form.

Interaction of Silibinin and CsA in Plasma and Bile. Figures 6 and 7 show unconjugated silibinin and total silibinin in plasma and bile for 30 mg/kg silibinin alone and 30 mg/kg silibinin coadministered with CsA. Each group of data is presented as the mean ± S.E.M. from six individual experiments.

A comparison with the 30 mg/kg silibinin alone group shows that
after CsA exposure before i.v. administration of 30 mg/kg silibinin, the unconjugated silibinin concentration and AUC in the plasma were markedly decreased. The AUC of total silibinin in the plasma of the 30 mg/kg silibinin coadministered with CsA group was significantly increased compared with the 30 mg/kg silibinin alone group. CsA was administered parenterally before silibinin to allow it to diffuse into the various biological fluid compartments before silibinin administration. Biliary excretion of total silibinin, expressed as AUC_bile/AUC_blood, was significantly decreased. The AUC of total silibinin in the plasma of the unconjugated silibinin concentration and AUC in the plasma were significantly reduced (46% decreased). The elimination half-life of unconjugated silibinin in bile was significantly increased, but the bile AUC and C_max were not significantly different from those for the group not treated with DMN. The total silibinin plasma AUC was significantly enhanced (83%), and the elimination half-life was significantly increased (104%) in the DMN-treated group.

The elimination half-life for total silibinin in bile was significantly increased, and the bile C_max was significantly decreased when silibinin was given to the liver cirrhosis group. The blood to bile excretion of total silibinin in the 50 mg/kg silibinin cirrhosis group was significantly decreased (approximately 52%) from that for the group not treated with DMN (Table 3).

Discussion

The majority of the silibinin present in plasma was found to be in either the glucuronide or sulfate conjugate form (Weyhenmeyer et al., 1992). A recent study indicates that silibinin undergoes multiple conjugative reactions, and silibinin monoglucuronide, silibinin diglucuronide, silibinin monosulfate, and silibinin diglucuronide sulfone were identified in human plasma (Hoh et al., 2006). Our data demonstrate that the conjugated form of silibinin accounts for 92 to 95% of total silibinin at the dose ranges of 10, 30, and 50 mg/kg. The pharmacological implication of conjugated silibinin is unclear. Although conjugated metabolites are usually devoid of pharmacological activity, morphine 6-glucuronide is an exception to this rule (Portenoy et al., 1992).

All of the unconjugated or total silibinin concentrations in bile were significantly higher than those at the sample time point in plasma at each dose. For doses of 10, 30, and 50 mg/kg, the total silibinin C_max values in bile were approximately 100 times higher than those found in plasma; this observation agrees well with a previous report (Frauschini et al., 2002). The high total hepatobiliary excretion (AUC_bile/AUC_blood) could be partially (68.7%) reduced from 31 ± 5.4 to 9.7 ± 1.3 after treatment with CsA (Table 2) suggesting that an active transport mechanism of hepatobiliary excretion might be regulated by P-gp.

After CsA exposure before silibinin administration, unconjugated plasma C_max and AUC were decreased, but the total silibinin levels were increased. The pharmacokinetic interaction between silibinin and CsA may be caused by the competition for protein binding. Of the 90 to 99.8% of circulation CsA bound to plasma protein, 85 to 90% is carried on lipoprotein (Dunn et al., 2001). The endogenous lipoprotein may play a role in the transport of silibinin from the liver to the extrahepatic tissues (Svagera et al., 2003). Displacement of protein binding may be significant when CsA and silibinin are coadmini-
tered, resulting in higher free silibinin concentrations, and free silibinin can be cleared faster or distributed to various tissues.

CsA plays the role of an inhibitor of P-gp transporter and has been reported to decrease the hepatobiliary excretion of substrates from the liver into bile (Chu et al., 1999). Generally, the hydrophilic glucuronide metabolites are beneficial to diffuse hepatocyte passively into either bile or sinusoidal blood after intrahepatic formation. Carrier- or transporter-mediated processes are associated to transport conjugated metabolites across either the canalicular or basolateral membrane (Zamek-Gliszczynski et al., 2006). Multidrug resistance protein 2 is responsible for the biliary excretion of a variety of endogenous and exogenous organic anions, including many glucuronide conjugates, some glutathione conjugates, and a few sulfate conjugates (Oude Elferink et al., 1995). In this study, a dose of CsA (20 mg/kg) for the hepatobiliary excretion of total silibinin in rats was sufficient to inhibit the pharmacokinetic interaction at the 30 mg/kg dose of silibinin. This result indicates that P-gp might act as a certain affinity component in the total silibinin excretion into bile.

CsA and its metabolites are mainly eliminated via bile (Christians et al., 1991), and an apparent rise with CsA in /H9252-glucuronidase activity was seen in the renal tissue in rats (Mohamadin et al., 2005) and human serum (Falkenbach et al., 1993). The Cmax of unconjugated silibinin in bile was decreased from 242 ± 55 to 37 ± 9.1 μg/ml, which reduced the bile elimination of unconjugated silibinin. β-Glucuronidase is present in all mammalian tissues and is especially abundant in the liver to process enzymatic deconjugation (Fishman et al., 1991).

Fig. 8. Concentration-time profiles for unconjugated silibinin in plasma (A) and bile (B) after silibinin administration (50 mg/kg i.v.) to the control group and the group DMN-induced cirrhosis. Each group of data is presented as the mean ± S.E.M. from six individual experiments.

Fig. 9. Concentration-time profiles for total silibinin in plasma (A) and bile (B) after silibinin administration (50 mg/kg i.v.) to the control group and the group with DMN-induced cirrhosis. Each group of data is presented as the mean ± S.E.M. from six individual experiments.

The unconjugated silibinin AUC_{bile}/AUC_{blood} ratio for the liver cirrhosis group was increased 73% compared with that for the control group (Table 3). Many drugs induce a significant release of more unconjugated fraction with chronic liver disease; such drugs may be metabolized more rapidly in patients with cirrhosis (Delcó et al., 2005). However, Pacifici et al. (1990) demonstrated that liver disease reduces the activities of sulfotransferase, acetyltransferase, glutathione transferase, and thiomethyltransferase. The plasma AUC of unconjugated silibinin was significantly reduced in the presence of liver cirrhosis, which might be due to a decrease in the enzyme activity. An extrahaepatic contribution to drug metabolism is often disregarded. Kidney, jejunum, and adrenal gland are most enriched in uridine diphosphate-glucuronosyltransferase activity. The kidney may contribute to glucuronidation of bilirubin in bile duct-ligated rats (Chowdhury et al., 1985).

The total silibinin AUC_{bile}/AUC_{blood} ratio was reduced 52% from 45 ± 4.4 for the control group to 21.6 ± 6.7 for the group with liver cirrhosis (Table 3). The extrahepatic biliary obstruction may be associated with reduced clearance of conjugated silibinin, probably because of impaired excretion of the conjugate in bile (Schandalk and Perucca, 1994). In chronic cholestatic liver diseases, the biliary excretion of cholephilic organic anions is impaired. Multidrug resistance protein 2 expression decreases with liver cirrhosis (Table 3). Many drugs induce a significant release of more conjugates. Multidrug resistance protein 2 expression decreases with liver cirrhosis (Table 3). Many drugs induce a significant release of more conjugates.

In conclusion, we have demonstrated the effect of CsA and liver cirrhosis on the pharmacokinetics of silibinin in rats. The high biliary levels of silibinin indicate that it may undergo active hepatobiliary excretion, and the bile elimination of silibinin was partially blocked by coadministration with CsA. The total plasma silibinin AUC for the group of cirrhotic rats was significantly greater than that for the control group. These results suggest that phase II conjugation and hepatobiliary excretion play a crucial role in the elimination of silibinin.

References


