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Short communication

# Analysis of silibinin in rat plasma and bile for hepatobiliary excretion and oral bioavailability application

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**Abstract**

Silibinin is an herbal ingredient isolated from milk thistle. The aim of this study was to develop a simple liquid chromatographic system to assay silibinin in plasma and bile for pharmacokinetic study. Silibinin was given oral and intravenously. The plasma sample (25 μL) was vortex-mixed with 50 μL of internal standard solution (naringenin 10 μg/mL in acetonitrile) to achieve protein precipitation. Silibinin in the rat plasma and bile was separated using a reversed-phase C18 column (250 mm × 4.6 mm, 5 μm) with a mobile phase of acetonitrile −10 mM monosodium phosphate (pH 5.45 adjusted with orthophosphoric acid) (50:50, v/v) and the flow-rate of 1 mL/min. The UV detection wavelength was 288 nm. The concentration–response relationship from the present method indicated linearity over a concentration range of 0.5–100 μg/mL. Intra- and inter-assay precision and accuracy of silibinin fell well within the predefined limits of acceptability (<15%). An ultrafiltration method was used in this experiment and the protein binding of silibinin was 70.3 ± 4.6%. After silibinin administration in rats, the disposition of silibinin in the plasma and bile fluid was due to rapid distribution and equilibration between the blood and hepatobiliary system, and the bile levels of unconjugated silibinin and total silibinin were greater than those in the plasma. The oral bioavailability of silibinin in rats was estimated to be 0.73%.
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*Keywords:* Bioavailability; Herbal medicine; Milk thistle; *Silybum marianum*; Silymarin

## 1. Introduction

Milk thistle (*Silybum marianum* L.) is a medicinal plant widely used in traditional European medicine [1]. Silymarin, a polyphenolic flavonoid isolated from milk thistle, primarily consists of four isomeric mixture of active flavonolignans: silychristin, silydianin, and two groups of diastereoisomeric flavonolignans, silibinin, and isosilibinin [2]. Silibinin (Fig. 1), a flavanone, is the major and most active component present in silymarin, represents about 60–70% [3].

Silymarin has clinical applications in the treatment of toxic hepatitis, fatty liver, cirrhosis, ischaemic injury, radiation toxicity and viral hepatitis as a result of its antioxidative, anti-lipid-peroxidative, antifibrotic, anti-inflammatory, immunomodulating and even liver regenerating effects [4]. It is a strong antioxidant capable of scavenging unconjugated radicals [5–7].

Several chromatographic methods have been reported for the separation or quantitative measurement of silibinin. Published methods include those based on thin-layer chromatography (TLC) [8], high-performance liquid chromatography (HPLC) separation with ultraviolet (UV) [9–15], column-switching with electrochemical [9], mass spectrometry (MS) [14] and capillary electrophoresis (CE) [15].

Despite a wide range of studies with both silymarin and silibinin and their clinical usage, there is no information available

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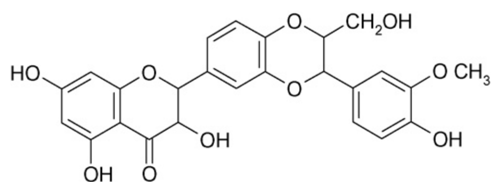


Fig. 1. Chemical structure of silibinin.

on the pharmacokinetics of silibinin administered orally and intravenously to investigate its hepatobiliary excretion and absolute bioavailability. The polyphenolic silibinin potentially go through phase II conjugated reaction with glucuronidation. The conjugated metabolites are more water-soluble, and they are possible in due course excreted in the urine and bile.

A number of experimental model systems have been used to examine the hepatobiliary functions, including isolated membrane vesicles, cell lines, primary hepatocytes, and liver slices [16]. The most direct method for examining biliary excretory function, however, is to collect bile via the common bile duct in anesthetized animals. In the present study, we develop a validated liquid chromatographic method to measure unconjugated silibinin and total silibinin in rat plasma and bile. Besides, protein binding, disposition mechanism and oral bioavailability were investigated.

## 2. Experimental

### 2.1. Chemical and reagent

Silibinin (molecular weight 482.44), naringenin (internal standard) and β-glucuronidase (Type H-1 from Helix pomatia, 338,000 unit/g) were purchased from Sigma Chemicals (St. Louis, MO, USA). The solvents and reagents for chromatography were obtained from BDH (Poole, UK). Triply de-ionized water from Millipore (Bedford, MA, USA) 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 de-ionized water according Taylor's report [17]. The β-glucuronidase enzyme solution 3000 unit/mL was freshly prepared using sodium acetate buffer (pH 5).

### 2.2. Liquid chromatography

Liquid chromatographic grade solvents and reagents were obtained from E. Merck (Darmstadt, Germany). The HPLC system consisted of a chromatographic pump (BAS PM-80, West Lafayette, IN, USA), equipped with a 20 μL sample loop, and a UV detector (Soma S-3702, Tokyo, Japan). Separation was achieved by a LiChrosorb RP-18 column (Merck, 250 × 4 mm i.d.; particle size 5 μm) protected by guard column (Purospher STAR RP-18e, 5 μm) at room temperature (24 ± 1 °C). The mobile phases consisted of acetonitrile −10 mM monosodium phosphate (pH 5.45 adjusted with orthophosphoric acid) (50:50, v/v). The flow-rate used was 1 mL/min. The detection wavelength was set at 288 nm. Output data from the detector were integrated using an EZChrom chromatographic data system (Scientific Software, San Roman, CA, USA).

### 2.3. Method validation

Calibration curves were established using blank plasma spiked with different amounts of silibinin. The peak area ratios of silibinin to the internal standard versus analyte concentrations were used to calculate linear regression. The concentration–response relationship from the present method indicated linearity over a concentration range of 0.5–100 μg/mL with the coefficient of estimation ($r^2$) of 0.995. The intra-assay and inter-assay variabilities were determined by quantitating six replicates at concentrations of 0.5, 1, 5, 10, 50 and 100 μg/mL using the HPLC method described above on the same day and six consecutive days, respectively. The lowest concentration of the linear regression defined the limit of quantitation (LOQ). The accuracy (% bias) was calculated from the mean value of observed concentration ($C_{obs}$) and the nominal concentration ($C_{nom}$) as follows: accuracy (% bias) = $[(C_{obs} - C_{nom})/C_{nom}] \times 100$. The relative standard deviation (R.S.D.) was calculated from the observed concentrations as follows: precision (% R.S.D.) = [standard deviation (S.D.)/$C_{obs}$] × 100.

### 2.4. Experimental animals

All experimental protocols involving animals were reviewed and approved by the institutional animal experimentation committee of the National Yang-Ming University. Male specific pathogen-unconjugated Sprague–Dawley rats weighing 280–320 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, USA) and water until 18 h prior to being used in experiments, and after that only food was removed.

Animals were divided into two groups, single i.v. and oral administrated groups. For single i.v. administered group, six Sprague–Dawley rats (280–320 g) were initially anesthetized with pentobarbital (50 mg/kg, i.p.), and remained anesthetized throughout the surgical operation and remained anesthetized throughout the experimental period. The right atrium through a catheter (cannula) implanted into the right external jugular vein of adult rats for blood samples [18], and a catheter (PE-10) was inserted into the proximal portion of the bile duct toward the liver for bile juice collection.

For oral administrated group, six Sprague–Dawley rats (280–320 g) were initially anesthetized with pentobarbital (50 mg/kg, i.p.), and remained anesthetized throughout the surgical operation. The right atrium through a catheter (cannula) was implanted into the right external jugular vein of adult rats for blood samples. During the period of surgery, the body temperature of each rat was maintained at 37 °C with a heating pad. After surgery, the rats were installed in the experimental cage and allowed to recover for 1 day.

### 2.5. Blood, bile sampling and sample preparation

Silibinin (100 mg/kg, i.v.) dissolved in ethanol and PEG 200 (1:1, v/v) solution was administered intravenously through the femoral vein and 500 mg/kg of the drug was given by gastrogavage for the route of oral administration. A 100 μL blood sample was withdrawn from the jugular vein into a heparin rinsed vial at 1 min, 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 90 min, 2 h, 3 h, 4 h, 5 h and 6 h after i.v. or oral administration. A 30 μL bile sample was withdrawn from the catheter (PE-10) of the bile duct at 1 min, 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 90 min, 2 h, 3 h, 4 h, 5 h and 6 h after i.v. administration.

Each blood sample was centrifuged at 2000 × *g* (PC-100 microcentrifuge, Bertec Enterrise, Taiwan) for 10 min. The small amount of plasma sample (25 μL) was vortex-mixed with 50 μL of internal standard solution (naringenin 10 μg/mL dissolved in acetonitrile). The protein precipitation was achieved by the internal standard solution and separated by centrifugation at 2000 × *g* for 30 min. An aliquot (20 μL) of the supernatant was directly injected onto the HPLC system for analysis.

The bile sample (15 μL) was vortex-mixed with 30 μL of internal standard solution and centrifuged at 2000 × *g* for 30 min. An aliquot (20 μL) of the supernatant was directly injected onto the HPLC system for analysis.

For the determination of total (unconjugated + conjugated) silibinin, 15 μL plasma or bile sample were incubated (1 h, 37 °C) with 45 μL β-glucuronidase enzyme solution at 3000 unit/mL. After incubation, the solution (30 μL) was vortex-mixed with 60 μL of internal standard solution. The protein precipitation was achieved using an internal standard solution and separated by centrifugation at 2000 × *g* for 30 min. An aliquot (20 μL) of the supernatant was directly injected onto the HPLC system for analysis.

### 2.6. Protein binding

The protein binding of silibinin was determined by ultrafiltration, the drug was given via i.v. bolus to rats with a dose of 100 mg/kg. Then, the blood sample (2 mL) was withdrawn from the heart puncture at 10 min after injection. The rat blood sample was centrifuged at 2000 × *g* for 10 min. The plasma was divided into two parts; 0.1 mL of plasma was used to measure the total form concentration of silibinin ($C_t$). The remaining plasma was transferred to an ultrafiltration tube (Centrifugal, Millipore, Bedford, MA, USA). Further centrifugation was performed at 2000 × *g* for 30 min for free form measurement ($C_f$). Measurement of silibinin was also carried out in the same way. The protein binding ($B$) of silibinin was calculated by following the equation: $B\ (\%) = [(C_t - C_f)/C_t] \times 100$.

### 2.7. Pharmacokinetic application

Calibration curves were constructed based on LC analyses of a standard mixture prior to each experiment. Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic software WinNonlin Standard Edition Version 1.1 (Scientific Consulting, Apex, NC, USA) by noncompartmental method. The area under the concentration–time curve (AUC) was calculated according to the linear trapezoidal method. The absolute oral bioavailability ($F$) of a drug is generally measured by comparing the respective AUCs after oral and intravenous administration according to the following equation: $F = (\text{AUC}_{\text{p.o.}}/\text{dose}_{\text{p.o.}})/(\text{AUC}_{\text{i.v.}}/\text{dose}_{\text{i.v.}})$.

## 3. Results and discussion

### 3.1. Chromatography

Silibinin in rat plasma was quantitated using LC coupled with ultraviolet detection. Fig. 2A shows a chromatogram of

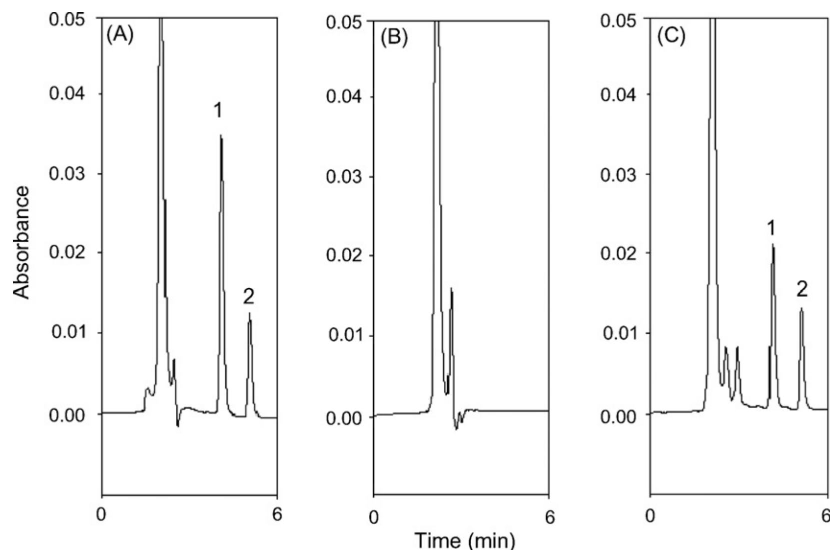


Fig. 2. Typical chromatograms of (A) standard silibinin (50 μg/mL) spiked in blank plasma, (B) blank plasma, and (C) plasma containing silibinin 32.9 μg/mL), collected from the rat plasma 15 min post silibinin administration (100 mg/kg i.v.). 1: silibinin, 2: naringenin (internal standard).

a standard of silibinin (50 μg/mL) spiked in blank plasma sample. Fig. 2B was a chromatogram of a drug-free plasma extract showing a clean, stable baseline without interfering endogenous peaks. Fig. 2C is the chromatogram of a plasma sample containing silibinin (32.9 μg/mL) collected from rat plasma 15 min after silibinin administration (100 mg/kg, i.v.). The analytes were well separated using the present chromatographic conditions. The retention times were 4.0 and 4.8 min for silibinin and internal standard, respectively, and no peak distortions were visible. In this method, no interferences from endogenous substances were observed in rat plasma and bile. Fig. 3A–C represents the chromatograms of a blank bile sample spiked with silibinin (50 μg/mL), blank bile sample, and a bile sample containing silibinin (32.2 μg/mL) collected 40–50 min after silibinin administration (100 mg/kg, i.v.), respectively.

### 3.2. Method validation

Calibration curves for silibinin with various biological matrices were considered linear with a correlation coefficient of determination ($r^2$) greater than 0.995 for all curves. Linear least-square regression analysis of the calibration graph on six different days demonstrated linearity between the response and the nominal concentration of silibinin over the range of 0.5–100 μg/mL. For calculating the plasma sample of silibinin from animal study, the calibration curve based on the peak area ratios (silibinin-to-internal standard) versus analyte concentration was constructed prior to assay. The data show the excellent repeatability of the sample analysis. The limit of detection (LOD) and the limit of quantitation (LOQ) 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 LOQ was defined as the linear lowest concentration of silibinin in plasma and bile sample that could be quantified with acceptable criteria (the inter-assay coefficient of variation ≤15% and accuracy within ±15%).

The intra- and inter-assay precision and accuracy values in the matrix of plasma and bile were presented in Tables 1 and 2, respectively. The overall mean precision, defined by the R.S.D., ranged from 8.30 to 0.33%. Analytical accuracy, expressed as the percentage difference of the mean observed values compared with known concentration varied from −6 to 8%.

Table 1
Method validation for the inter-assay precision (% R.S.D.) and accuracy (% bias) of the HPLC method for the determination of silibinin in plasma

| Nominal concentration | Observed concentration (μg/mL) | Precision R.S.D. (%) | Accuracy bias (%) |
|---|---|---|---|
| Intra-assay | | | |
| 0.5 | 0.48 ± 0.04 | 8.30 | −4 |
| 1 | 0.94 ± 0.02 | 2.13 | −6 |
| 5 | 4.96 ± 0.21 | 4.23 | −0.8 |
| 10 | 10.12 ± 0.44 | 4.35 | 1.2 |
| 50 | 50.03 ± 0.73 | 1.46 | 0.06 |
| 100 | 99.97 ± 0.33 | 0.33 | −0.3 |
| Inter-assay | | | |
| 0.5 | 0.54 ± 0.04 | 7.41 | 8 |
| 1 | 1.02 ± 0.08 | 7.84 | 2 |
| 5 | 5.14 ± 0.05 | 0.97 | 2.8 |
| 10 | 10.07 ± 0.38 | 3.77 | 0.7 |
| 50 | 49.53 ± 0.77 | 1.55 | −0.94 |
| 100 | 100.21 ± 0.34 | 0.34 | 0.21. |

R.S.D.: relative standard deviation.

Table 2
Method validation for the inter-assay precision (% R.S.D.) and accuracy (% bias) of the HPLC method for the determination of silibinin in bile

| Nominal concentration | Observed concentration (μg/mL) | Precision R.S.D. (%) | Accuracy bias (%) |
|---|---|---|---|
| Intra-assay | | | |
| 0.5 | 0.53 ± 0.03 | 5.66 | 6 |
| 1 | 1.07 ± 0.03 | 2.80 | 7 |
| 5 | 5.01 ± 0.37 | 7.39 | 0.2 |
| 10 | 10.08 ± 0.30 | 2.98 | 0.8 |
| 50 | 49.65 ± 0.85 | 1.71 | −0.7 |
| 100 | 100.16 ± 0.39 | 0.39 | 0.16 |
| Inter-assay | | | |
| 0.5 | 0.52 ± 0.04 | 7.69 | 4 |
| 1 | 1.06 ± 0.02 | 1.89 | 6 |
| 5 | 4.98 ± 0.40 | 8.03 | −0.4 |
| 10 | 10.02 ± 0.38 | 3.79 | 0.2 |
| 50 | 49.86 ± 1.15 | 2.31 | −0.28 |
| 100 | 100.05 ± 0.52 | 0.53 | 0.05 |

R.S.D.: relative standard deviation, observed concentration data are expressed as mean ± S.D. Accuracy (% bias) = $[(C_{obs} - C_{nom})/C_{nom}] \times 100$, precision (% R.S.D) = $[S.D./C_{obs}] \times 100$.

### 3.3. Recovery

The one-step extraction procedure was fairly rapid. The solvent acetonitrile for protein precipitation gave good recovery and the average absolute recoveries of silibinin from plasma was 96.3 ± 3.8% at concentrations of 5, 10 and 50 μg/mL as shown in Table 3.

Table 3
Recovery of silibinin in rat plasma

| Concentration (μg/mL) | Recovery (%) |
|---|---|
| 1 | 96 ± 7.3 |
| 5 | 103 ± 6.3 |
| 10 | 90 ± 4.0 |

Data expressed as mean ± S.D.

### 3.4. Protein binding

Methods for studying protein binding, including equilibrium dialysis [19] and ultrafiltration [20], make use of a semipermeable membrane that separates the protein and protein-bound drug from the free or unbound drug. The protein-bound drug is a large complex that cannot easily transverse cell or possibly even capillary membranes and therefore has a restricted distribution. An ultrafiltration method was used in this experiment and the protein binding ($B$) of silibinin was calculated by $B$ (%) = $[(C_t - C_f)/C_t] \times 100$. The protein binding in rat plasma was 70.3 ± 4.6%. To our knowledge, this is the first report about

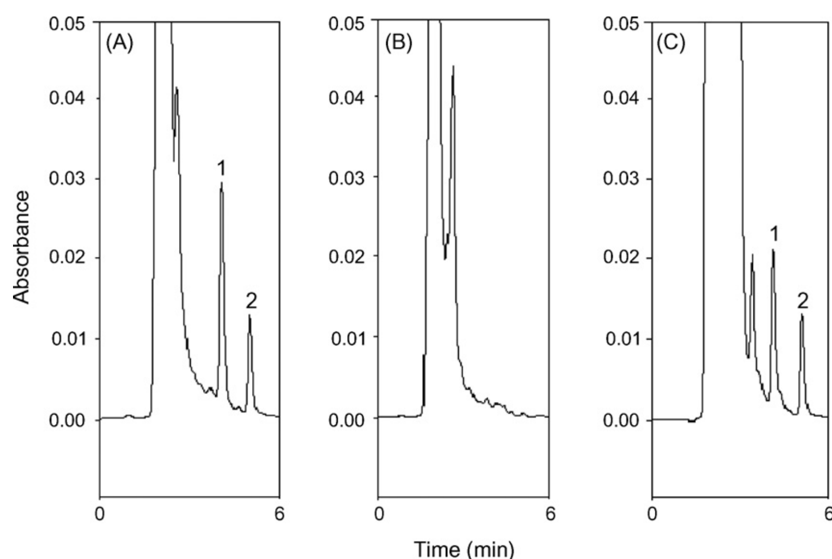


Fig. 3. Typical chromatograms of (A) standard silibinin (50 μg/mL) spiked in blank bile, (B) blank bile, and (C) bile containing silibinin (30.20 μg/mL), collected from the rat bile 45 min post silibinin administration (100 mg/kg i.v.). 1: silibinin, 2: naringenin (internal standard).

protein binding of silibinin. The endogenous lipoprotein may play a role in the transport of silibinin from the liver to the extra-hepatic tissues [21]. The extent of protein binding is important to know because binding of a drug to plasma proteins limits its concentration in tissue and its pharmacological action, since only the unbound drug in equilibrium across membranes, fraction of a drug, bind to a receptor or an enzyme and along with this to induce a pharmacological effect. In particular, the competition between two drugs for their binding to protein can significantly affect the disposition of both drugs, which possibly serious physiological consequences. These phenomena may result in the case of tightly bound drugs (over 90% bound to plasma proteins) [22], since silibinin–drug interactions cannot be excluded.

### 3.5. Pharmacokinetics of silibinin after i.v. administration

The mean concentration–time profiles of the silibinin in rat plasma and bile after 100 mg/kg, i.v. and 500 mg/kg, p.o. were shown in Fig. 3, and their relevant pharmacokinetic parameters are presented in Table 4.

Barzaghi et al. demonstrates that the pharmacokinetics of human subjects shows a pattern similar to rats [23]. The pharmacokinetic curves for the unconjugated silibinin reflect the fact that the disposition of unconjugated silibinin in rat bile exhibited a peak concentration at 5.1 ± 1.0 min after silibinin administration (100 mg/kg). The data demonstrate that silibinin distributed quickly to biliary and followed a slow elimination phase. The AUCs of unconjugated silibinin in the plasma and bile were 5.5 ± 0.73 and 11.1 ± 2.84 min mg/mL, respectively. After enzymatic hydrolysis by β-glucuronidase, the AUCs of total silibinin in the plasma and bile were 35.5 ± 5.9 and 1202 ± 186 min mg/mL, respectively. The peak time of total silibinin in bile was in 50 ± 18 min (Table 4).

The hepatobiliary excretion of unconjugated and total silibinin were 3.1 ± 0.95 and 30 ± 9.4, respectively, which was defined as the bile-to-blood excretion ($k$ value) calculated by AUC ratio ($k = AUC_{bile}/AUC_{blood}$) [24]. Since the metabolism of

Table 4
Pharmacokinetic parameters after silibinin administration (100 mg/kg, i.v. and 500 mg/kg oral)

| | Silibinin (100 mg/kg i.v.) | | Silibinin (500 mg/kg p.o.) | |
|---|---|---|---|---|
| | Unconjugated | Total | Unconjugated | Total |
| Blood | | | | |
| AUC (min mg/mL) | 5.5 ± 0.73 | 35.5 ± 5.9 | 0.26 ± 0.07 | 7.17 ± 1.42 |
| $C_{max}$ (μg/mL) | 256 ± 36 | 239 ± 19 | 8.5 ± 2.6 | 76 ± 15 |
| $t_{1/2}$ (min) | 68 ± 7.2 | 105 ± 29 | 12.2 ± 1.8 | 77 ± 3.2 |
| $T_{max}$ | 0 | 8.2 ± 4.5 | 11 ± 1.8 | 30 ± 4.7 |
| Bile | | | | |
| AUC (min mg/mL) | 11.1 ± 2.84 | 1202 ± 186 | | |
| $C_{max}$ (μg/mL) | 517 ± 85 | 7910 ± 2080 | | |
| $t_{1/2}$ (min) | 33 ± 8.3 | 97 ± 28 | | |
| $T_{max}$ | 5.1 ± 1.0 | 50 ± 18 | | |
| $AUC_{bile}/AUC_{blood}$ | 3.1 ± 0.95 | 30 ± 9.4 | | |

Data are expressed as mean ± S.E. of mean ($n = 6$).

silibinin is reported to be almost exclusively conjugative [25], all plasma and bile samples were hydrolyzed with β-glucuronidase in order to evaluate the biliary excretion. Moreover, identification of drug in bile provides direct evidence for the involvement of hepatobiliary elimination.

After i.v. administration, the concentrations of silibinin in the bile were higher than those in the blood for the same period of time, indicating that silibinin was concentrated in the bile by an active transport mechanism. Active biliary excretion would be compatible with the significantly great AUCs in the bile than in the blood.

### *3.6. Pharmacokinetics of silibinin after oral administration*

The peak plasma levels of unconjugated and total silibinin were 8.5 ± 2.6 and 76 ± 15 μg/mL, respectively, after an oral dose of 500 mg/kg. An in vitro study indicates that the inhibitory concentration 50% ($IC_{50}$) of silibinin is 1.4 μM (0.68 μg/mL) for the selective inhibition of uridinediphosphoglucuronate-glucuronosyltransferase-1A1 (UGT-1A1) [26]. The $IC_{50}$ level can be reachable during 60 min and 360 min after silibinin oral and i.v. administration (Figs. 4 and 5).

The absolute oral bioavailability of silibinin in rats was calculated to be about 0.95%. The poor bioavailability may be due to the high reactivity with phase II conjugation and poor absorption rate.

In conclusion, this HPLC method is not hindered by interference from endogenous materials and provides sufficient sensitivity for the determination of silibinin in plasma and bile. The plasma and bile pharmacokinetic data of silibinin presented that silibinin is rapidly and readily excreted into the bile, thus producing bile concentrations higher than those in blood. Furthermore, the distribution ratio of $AUC_{bile}/AUC_{blood}$ of silibinin suggests that the hepatobiliary elimination of silibinin may be regulated by an active transport.

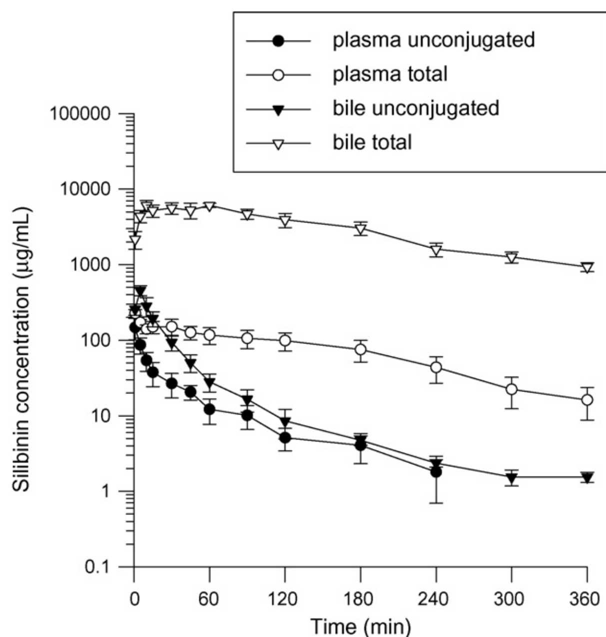


Fig. 4. Concentration–time profiles for silibinin in plasma and bile after silibinin administration (100 mg/kg, i.v.). Each group of data is represented as means ± S.E. mean from six individual experiments.

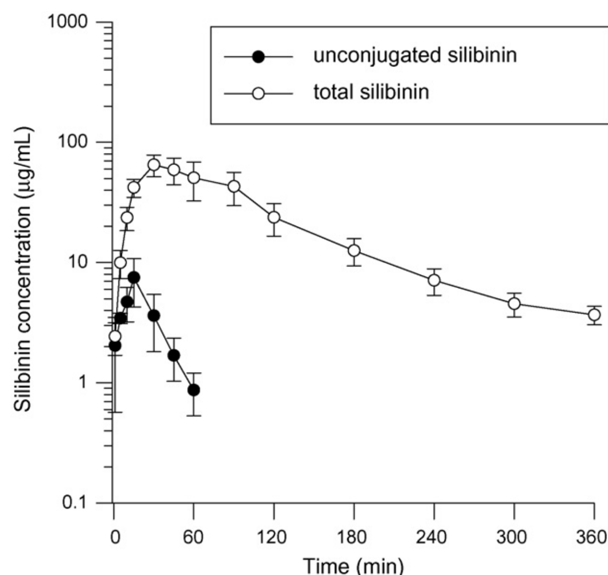


Fig. 5. Concentration–time profiles for silibinin in plasma after silibinin administration (500 mg/kg, p.o.). Each group of data is represented as means ± S.E. mean from six individual experiments.

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