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Determination and pharmacokinetic analysis of salvianolic acid B in rat blood and bile by microdialysis and liquid chromatography

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Abstract

Salvianolic acid B is an herbal ingredient isolated from *Salvia miltiorrhiza*. An in vivo microdialysis sampling method coupled to high-performance liquid chromatography has been developed for continuous monitoring of protein-unbound salvianolic acid B in rat blood and bile. Microdialysis probes were inserted into the jugular vein/right atrium and bile duct of Sprague–Dawley rats, and a dose of 100 mg/kg salvianolic acid B was then administered via the femoral vein. Dialysates were collected and directly injected into a liquid chromatographic system. Salvianolic acid B was eluted using a microbore reversed-phase ODS 5 μm (150 mm × 1 mm I.D.) column. Isocratic elution of salvianolic acid B was achieved within 10 min using the liquid chromatographic system. The chromatographic mobile phase consisted of acetonitrile–methanol–20 mM monosodium phosphoric acid (pH 3.5) (10:30:60, v/v/v) containing 0.1 mM 1-octanesulfonic acid with 0.05 ml/min. The wavelength of the UV detector was set at 290 nm. Salvianolic acid B in both blood and bile dialysates was adequately determined using the liquid chromatographic conditions described, although the blank bile pattern was more complex. The retention times of salvianolic acid B in rat blood and bile dialysates were found to be 7.2 min. Peak-areas of salvianolic acid B were linear ($r^2 > 0.995$) over a concentration range of 0.1–50 μg/ml. In vivo recoveries of microdialysis probes of salvianolic acid B in rat blood and bile averaged 22 ± 2% and 41 ± 1%, respectively. This study indicates that salvianolic acid B undergoes hepatobiliary excretion.

Keywords: Salvianolic acid B; Microdialysis; Liquid chromatography

1. Introduction

Salvia miltiorrhiza Bunge (Chinese herb: Dan-Shen) has been used widely in traditional Chinese medicine for the treatment of coronary artery disease [1], angina pectoris [2], atherosclerosis [3], myocardial ischemia [4], liver fibrosis and cirrhosis [5]. Recently the active components of this herb have been isolated and structurally identified.

Salvianolic acid B (Fig. 1), a water-soluble polyphenolic antioxidant isolated from the roots of this plant [6,7], was previously found to scavenge 1,1-diphenyl-2-picrylhydrazyl radicals and inhibit LDL oxidation more effectively than probucol [3]. Purified salvianolic acid B was obtained from Professor Shiao (Veterans General Hospital, Taipei, Taiwan). By extraction with ethanol–water from *S. miltiorrhiza* Bunge yields 342 mg salvianolic acid B at about 98% purity from 500 mg of the crude extract in a one-step separation [6].

Based on the pharmacokinetics, herb-drug interaction takes place in the treatment of *S. miltiorrhiza* extract. The effects of *S. miltiorrhiza* extract on the pharmacokinetics and pharmacodynamics of warfarin were studied in rats. In the pharmacokinetic study, single oral doses of warfarin were administered to rats or after 3 days treatment with *S. miltiorrhiza* extract intraperitoneally twice daily. The absorption rate (K_a), volume of distribution (V_d) and elimination half-life ($t_{1/2}$) of warfarin were significantly decreased, whereas C_{max} and T_{max} were significantly increased after treatment

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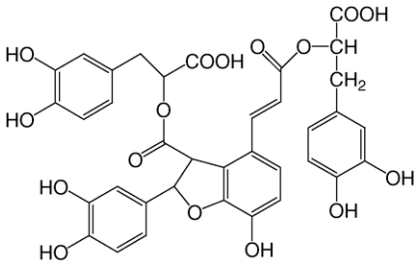

Fig. 1. Chemical structure of salvianolic acid B.

with *S. miltiorrhiza* extract. There was no significant change in prothrombin time (PT) during the *S. miltiorrhiza* extract treatment period, whereas the PTs were increased significantly in the first two days after warfarin doses. The results suggested that *S. miltiorrhiza* extract can increase the initial bioavailability of warfarin and also affect the elimination of warfarin. The pharmacokinetic and pharmacodynamic interactions observed in this study indicate a clinically important interaction between *S. miltiorrhiza* extract and warfarin if these two agents are taken together [8]. The pharmacokinetic and pharmacodynamic interactions of warfarin during co-treatment with *S. miltiorrhiza* extract observed in this study indicate an explanation for the clinically observed incidents of exaggerated warfarin adverse effects when traditional Chinese medicinal herbs or herbal products such as *S. miltiorrhiza* extract were co-administered [9].

Recently, for many endogenous and exogenous compounds, it has been reported that carrier-mediated transport contributes to the hepatic uptake and/or biliary excretion [10,11]. The elimination process through the primary active transport mechanisms is now designated as "Phase III" [12] in the detoxification for xenobiotics, in addition to Phase I by cytochrome P450 and Phase II by conjugation.

To our knowledge, there is little information for unbound pharmacokinetics of salvianolic acid B. In this study, we provide a microdialysis sampling method to study the protein-free pharmacokinetics of salvianolic acid B. Microdialysis provides the advantage of clean samples that do not require cleanup prior to analysis. And the microdialysis technique involves no biological fluid loss from the body, higher temporal resolution of the sampling interval and continuous sampling over long periods of time. Since no biological fluid is removed from or introduced into the body during the process of microdialysis, minimal perturbation can be achieved. Moreover, multiple sites sampling provide detailed pharmacokinetic information.

This study develops a liquid chromatographic method coupled to a microdialysis system for additional investigation of hepatobiliary excretion of salvianolic acid B. Until now, only scattered information has been available regarding the pharmacokinetics of this herbal ingredient and its hepatobiliary excretion based on the perspective of pharmacokinetics.

2. Experimental

2.1. Animals

Adult, male Sprague–Dawley rats (280–350 g) were obtained from the Laboratory Animal Center at National Yang-Ming University (Taipei, Taiwan). These animals were specifically pathogen-free and allowed to acclimate to their environmentally controlled quarters ($24 \pm 1\,^{\circ}C$ and 12:12 h light–dark cycle). Before experimentation, animals were allowed a one-week acclimation period 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 no. 5P14, PMI Feeds, Richmond, IN, USA) and water. On the day of experiments, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and supplements of sodium pentobarbital were given as needed throughout the experimental period.

2.2. Chromatographic conditions

The HPLC system consisted of a chromatographic pump (BAS PM-80, Bioanalytical System, West Lafayette, IN, USA), an on-line injector (CMA/160, Stockholm, Sweden) equipped with a 10 μl sample loop and Dynamax UV–Vis absorbance detector (Walnut Creek, CA, USA). Salvianolic acid B was eluted using a microbore reversed-phase Intertsil-2 C_{18} (150 mm × 1 mm I.D., 5 μm) column maintained at ambient temperature ($24 \pm 1\,^{\circ}C$). The mobile phase was comprised of acetonitrile–methanol–20 mM monosodium phosphoric acid, pH 3.5 (10:30:60, v/v/v), and 0.1 mM octanesulfonic acid. The mobile phase was filtered through a Millipore 0.22 μm filter and degassed prior to use and the flow rate of mobile phase was 0.05 ml/min. The UV wavelength was set at 290 nm for the detection. The HPLC-UV output signal was recorded via an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

2.3. Method validation

External standard method was used in this experiment. Calibration curves of salvianolic acid B were made prior to the experiments with correlation values (r^2) of at least 0.995. The intra- and inter-day variabilities of salvianolic acid B were assayed ($n = 6$) at concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, and 50 μg/ml on the same day and on six sequential days, respectively. The accuracy (% bias) was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentration (C_{obs}) as follows: bias (%) = $[(C_{obs} - C_{nom})/(C_{nom})] \times 100$. The precision (relative standard deviation; RSD) was calculated from the observed concentrations as follows: % RSD = [standard deviation (SD)/C_{obs}] × 100. In the results accuracy (% bias) and precision (% RSD) values of within ±15% covering the range of actual experimental concentrations were considered acceptable.

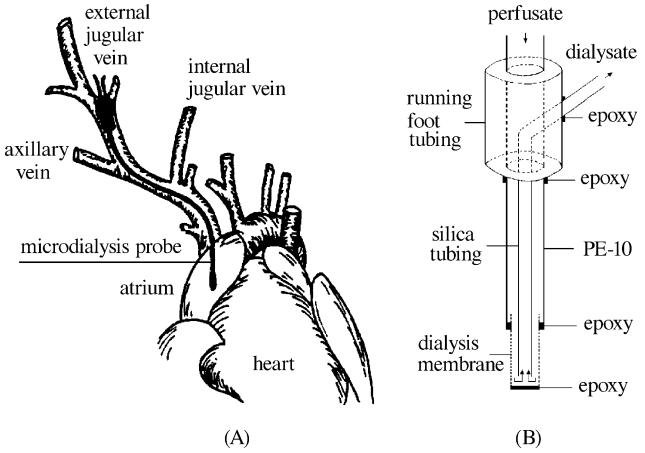

Fig. 2. The microdialysis probe used in the rat blood: (A) a microdialysis probe inserted into the rat jugular vein/right atrium; (B) the detailed structure of the homemade microdialysis probe.

2.4. Microdialysis in rat blood and bile

The microdialysis system consisted of a microinjection pump (CMA/100, Stockholm, Sweden), an on-line injector (CMA/160) and a microdialysis probe for sampling. In brief, a 10 mm section of capillary silica tubing (40 μm I.D., 140 μm O.D.; SGE, Australia) was inserted into a 1-cm running foot tubing (0.381 mm I.D., 2.21 mm O.D.). A 3 cm section of polyethylene PE-10 tube (0.28 mm I.D., 0.61 mm O.D.; Clay Adams, MD, USA) and a 10-mm piece of dialyzing membrane (nominal molecular weight cut-off 13,000 Da) were concentrically inserted into the running foot tubing (Fig. 2) [13]. All tubing unions and the end of the dialysis membrane were cemented with epoxy. At least 24 h were allowed for the epoxy to react. Under pentobarbital anesthesia, the blood microdialysis probe was inserted into the right jugular vein and then perfused with anticoagulant ACD solution (citric acid 3.5 mM; sodium citrate 7.5 mM; dextrose 13.6 mM) at a flow-rate of 1 μl/min, using a microinjection pump. After dialysate levels had stabilized (approximately 2 h), drug-free control samples were collected into the on-line injector and then salvianolic acid B (100 mg/kg) was intravenously administered via a femoral cannula. Dialysis samples were collected every 10 min and 10 μl of the dialysate was injected into the HPLC for salvianolic acid B assay.

The bile duct microdialysis probe (Fig. 3) was also constructed in our laboratory [13–16]. In brief a 7-cm piece of dialysis membrane was inserted into a section of polyethylene tubing (PE-60; 0.76 mm I.D., 1.22 mm O.D.) with the ends of the dialysis membrane connected to a silica tubing (40 μm I.D., 140 μm O.D.; SGE, Australia). A piece of PE-10 tubing (0.28 mm I.D., 0.61 mm O.D.) was then attached to both ends of the PE-60 tubing and all unions were cemented with epoxy. At least 24 h were allowed for the epoxy to react. After bile duct cannulation, the probe was perfused with Ringer's solution. Following a stabilization after the post surgical procedure (approximately 2 h), drug-free blank samples were collected and then salvianolic acid B (100 mg/kg) was intravenously administered via a femoral canula. Each dialysate sample (10 μl) was assayed immediately using the high-performance liquid chromatographic system. The microsyringe and the 10 μl loop of injector were washed with methanol between samples.

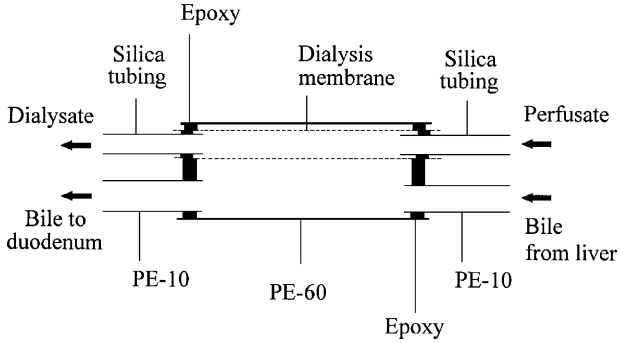

Fig. 3. Detailed description of the homemade bile microdialysis probe.

The in vivo recovery of microdialysis probe was determined by estimating the loss (the extraction ratio) of the salvianolic acid B, which was calculated from the concentration in the dialysate (C_{out}) relative to the concentration of the salvianolic acid B in the perfusate (C_{in}). Recovery (R_{dial}) was expressed using the following equation: $R_{dial} = 1 - (C_{out}/C_{in})$ [14,17].

2.5. Pharmacokinetic study

Calibration curves were constructed based on LC analyses of a standard mixture prior to each experiment. Following a 2 h post-surgical stabilization period subsequent to probe implantation, salvianolic acid B was administered (100 mg/kg, i.v.). The protein-unbound salvianolic acid B concentrations in rat blood and bile dialysates were determined. Salvianolic acid B concentration (C) in extracellular fluid was calculated from its concentration in the dialysate by the following equation: $C = C_{dialysate}/R_{dial}$.

Pharmacokinetic parameters were calculated on each set of data. Pharmacokinetic calculations were obtained using the WinNonlin Standard Edition Version 1.1 (Pharsight Corp., Mountain View, CA, USA). The incremental areas under the concentration curves (AUC) were calculated using the linear trapezoid method. $AUC = AUC_{last} + C_{last}/\lambda_z$; where C_{last} and t_{last} are the last observed concentration and time, respectively; and λ_z is the terminal slope, which is estimated by linear regression of the logarithmic value of the last observed data. The clearance (Cl) was estimated as follows: Cl = dose/AUC.

3. Results and discussion

Salvianolic acid B is not separated well from biological fluid in a reversed phase column without an appropriate buffer

because of its natural higher hydrophilicity and acidity. In this study, methanol, acetonitrile and phosphate buffer are used concurrently and so salvianolic acid B was adequately separated with the highest peak symmetries, particularly at an optimal mobile phase of acetonitrile–methanol–20 mM NaH_2PO_4 (pH 3.5) (10:30:60, v/v/v) and containing 0.1 mM 1-octanesulfonic acid with 0.05 ml/min. A little peak tailing and some endogenous interference were observed when we use only methanol and 20 mM monosodium phosphoric acid as the mobile phase. After we added a portion of acetonitrile to the mobile phase the peak become sharper and no interference was observed during the retention time of salvianolic acid B. The present validated liquid chromatographic method was coupled to the microdialysis technique and employed to determine salvianolic acid B disposition from rat jugular vein and bile duct following drug administration. The method demonstrated excellent chromatographic selectivity with no endogenous interferences at the peak for salvianolic acid B. Retention time of salvianolic acid B was about 7.2 min (Figs. 4 and 5). Salvianolic acid B in both blood and bile dialysates was adequately resolved using the liquid chromatographic conditions described, although the blank bile pattern was more complex.

Peak-areas of salvianolic acid B were linear ($r^2 > 0.995$) over a concentration range of 0.1–50 μg/ml. A typical chromatogram of a blood dialysate shows that the chromatographic conditions revealed no observable peaks that would significantly interfere with the determination of salvianolic acid B (Fig. 4B). Fig. 4C depicts a chromatogram of salvianolic acid B (1.87 μg/ml) obtained from a blood dialysate 20 min after salvianolic acid B administration (100 mg/kg, i.v.). Fig. 5A–C are chromatograms of salvianolic acid B standard, blank bile dialysate and bile dialysate obtained 40 min after salvianolic acid B administration. A bulky solvent front has been found in the chromatogram of 5C. These large peaks may contain several undefined peaks which may be the metabolites of salvianolic acid B after drug administration. Additional study is required to determine these unidentified peaks.

Intra- and inter-assay (Table 1) precision and accuracy values for salvianolic acid B fell well within predefined limits of acceptability (<15%) with a detection limit for salvianolic acid B of 0.05 μg/ml at signal-to-noise ratio of 3. The lowest acceptable reproducibility concentration for salvianolic acid B was 0.1 μg/ml, which was sufficiently sensitive to allow measurement of salvianolic acid B in rat blood for pharmacokinetic study. In vivo recoveries of microdialysis probes of salvianolic acid B in rat blood and bile averaged 22 ± 2% and 41 ± 1%, respectively. The dialysate is a protein-free solution that the biological matrix does not affect the analysis of salvianolic acid B. Therefore, we did not spike the dialysate of biological matrix with standards to validate intra- and inter-

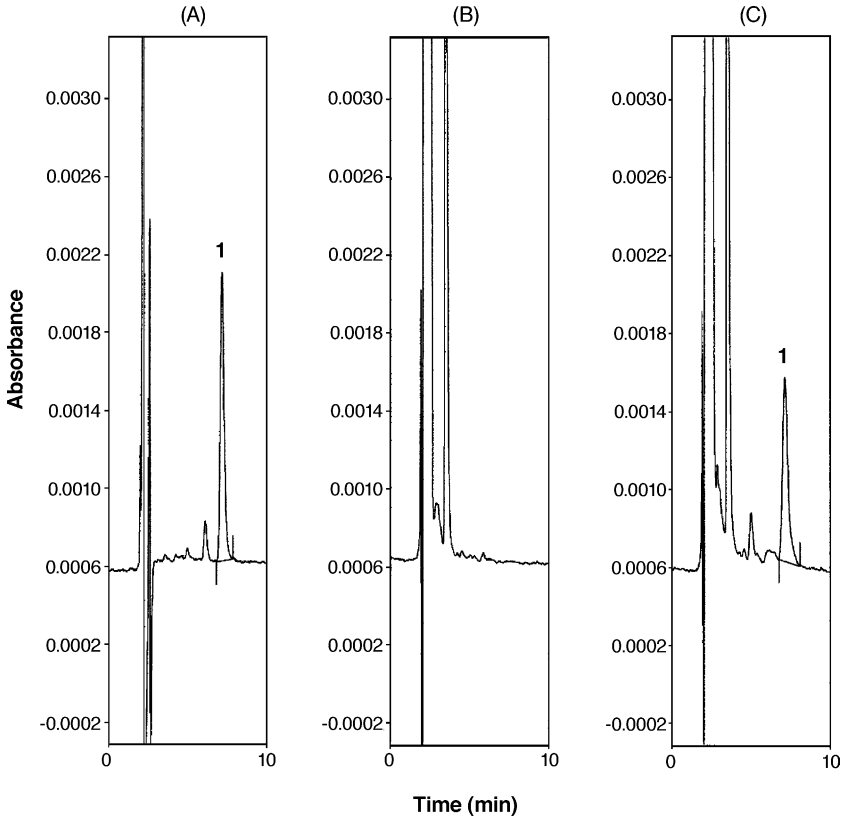

Fig. 4. Typical chromatograms of: (A) standard a salvianolic acid B (2 μg/ml); (B) blank blood dialysate; and (C) blood dialysate sample containing salvianolic acid B (1.87 μg/ml) collected from jugular vein at 20 min after salvianolic acid B administration (100 mg/kg, i.v.). 1: Salvianolic acid B.

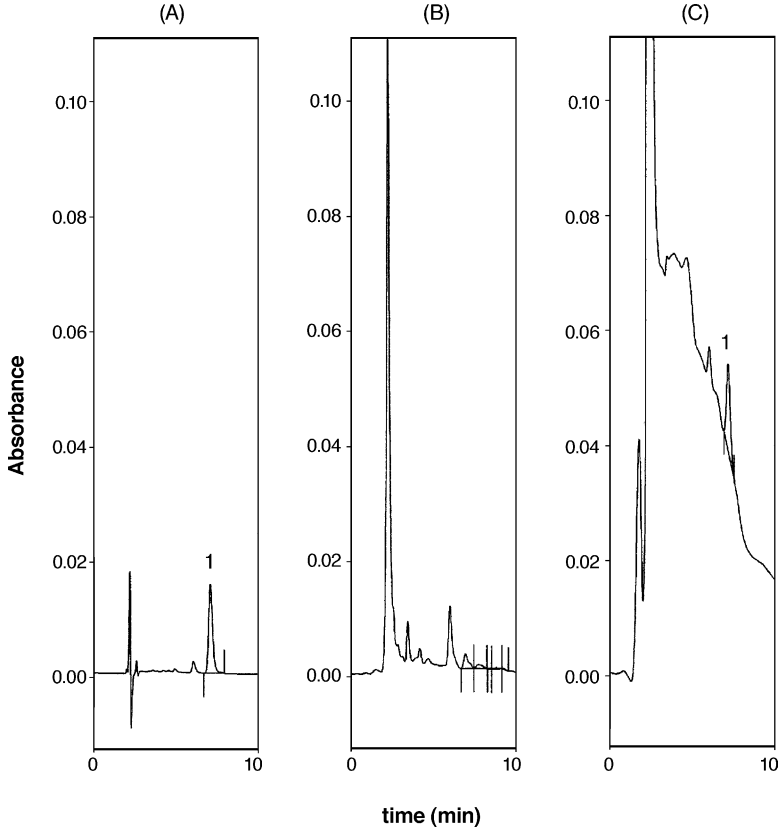

Fig. 5. Typical chromatograms of: (A) standard a salvianolic acid B (20 μg/ml); (B) blank bile dialysate; and (C) bile dialysate sample containing salvianolic acid B (20.17 μg/ml) collected from jugular vein at 40 min after salvianolic acid B administration (100 mg/kg, i.v.). 1: Salvianolic acid B.

Table 1
Intra- and inter-assay of salvianolic acid B as measured by the LC-UV system

Nominal concentration (μg/ml)	Observed concentration (μg/ml)*	RSD (%)	Accuracy (% bias)
Intra-assay			
0.1	0.11 ± 0.006	5.5	9.0
0.2	0.18 ± 0.009	4.9	−8.5
0.5	0.48 ± 0.030	6.2	−3.0
1.0	1.08 ± 0.082	7.6	8.5
2.0	2.16 ± 0.05	2.3	8.0
5.0	4.83 ± 0.21	4.3	−3.4
10	9.0 ± 0.40	4.4	−10.0
20	20.1 ± 0.44	2.2	0.6
50	50.1 ± 0.18	0.4	0.2
Inter-assay			
0.1	0.10 ± 0.009	8.6	4.0
0.2	0.19 ± 0.017	8.9	−4.5
0.5	0.52 ± 0.047	9.1	3.6
1.0	1.07 ± 0.07	6.5	7.0
2.0	2.12 ± 0.04	1.9	6.0
5.0	4.66 ± 0.13	2.8	−6.8
10	9.34 ± 0.35	3.7	−6.6
20	20.7 ± 0.49	2.4	3.3
50	49.9 ± 0.24	0.5	−0.3

* Observed concentration data are expressed as means ± SD ($n = 6$).

assay. Our result consists with previous report [18]; the recovery is independent of the matrix of the analysis of analyte for this experiment. Therefore, subsequent validation was only performed using Ringer's solution.

A conventional reversed phase C_{18} column (250 mm × 4.6 mm, I.D., 5 mm) with gradient elution system has been reported to measure salvianolic acid B (detectable at 3.5 μg/ml, with loaded amount 70 ng for 20 μl injection loop) from medicinal plant *S. miltiorrhiza* [6]. Compared with the conventional column, this microbore chromatographic system provides higher sensitivity to detect salvianolic acid B from biological dialysates.

samples collected over the first 2 h were discarded to compensate for recovery from acute effects of the surgical and the insertion procedures of a microdialysis probe. Then microdialysis-liquid chromatography was applied to determine dialysates of rat blood and bile. Dialysis samples were collected at 10-min intervals over the entire experimental period. Fig. 6 shows the concentration-time profile of unbound salvianolic acid B in the rat blood (corrected by in vivo recovery) after salvianolic acid B (100 mg/kg, i.v.) administration. The pharmacokinetic parameters of salvianolic acid B are shown in Table 2. The ester and phenolic structures of salvianolic acid B may go through phase I hydroxylation and phase II conjugation in the body, which may cause the possibility of the rapid disposition of salvianolic acid B. Hydroxyl

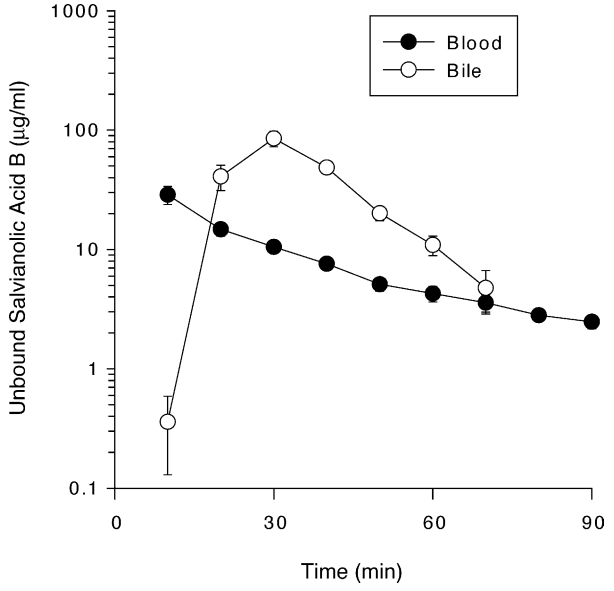

Fig. 6. Unbound salvianolic acid B concentration-time profile in blood and bile after salvianolic acid B administration (100 mg/kg, i.v., $n = 6$).

Table 2
Estimated pharmacokinetic parameters in rat blood and bile after salvianolic acid B administration (100 mg/kg, i.v.)

Parameters	Estimated
Blood	
$t_{1/2,\beta}$ (min)	53 ± 15
AUC (min μg/ml)	1340 ± 167
Cl (ml/kg/min)	79 ± 9
Bile	
C_{max} (μg/ml)	85.2 ± 12.7
T_{max} (min)	30 ± 0
$t_{1/2,\beta}$ (min)	9 ± 1
AUC (min μg/ml)	2080 ± 278
Bile-to-blood distribution	
AUC_{bile}/AUC_{blood}	1.55 ± 0.21

Data are expressed as means ± SEM ($n = 6$).

groups are conjugated with glucuronic acid or sulphate in the liver [19]. Bacteria in the colon hydrolyze conjugated glycosides by which the degraded metabolite can be reabsorbed into the portal circulation with enterohepatic circulation [20].

The results indicate that the concentration of salvianolic acid B in bile gradually increased, reaching a peak concentration at about 30 min (Fig. 6). Most of the concentrations of salvianolic acid B in bile were significantly higher than those in blood, suggesting an active transport of salvianolic acid B that might be excreted from blood vessels through liver into bile duct (Table 2). The hepatobiliary excretion of salvianolic acid B was defined as the bile-to-blood distribution (k value), which was calculated by dividing the salvianolic acid B AUC in bile by that in blood ($k = AUC_{bile}/AUC_{blood}$) [21]. The bile-to-blood distribution ratio was 1.55 ± 0.21 at the dose of 100 mg/kg.

In conclusion, a rapid and sensitive microbore liquid chromatographic system for the determination of unbound salvianolic acid B in rat blood and bile was developed. The blood and bile pharmacokinetic data of salvianolic acid B presented here are important in the expectation that salvianolic acid B 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 salvianolic acid B suggests that the hepatobiliary elimination of salvianolic acid B may be regulated by an active transport.

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