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Determination of extracellular hesperidin in blood and bile of anaesthetized rats by microdialysis with high-performance liquid chromatography: a pharmacokinetic application

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Abstract

A method coupled with microdialysis technique and liquid chromatography was applied in the continuous and concurrent in vivo monitoring of extracellular hesperidin in the blood and bile of anaesthetized rats. Hesperidin was intravenously administered via the femoral vein. Sampling was achieved using two microdialysis probes, which were implanted into the jugular vein and into the bile duct. Dialysates of blood and bile were both directly injected onto the liquid chromatographic system, so no further clean-up procedures were required. Separation was performed using a reversed phase ODS-2 microbore column 150 mm × 1 mm i.d., particle size 5 μm with mobile phase of acetonitrile–0.1 M ammonium acetate (30:70, v/v) at flow-rate of 0.05 ml/min. The UV detection for hesperidin was set at a wavelength of 283 nm. This method was used to determine the pharmacokinetics of hesperidin and its interaction in the presence of cyclosporin A, which is a *P*-glycoprotein modulator. The results indicate that the curve of area under the concentration versus time (AUC) for hesperidin in bile was significantly greater than that for hesperidin in blood at the dose of 30 mg/kg. The blood-to-bile distribution ratio ($k = AUC_{bile}/AUC_{blood}$) was 8.9 ± 2.5 for hesperidin at 30 mg/kg. Following cyclosporin A treatment, the distribution ratio was reduced to 3.2 ± 0.6. In conclusion, hesperidin goes through hepatobiliary elimination against the concentration gradient from blood to bile, and this hepatobiliary excretion of hesperidin may be regulated by the *P*-glycoprotein.
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Keyword: Hesperidin

1. Introduction

Hesperidin (Fig. 1) is a bioactive flavonoid which is contained principally in the various species of the Citrus (family Rutaceae). Hesperetin is an aglycone and a metabolite of hesperidin, and both of them have been reported to possess antioxidant properties to reduce superoxide in electron transfer, as well as concerted proton transfer reaction in vitro [1]. After oral administration, hesperidin is absorbed through the gastrointestinal tract, however cumulative urinary recovery indicates low bioavailability (<25%). Of the aglycones, hesperetin, has been detected in both urine and plasma [2], and additional investigation found that absorbed Citrus flavanones went thought glucuronidation before urinary excretion [2]. In addition, due to the low gastrointestinal absorption rate, urinary excretion was lower than fecal excretion after oral ingestion. However, both urinary and fecal excretion ratios were comparable after intravenous injection of hesperidin methylchalcone [3].

Previous reports have demonstrated that high-performance liquid chromatography (HPLC) is a suitable instrument for the determination of hesperidin in citrus and plant extracts [4–7], biological fluids [8,9] and pharmaceutical formulations [10,11]. Recently, capillary electrophoresis [12] and liquid chromatography with tandem mass spectrometry have been used in the measurement of hesperidin in various matrixes [13,14]. However, no previous studies have attempted to monitor the unbound form of hesperidin in biological fluids. The unbound form of a drug is considered the pharmacologically active portion because it can

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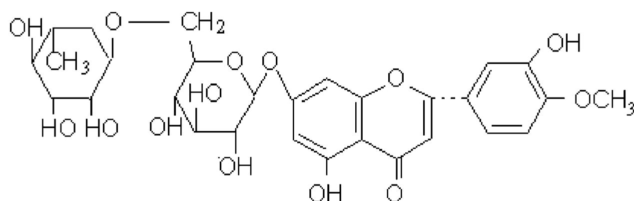

Fig. 1. Chemical structure of hesperidin.

diffuse out of the vascular compartment through cell membranes to reach target tissues. Furthermore, plasma protein binding can significantly affect important pharmacokinetic processes, such as distribution and elimination by renal and/or hepatic mechanisms. Thus, for many drugs, the unbound drug concentration has important pharmacokinetic and pharmacodynamic implications [15].

Over the past several years, microdialysis has been increasingly used in various animal experiments, for the in vivo sampling of unbound endogenous or exogenous compounds present in blood, brain or tissue, etc. [16,17]. Sampling by this technique involves continuous perfusion of fluid through microdialysis probes implanted in the appropriate tissue space.

According to classical pharmacokinetics, several factors influence distribution or excretion, including drug chemical properties and the ability to cross membrane. In addition, hepatic blood flow, protein binding and hepatic intrinsic clearance also play important roles in the pharmacokinetics of drugs. In particular, *P*-glycoprotein in the liver plays a significant role in the excretion of drugs into the bile, and this physiological function may be a control mechanism to accelerate the processes of hepatobiliary excretion. *P*-Glycoprotein is located in canalicular structures that are formed in adult rat hepatocytes for the regulation of transportation function [18]. More recently, *P*-glycoprotein mediated transport has been shown to be responsible for the excretion of xenobiotics via the canalicular membrane of hepatocytes into the bile for hepatobiliary elimination of drugs [19]. The purpose of this study is to develop a system for measuring unbound concentration of hesperidin and provide further investigation the pharmacokinetics of hesperidin and its hepatobiliary excretion mechanism.

2. Experimental

2.1. Experimental animals

All experimental protocols involving animals were reviewed and approved by the institutional animal experimentation committee of the National Research Institute of Chinese Medicine. Male, specific pathogen-free Sprague–Dawley rats weighing 250–300 g were obtained from the Laboratory Animal Center of the National Yang-Ming University, Taipei, Taiwan. On arrival at the animal facilities, the animals were acclimatized for about 7 days, during which time they had free access to food (Laboratory Rodent Diet 5P14, PMI Feeds Inc., Richmond, IN, USA) and water until 18 h prior to being used in experiments, and after that only food was removed. The rats were initially anesthetized with urethane 1 g/ml and α-chloralose 0.1 g/ml (1 ml/kg, i.p.), and remained anesthetized throughout the experimental period. The femoral vein was exposed for further drug administration. The rat's body temperature was maintained at 37 °C with a heating pad during the experiment.

2.2. Chemicals and reagents

Hesperidin (97%) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Cyclosporin A (Sandimmun) was obtained from Novartis Pharma (Basle, Switzerland). The solvents and reagents for chromatography were purchased from E. Merck (Darmstadt, Germany). The standard solution of hesperidin was storage in the methanol at 4 °C refrigerator. Triply de-ionized water from Millipore (Bedford, MA, USA) was used for all preparations.

2.3. 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), an on-line injector (CMA 160, Stockholm, Sweden) equipped with a 10 μl sample loop to inject the sample from blood dialysate each 10 min and an ultraviolet detector (Varian, Walnut Creek, CA, USA). An off-line fraction collector (CMA 140, Stockholm, Sweden) was used to collect the sample each 10 min from the bile dialysate. Hesperidin and dialysate were separated using a GSK ODS-2 microbore column (150 mm × 1 mm i.d.; particle size 5 μm; Bedford, MA, USA). The mobile phase was comprised of acetonitrile–0.1 M ammonium acetate (30:70, v/v) and the flow-rate of the mobile phase was 0.05 ml/min. The mobile phase was filtered through a Millipore 0.22 μm filter and degassed prior to use. The optimal UV detection for hesperidin was set at a wavelength of 283 nm. Output data from the detector were integrated via an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA). The collected samples were analyzed on the same sampling day.

2.4. Method validation

The intra- and inter-assay variabilities for hesperidin were assayed (six replicates) at concentrations of 0.10, 0.50, 1.00, 5.00, 10.00 and 50.00 μg/ml on the same day of drug analysis and on 6 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 (R.S.D.) was calculated

from the observed concentrations as follows: % R.S.D. = [standard deviation (S.D.)/C_{obs}] × 100. Accuracy (% bias) and precision (% R.S.D.) value for the lowest acceptable reproducibility concentration was pre-defined as ±15%.

2.5. Microdialysis experiment

Blood and bile microdialysis systems consisted of a CMA/100 microinjection pump (CMA, Stockholm, Sweden) and microdialysis probes. The dialysis probes for blood (1 cm in length) [20] were made of silica capillary in a concentric design with the tips covered by dialysis membrane (Spectrum, 150 μm outer diameter with a cut-off at nominal molecular mass of 13,000 Da, Laguna Hills, CA, USA). The blood microdialysis probe was positioned within the jugular vein/right atrium (toward the heart) and then perfused with anticoagulant citrate dextrose, ACD solution (citric acid 3.5 mM; sodium citrate 7.5 mM; dextrose 13.6 mM) at a flow-rate of 1.3 μl/min.

The bile duct microdialysis probes were developed in our laboratory [21,22], according to the design originally described by Scott and Lunte [23]. 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, NJ, USA). The ends of the dialysis membrane and PE-60 were inserted into a silica tubing (40 μm i.d; 140 μm o.d., SGE, Australia) and PE-10 (0.28 mm i.d.; 0.61 mm o.d.), respectively. Both the ends of tubing and the union were cemented with epoxy, which was allowed to dry for a period of 24 h. After bile duct cannulation, each probe was perfused with Ringer's solution at a flow-rate of 1.3 μl/min. Bile dialysate was then analyzed by the HPLC system. The dialysates were collected each 10 min and measured on the same experimental day.

2.6. In vivo recovery

Following the general surgical procedures described above, the probes were perfused with drug-free perfusion fluid of ACD and Ringer solutions into blood and bile probes, respectively. After a 2-h post-surgical stabilization period, drug-free perfusion fluid was then changed to perfusate containing hesperidin (5 μg/ml) (C_{perf}) for in vivo calibration. The perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of hesperidin were determined by HPLC. The relative in vivo recovery (R_{dial}) of hesperidin across the microdialysis probes inserted in the rat blood and bile were calculated according to the following equation, $R_{dial} = (C_{perf} - C_{dial})/C_{perf}$. In order to clarify the effect of cyclosporin A on the hepatobiliary excretion of hesperidin, the in vivo bile recovery of hesperidin was performed by two groups in rats. The control group was perfused hesperidin (5 μg/ml) alone into bile probe. For the cyclosporin A group, the cyclosporin A 10 mg/kg was injected into femoral vein 10 min before hesperidin (5 μg/ml) perfusion.

2.7. Pharmacokinetic analysis

The concentrations of hesperidin in rat dialysates were determined from the calibration curves. The midpoint of the 10-min periods was used as the sample time for blood and bile hesperidin microdialysate concentration-time profiles. After a 2-h post-surgical stabilization period, hesperidin (30 mg/kg, i.v.) was administered to the control group (n = 6). For cyclosporin A-treated groups, a 20 mg/kg of cyclosporin A was concomitantly injected via the left femoral vein 10 min prior to hesperidin injection. The volume of each injection was 1 ml/kg. Blood and bile dialysates were measured by liquid chromatography during the same experimental day. Hesperidin concentrations in blood and bile were corrected by the estimated in vitro recoveries from the respective microdialysis probes.

Microdialysate concentrations (C_m) of hesperidin were converted to unbound concentration (C_u) as follows: $C_u = C_m/R_{dial}$. The microdialysate recovery and concentration calculation were performed according to our previous report [20–22]. Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic calculation software WinNonlin Standard Edition Version 1.1 (Scientific Consulting Inc., Apex, NC, USA) by the non-compartmental method.

2.8. Statistics

The results are represented as mean ± S.E. of the mean. The statistical analysis was performed with SPSS version 10.0 (SPSS Inc. Chicago, IL, USA). Student's t-test was used to compare between the control (hesperidin treated alone) and cyclosporin A-treated groups. All statistical tests were performed for the two-sided 5% level of significance.

3. Results and discussion

The present validated liquid chromatographic method was coupled with the microdialysis technique and employed to determine hesperidin disposition from rat jugular vein and bile duct following drug administration. Typical resulting chromatograms of hesperidin in rat blood and bile are shown in Figs. 2 and 3, respectively. Separation of hesperidin from some endogenous chemicals in the blood, and bile dialysates were achieved in a mobile phase containing 30% acetonitrile and 70% 0.1 M monosodium phosphate at pH 3.0. Retention time of hesperidin was 5.9 min. The calibration curve of hesperidin was obtained prior to LC analysis of dialysates over concentration ranges of 0.1–50 μg/ml. However, the bile dialysate affect the lower concentration of hesperidin.

Fig. 2A shows a standard injection of hesperidin (1 μg/ml), and Fig. 2B shows the chromatogram of a blank blood dialysate. None of the observed peaks interfered with the analyte. Fig. 2C shows the chromatogram of a blood

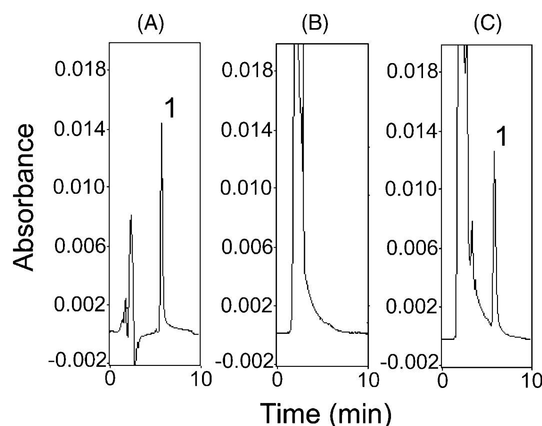

Fig. 2. Typical chromatograms of: (A) standard hesperidin (1 μg/ml), (B) blank blood dialysate, and (C) blood sample containing hesperidin (0.82 μg/ml) collected from jugular vein at 60 min after hesperidin administration (30 mg/kg, i.v.). 1: hesperidin.

dialysate sample containing hesperidin (0.82 μg/ml) collected from a rat blood microdialysis probe 50–60 min after hesperidin administration (30 mg/kg, i.v.).

Furthermore, based on the same chromatographic conditions, none of the observed peaks interfered with the analyte in the chromatogram of the bile sample within the run time. Fig. 3A shows a standard injection of hesperidin (5 μg/ml). Fig. 3B shows a chromatogram of a blank bile dialysate sample obtained from the bile duct microdialysis probe before the drug administration. Fig. 3C shows the chromatogram of a bile dialysate sample obtained with hesperidin (4.92 μg/ml) collected from the bile duct microdialysis probe 70–80 min after hesperidin administration (30 mg/kg, i.v.).

The intra- and inter-assay precision and accuracy of hesperidin were well both within the predefined limits of acceptability (Table 1). Telting-Diaz et al. [24] indicated that

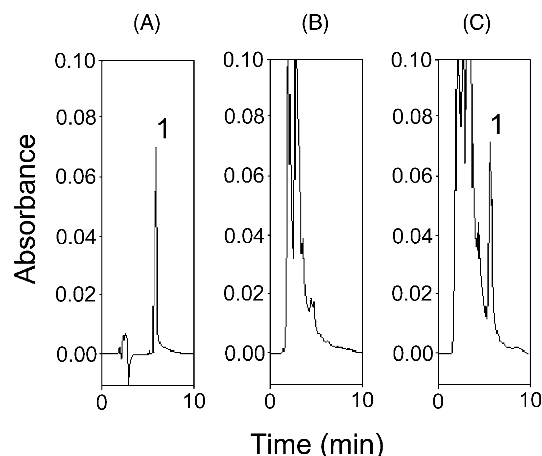

Fig. 3. Typical chromatograms of: (A) standard hesperidin (5 μg/ml), (B) blank bile dialysate, and (C) bile sample containing hesperidin (4.92 μg/ml) collected from bile duct at 80 min after hesperidin administration (30 mg/kg, i.v.). 1: hesperidin.

the recovery is independent of the matrix. Based on that report, the precision, accuracy and microdialysis probes for blood sampling only calibrated using the Ringer's solution. The average in vivo microdialysate recovery of hesperidin (5 μg/ml) for blood microdialysis probes was 53.5 ± 3.3%. The lowest acceptable reproducibility concentration for hesperidin was 0.1 μg/ml, which was sufficiently sensitive to allow measurement of hesperidin in rat blood for pharmacokinetic study. According to the apparent interference in the chromatogram of bile (Fig. 3), the microdialysate was collected from bile for additional precision exam. The lower concentration of hesperidin add in the bile dialysate was 0.5 μg/ml.

In order to clarify the effect of cyclosporin A on the hepatobiliary excretion of hesperidin, the in vivo recovery of hesperidin in bile was performed by two groups in rats. The control group was perfused hesperidin (5 μg/ml) alone into microdialysis probe for bile perfusion and the recovery was 79.3 ± 5.4%. For the cyclosporin A group, the cyclosporin A 10 mg/kg was injected into femoral vein 10 min before hesperidin (5 μg/ml) perfusion. The in vivo recovery for bile was significantly increased from up to 86.3 ± 4.4% ($P < 0.05$, $n = 6$), which may cause by the effect of cyclosporin A administration. The concentrations versus of time curves of hesperidin in biological fluids were therefore corrected by the data of in vivo recovery.

Microdialysis, which excludes large molecules and thus simplifies sample clean-up procedures often necessary before HPLC analysis, provided an excellent means by which unbound drugs in the blood and bile could be continuously monitored [25]. As a result, the dialysates were quickly and adequately resolved. Mean hesperidin blood concentrations versus time profiles at the dosage of 30 mg/kg are presented in Fig. 4.

The concentration of hesperidin in bile gradually increased, reaching a peak concentration at about 20 min (Fig. 5). The concentration of hesperidin in bile was significantly higher than that in blood, suggesting an active transport of hesperidin that might excrete from blood to bile (Table 2). The hepatobiliary excretion of hesperidin was defined as the blood-to-bile distribution (k-value), which was calculated by dividing the hesperidin AUC in bile by that in blood ($k = AUC_{bile}/AUC_{blood}$). The blood-to-bile distribution ratio was 8.9 ± 2.5 at the dose of 30 mg/kg. After cyclosporin A was injected prior to hesperidin administration, hesperidin concentrations in blood were significantly altered (Fig. 4).

The blood AUC of hesperidin both alone and in the cyclosporin A-treated group were 463 ± 114 min μg/ml and 603 ± 84.8 min μg/ml, respectively, at a dosage of 30 mg/kg hesperidin administration. The bile AUC of hesperidin (30 mg/kg) alone and with cyclosporin A coadministration were 2910 ± 631 min μg/ml and 1710 ± 179 min μg/ml, respectively. The blood-to-bile distribution ratio of hesperidin was reduced from 8.9 ± 2.5 to 3.2 ± 0.6 when cyclosporin A was coadministered. These results reveal that the efflux

Table 1
Intra- and inter-assay precision (R.S.D.) and accuracy (bias) of hesperidin with Ringer solution and the matrix of bile microdialysate

Nominal concentration (μg/ml)	Observed concentration (μg/ml) in Ringer	R.S.D. (%)	Bias (%)	Observed concentration (μg/ml) in bile microdialysate	R.S.D. (%)	Bias (%)
Intra-assay						
0.10	0.103 ± 0.002	1.9	3.0	–		
0.50	0.482 ± 0.021	4.4	−3.6	0.53 ± 0.05	7.8	9.2
1.00	0.998 ± 0.032	3.2	−0.1	0.96 ± 0.07	7.2	−3.5
5.00	5.27 ± 0.11	2.1	5.4	4.98 ± 0.12	2.4	−0.4
10.00	10.54 ± 0.50	4.7	5.4	9.90 ± 0.46	4.6	−1.0
50.00	49.86 ± 0.09	0.2	−0.3	50.0 ± 0.03	0.06	0.08
Inter-assay						
0.10	0.104 ± 0.010	9.6	4.0	–		
0.50	0.484 ± 0.039	8.1	−3.2	0.54 ± 0.08	15	8.4
1.00	1.005 ± 0.017	1.7	0.5	0.98 ± 0.07	7.5	−2.2
5.00	4.90 ± 0.36	7.3	−2.0	4.93 ± 0.09	1.8	−1.4
10.00	10.17 ± 0.86	8.5	1.7	10.0 ± 0.09	1.8	0
50.00	49.96 ± 0.18	0.4	−0.08	49.9 ± 0.04	0.08	−0.2

Data expressed as means ± S.D. ($n = 6$).

transport system of hesperidin was inhibited by the treatment of cyclosporin A (Table 2).

Active hepatobiliary excretion would be compatible with the significantly larger AUC in bile than in blood. In addition, many studies have shown that *P*-glycoprotein may play a transportation role in excreting some drugs from the liver to the bile [26]. The convenience of microdialysis applied in the determination of hepatobiliary excretion study can provide results with the least possible disturbance of the physiological state of enterohepatic circulation in individual experimental animals [27]. The present studies combining cyclosporin A with hesperidin have clarified the elimination of hesperidin is significantly altered by *P*-glycoprotein.

It is well known that *P*-glycoprotein mediates the transcellular transport of many drugs and is involved with multiple-drug resistance during anticancer treatment [28]. Because hesperidin is a very common ingredient contained in fruit drinks and herbs, food–drug interaction and herb–drug interaction should be considered. However, to date the precise mechanism of this pharmacokinetic food–drug interaction has not been appreciated, and warnings have not been promulgated.

In conclusion, the bile and blood pharmacokinetic data presented here are important in confirming the expectation that hesperidin is rapidly and readily excreted into the bile, thus producing bile concentrations higher than those in

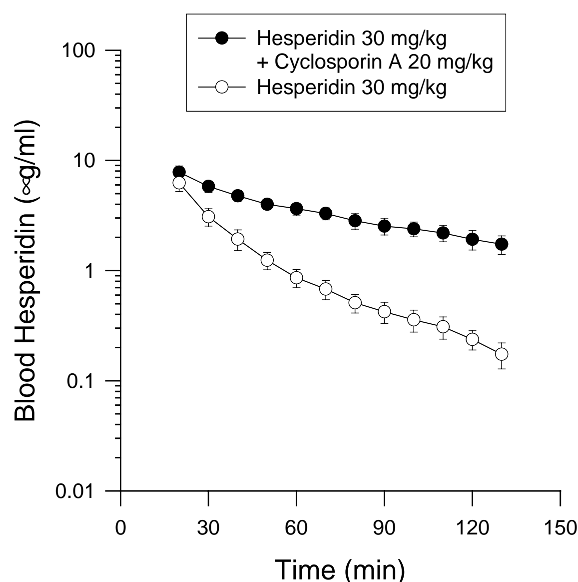

Fig. 4. Concentration-time profiles for hesperidin in blood after hesperidin i.v. administration at dosage of 30 mg/kg, with and without cyclosporin A (20 mg/kg) administration. Each group of data is represented as means ± S.E. from six individual microdialysis experiments.

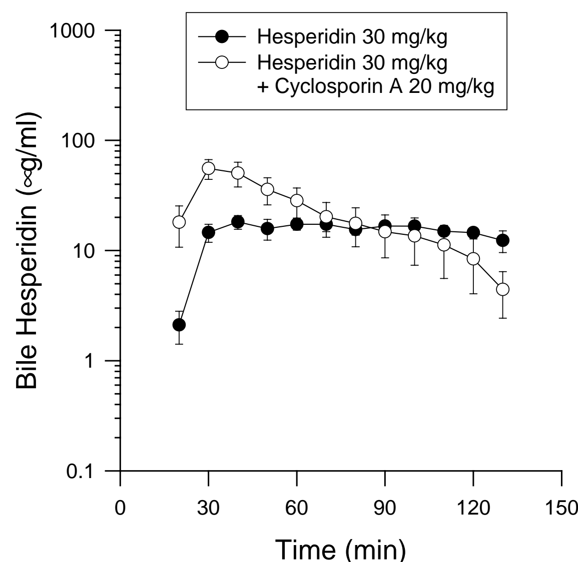

Fig. 5. Concentration-time profiles for the group with hesperidin in bile after hesperidin i.v. administration at dosage of 30 mg/kg, and the group with cyclosporin A (20 mg/kg) treatment. Each group of data is represented as means ± S.E. from six individual microdialysis experiments.

Table 2
Pharmacokinetic data of hesperidin (30 mg/kg) in rat blood and bile, both with and without treatment with cyclosporin A (20 mg/kg)

	Without cyclosporin A	With cyclosporin A
Blood		
AUC (min μg/ml)	463 ± 114	603 ± 84.8
Cl (ml/kg min)	75.5 ± 14.3	44.5 ± 8.1
MRT (min)	57.6 ± 38.2	83.3 ± 10.0
Bile		
AUC (min μg/ml)	2910 ± 631	1710 ± 179
T_{max} (min)	31.7 ± 1.7	73.3 ± 11.5*
C_{max} (μg/ml)	67.0 ± 12.6	20.8 ± 2.3*
MRT (min)	50.4 ± 4.5	75.6 ± 1.8*
Blood-to-bile distribution		
AUC_{bile}/AUC_{blood}	8.9 ± 2.5	3.2 ± 0.6*

Data are expressed as mean ± S.E. ($n = 6$).
* $P < 0.05$ indicates significant difference from the group without cyclosporin A.

blood. Furthermore, the addition of cyclosporin A showed significant effect both on the pharmacokinetic parameters and on the distribution ratios of AUC_{bile}/AUC_{blood} of hesperidin, which suggests that the hepatobiliary elimination of hesperidin may be regulated by *P*-glycoprotein.

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