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Concurrent measurement of unbound genistein in the blood, brain and bile of anesthetized rats using microdialysis and its pharmacokinetic application

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Abstract

Genistein, the major isoflavone in soybeans, has been shown to have a wide range of effects. We used an HPLC–UV combined with microdialysis method to detect unbound genistein in rat blood, brain and bile. Genistein dialysates were eluted with a mobile phase containing acetonitrile–water (40:60, v/v, pH 3.5 adjusted by 0.1% acetic acid). Samples were separated using a phenyl (5 μm) column maintained at ambient temperature. The UV detector wavelength was set at 259 nm. The flow rate was 1.0 ml/min. The limit of quantitation for genistein was 50 ng/ml. The in vitro recoveries of genistein were 31 ± 1, 13 ± 1 and 59 ± 4% in microdialysis probes of blood, brain and bile, respectively ($n = 4$). Inter- and intra-assay accuracy and precision of the analysis were less than 10% in the concentration ranges of 0.05–5.0 μg/ml. A small ratio of genistein penetrates the blood–brain barrier (BBB) and goes through hepatobiliary excretion after genistein administration (10 or 30 mg/kg, i.v.). The brain-to-blood (AUC_{brain}/AUC_{blood}) and bile-to-blood (AUC_{bile}/AUC_{blood}) distribution ratios were 0.04 ± 0.01 and 1.85 ± 0.42, respectively for the dosage of genistein 30 mg/kg. After co-administration of cyclosporine, a P-glycoprotein (P-gp) inhibitor, the distribution ratios of genistein in brain and bile were not significantly altered. These results suggest that the BBB penetration and hepatobiliary excretion of genistein may not regulated by P-gp.

Keywords: Genistein; Hepatobiliary excretion; Microdialysis; Pharmacokinetics

1. Introduction

Genistein (4',5,7-trihydroxyisoflavone; Fig. 1), one of the major isoflavones in soybeans, has been considered as potential remedy for a wide types of diseases such as osteoporosis, cardiovascular diseases and menopausal symptoms [1]. In general, soy products can lower coronary heart disease by lowering blood cholesterol levels. Following oral administration of a single soy serving for a human, genistein plasma concentration rose slowly and reached mean maximum values at about 8 h [2]. Genistein goes through phase II glucuronidation in the intestine concomitant with absorption [3].

A wide variety of analytical techniques have been applied for the quantitation of soy isoflavones in foods and biological fluids, including high-performance liquid chromatography (HPLC) with UV [4,5] and electrospray ionization mass spectrometry (LC–ESI-MS) [6], gas chromatography–mass spectrometry (GC–MS) [7,8] and capillary electrophoresis [9]. These methods require much time for sample preparation such as solid-phase extraction, liquid–liquid extraction, protein precipitation on the genistein analysis from biological samples. In contrast, the samples of microdialysate are protein-free, making it possible for direct injection onto the liquid chromatographic system with no sample clean-up required.

Genistein has been reported to interact with P-gp and inhibit P-gp-mediated drug transport [10]. However, Versantvoort et al. [11] reported that no effects of genistein were

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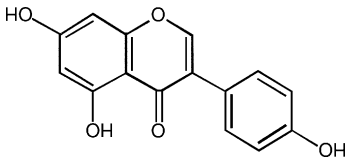

Fig. 1. Chemical structure of genistein.

observed in P-gp expressing cells. Hence, we were interested to study the interaction of genistein and cyclosporine, a P-gp inhibitor. In this study, we describe a rapid microdialysis procedure coupled with a liquid chromatographic system for the determination of unbound genistein in rat blood, brain and bile, together with its interaction with cyclosporine.

2. Experimental

2.1. Chemicals and reagents

Genistein was purchased from Sigma (St. Louis, MO, USA). Solvents and reagents of liquid chromatographic grade were obtained from E. Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. 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 were obtained from the Laboratory Animal Center of the National Yang Ming University, Taipei. The animals had free access to food (Laboratory rodent diet 5P14, PMI Feeds, Richmond, IN, USA) and water until 18 h prior to being used in experiments, at which time only food was removed. Six Sprague–Dawley rats (280–320 g) 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 genistein administration (10 or 30 mg/kg) and for treatment with cyclosporine 10 mg/kg 10 min before genistein administration (30 mg/kg). The body temperature of the rats was maintained at 37 °C with a heating pad.

2.3. Liquid chromatography

HPLC was performed with a chromatographic pump (BAS PM-80, West Lafayette, IN, USA), a Rheodyne Model 7125 injector equipped with a 20 μl sampling loop and an ultraviolet detector (Linear Model 340, San Jose, CA, USA). Separation was achieved by an Alltima phenyl column (150 mm × 4 mm i.d.; particle size 5 μm). The mobile phase consisted of acetonitrile–water (40:60, v/v, pH 3.5 adjusted by 0.1% acetic acid) at flow-rate of 1 ml/min. The detection wavelength was 259 nm. Output data from the detector were integrated using an EZChrom chromatographic data system (Scientific Software, San Roman, CA, USA).

2.4. Assay validation

All calibration curves were required to have a correlation value of at least 0.999. The intra- and inter-assay variabilities were determined by quantitating six replicates at concentrations of 0.01, 0.05, 0.1, 0.5, 1 and 5 μg/ml using the HPLC method described above on the same day and six consecutive days, respectively. 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.

2.5. Microdialysis experiment

Blood, brain and bile microdialysis systems consisted of a microinjection pump (CMA/100, Stockholm, Sweden), microdialysis probes and stereotaxic frame. The dialysis probes for blood (10 mm in length), brain (3 mm in length) and bile (7 cm in length) 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 the nominal molecular mass of 18,000, Laguna Hills, CA, USA). The blood microdialysis probe was positioned within the jugular vein toward the right atrium 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 2.5 μl/min.

The bile duct microdialysis probe was constructed in our laboratory, based on the design originally described by Scott and Lunte [14]. A 7 cm dialysis membrane was inserted into polyethylene tubing (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 silica tubing (40 mm i.d × 140 mm o.d., SGE, Australia) and PE-10 (0.28 mm i.d. × 0.61 mm o.d.), respectively. Both ends of the tubing and the union were cemented with epoxy and the epoxy was allowed to dry at least for 24 h. For post bile duct cannulation, the microdialysis probe was then perfused with Ringer's solution (147 mM Na^+; 2.2 mM Ca^{2+}; 4 mM K^+; pH 7.0) at 2.5 μl/min flow rate.

After the implantation of blood and bile microdialysis probes, the rat was immobilized in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The skull was surgically exposed, and a hole was trephined into the skull based on stereotaxic coordinates. The brain microdialysis probe was placed into the right striatum (0.2 mm anterior to bregma, 3.0 mm lateral to midline and 7.5 mm lower to tip). The brain microdialysis probe was perfused with Ringer's solution at the flow-rate of 2.5 μl/min.

2.6. Drug administration

After a 2 h post-surgical stabilization period subsequent to the implantation of probes, geniposide (10 or 30 mg/kg, i.v.) was administered via the femoral vein. The volume of each injection was 1 ml/kg. The dialysates from the blood, brain and bile were collected by a fraction collector (CMA/140) at 10 min intervals and immediately analyzed by a validated microbore HPLC system.

2.7. Recovery of microdialysate

The in vitro recovery was determined by recovery by gain (the extraction ratio) of the genistein, which was immersing the microdialysis probes in unstirred Ringer's solution containing genistein (0.1, 0.5 or 1 μg/ml) as a dialysis medium (C_{in}) at 37 °C. The bile microdialysis probe was perfused with the genistein standard solution contains 0.1, 0.5 or 1 μg/ml with flow-rate 20 μl/ml. Ringer's solution was used as perfusate at a constant flow rate of 2.5 μl/min using an infusion pump (CMA 100). The concentration of dialysate (C_{dial}) was determined by HPLC. The in vitro relative recovery (R) of genistein across the microdialysis probe was calculated by the equation $R = C_{dial}/C_{in}$.

2.8. Pharmacokinetic application

Genistein microdialysate concentrations (C_m) were converted to unbound concentrations (C_u) as follows: $C_u = C_m/R_{dial}$. Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic software WinNonlin Standard Edition Version 1.1 (Pharsight, Mountain View, CA, USA) by noncompartmental method. The area under the concentration curves (AUC), the area under the first moment curve (AUMC) and the mean residence time (MRT) were each calculated using statistical moments.

The area under the concentration–time curve (AUC) and the area under the first moment curve (AUMC) were calculated according to the log linear trapezoidal method. The clearance (Cl) and mean residence time (MRT) were calculated as follows: Cl = dose/AUC; MRT = AUMC/AUC. The blood to tissue distribution was calculated as follows: AUC_{tissue}/AUC_{blood}. All data were presented as mean ± standard error mean. Comparisons of pharmacokinetic data were performed by t-test and the statistically significant difference was set at $p < 0.05$.

3. Results and discussion

3.1. Chromatography

Separation of genistein from endogenous substances in biological dialysates were achieved in a phenyl column (150 mm × 4 mm i.d.; particle size 5 μm) and the optimal mobile phase containing 40% of acetonitrile and 60% of water (pH 3.5 adjusted by 0.1% acetic acid) with the flow rate of 1 ml/min. It can be seen that the genistein was well resolved and free of interference from endogenous substances in the rat blood, brain and bile, with retention time of 3.7 min (Figs. 2–4). Retention time was not influenced obviously by ambient temperature during the experiment. Run time for the blood and brain dialysates was 6 min.

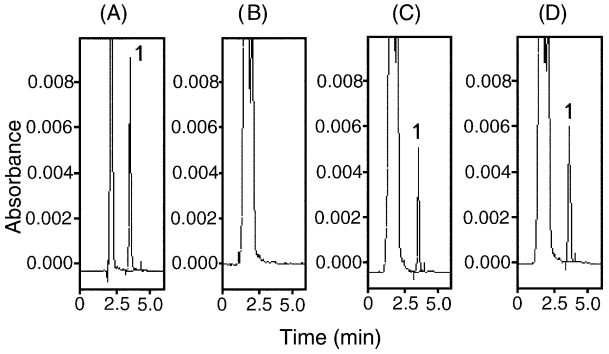

Fig. 2. Typical chromatograms of (A) standard genistein (0.5 μg/ml), (B) blank blood dialysate, (C) blood sample containing genistein (0.29 μg/ml) collected from jugular vein at 10 min after genistein administration (30 mg/kg, i.v.), and (D) chromatogram of genistein (0.32 μg/ml) 10 min after genistein (30 mg/kg, i.v.) and cyclosporine (10 mg/kg, i.v.) coadministration. 1: Genistein.

In comparison the measurement of genistein, Ksycinska et al. [12] using liquid–liquid extraction method extracts genistein from culture media. Mitani et al. [5] applied an automated on-line in-tube solid-phase microextraction method determine genistein from soybean foods. A gas chromatography coupled to mass spectrometry was used to measure genistein from serum, urine and tissue samples [13]. A solid-phase extraction method coupled to LC–ESI-MS was used to assay spiked genistein of urine with lower detection limit of 2.70 pg/ml [6]. Thomas et al. [4] developed a reversed-phase LC–UV method to investigate the genistein and its major conjugated metabolites in human plasma and urine. Sophisticated extraction methods should be required in the above methods. The microdialysis membrane excludes large molecules and thus simplifies sample clean-up procedures

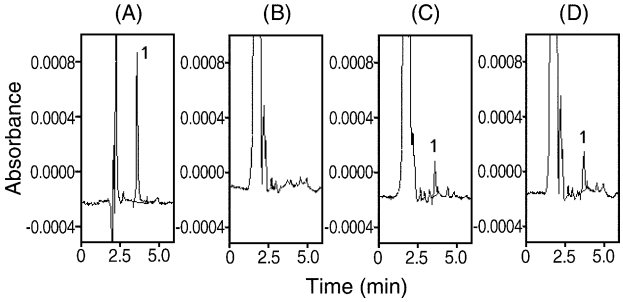

Fig. 3. (A) Chromatogram of a standard genistein (0.05 μg/ml). (B) Chromatogram of a blank brain dialysate. (C) Chromatogram of brain dialysate sample containing genistein (0.01 μg/ml) collected 20 min after genistein administration (30 mg/kg). (D) Chromatogram of genistein (0.01 μg/ml) brain dialysate 20 min after genistein (30 mg/kg, i.v.) and cyclosporine (10 mg/kg, i.v.) coadministration. 1: Genistein.

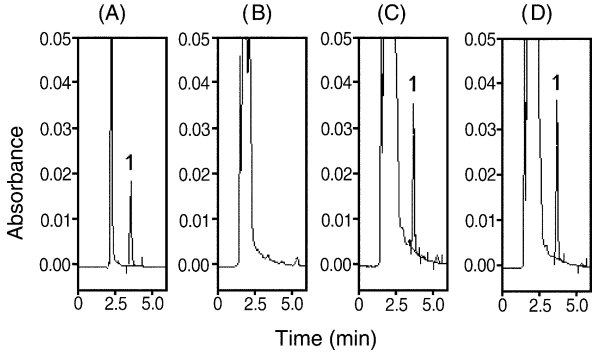

Fig. 4. (A) Chromatogram of a standard genistein (1 μg/ml). (B) Chromatogram of a blank bile dialysate. (C) Chromatogram of bile dialysate sample containing genistein (1.74 μg/ml) collected 20 min after genistein administration (30 mg/kg). (D) Chromatogram of genistein (1.89 μg/ml) bile dialysate 20 min after genistein (30 mg/kg, i.v.) and cyclosporine (10 mg/kg, i.v.) coadministration. 1: Genistein.

for continuous analysis of unbound drugs in various sites of tissues and organs [15]. The dialysates were not needed for further preparation for chromatographic analysis. In this study, microdialysis technique was applied to measure genistein from blood, brain and bile in anesthetized rats (Figs. 2–4).

Fig. 2 shows typical chromatograms resulting from dialysate of blood sample. Fig. 2A shows the standard injection of genistein (0.5 μg/ml). No interference with constituents from the blood dialysate matrix was observed in Fig. 2B. Fig. 2C shows the chromatogram of a blood dialysate sample containing genistein (0.29 μg/ml) 10 min after genistein administration (30 mg/kg, i.v.). Fig. 2D shows the chromatogram of genistein (0.32 μg/ml) 10 min after genistein (30 mg/kg, i.v.) and cyclosporine (10 mg/kg, i.v.) coadministration.

Fig. 3A shows the chromatogram of a standard genistein (0.05 μg/ml), and Fig. 3B shows the chromatogram of a blank brain dialysate. None of the observed peaks interfered with the analyte. Fig. 3C shows the chromatogram of a brain dialysate sample containing genistein (0.01 μg/ml) collected 20 min after genistein administration (30 mg/kg). Fig. 3D shows the chromatogram of genistein (0.01 μg/ml) brain dialysate 20 min after genistein (30 mg/kg, i.v.) and cyclosporine (10 mg/kg, i.v.) coadministration.

Fig. 4A shows the chromatogram of a standard genistein (1 μg/ml), and Fig. 4B shows the chromatogram of a blank bile dialysate. None of the observed peaks interfered with the analyte. Fig. 4C shows the chromatogram of a bile dialysate sample containing genistein (1.74 μg/ml) collected 20 min after genistein administration (30 mg/kg). Fig. 4D shows the chromatogram of genistein (1.89 μg/ml) bile dialysate 20 min after genistein (30 mg/kg, i.v.) and cyclosporine (10 mg/kg, i.v.) coadministration.

Cyclosporine does not demonstrate any interference peaks in the chromatogram of blood, brain and bile dialysates when genistein (30 mg/kg) was co-administered with cyclosporine (10 mg/kg).

Table 1
Standard curves of genistein assay

	Slope	Intercept	r
Day 1	7.39×10^{-6}	−0.018	0.999
Day 2	7.21×10^{-6}	−0.0079	0.999
Day 3	7.39×10^{-6}	−0.013	0.999
Day 4	7.12×10^{-6}	−0.0062	0.999
Day 5	8.03×10^{-6}	−0.0085	0.999
Day 6	7.90×10^{-6}	−1.041	0.999
Mean	7.51×10^{-6}	−0.182	0.999
S.D.	3.72×10^{-7}	−0.421	0

3.2. Validation of the method

3.2.1. Linearity

Linear least-square regression analysis of the calibration graph on six different days demonstrated linearity between the response and the nominal concentration of genistein over the range of 0.05–5.0 μg/ml. Table 1 shows the equations of the standard curves of genistein on six different days. The results of linear regression analysis show that the correlation coefficients of all standard curves were better than 0.995. The data show the reproducibility of the sample analysis.

3.2.2. Accuracy and precision

Inter- and intra-assay accuracy and precision of the analysis were less than 10% in the range of 0.05–5.0 μg/ml. The inter- and intra-assay precision and accuracy were determined at five concentrations of genistein and results are presented in Table 2.

3.2.3. Recovery for microdialysis probes

The in vitro recoveries of genistein in various types of microdialysis probes were 31 ± 1% in blood, 13 ± 1% in brain, and 59 ± 4% in bile (Table 3). Each value was evaluated by four independent microdialysis probes.

Table 2
Intra- and inter-assay precision (R.S.D.) and accuracy (bias) of the HPLC method for the determination of genistein

Nominal concentration (μg/ml)	Observed concentration (μg/ml)	R.S.D. (%)	Bias (%)
Inter-assay			
0.05	0.049 ± 0.005	10.2	−2.0
0.10	0.103 ± 0.010	9.7	3.0
0.50	0.492 ± 0.024	4.9	−1.6
1.00	1.009 ± 0.015	1.5	0.9
5.00	5.000 ± 0.00	0	0
Intra-assay			
0.05	0.050 ± 0.004	8.0	0
0.10	0.098 ± 0.002	2.0	−2
0.50	0.495 ± 0.014	2.8	−1
1.00	1.017 ± 0.037	3.6	1.7
5.00	4.982 ± 0.045	0.9	−0.4

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

Table 3
In vitro recoveries of genistein in various types of microdialysis probes

Concentrations (μg/ml)	Recovery (%)		
	Blood	Brain	Bile
0.1	33 ± 1	15 ± 1	62 ± 4
0.5	29 ± 2	12 ± 1	61 ± 4
1	31 ± 1	13 ± 1	56 ± 4
Average	31 ± 1	13 ± 1	59 ± 4

Data expressed as mean ± S.E. ($n = 4$).

3.3. Pharmacokinetic parameters

The dialysate samples collected over the first 60 min were discarded to allow for recovery from the acute effects of the surgical procedure. Figs. 5–7 show the extracellular concentrations of genistein after intravenous administration of genistein. The pharmacokinetic calculations used the non-compartment model. The pharmacokinetic parameters, as derived from these data and calculated by WinNonlin program, are shown in Table 4. The distribution ratios of genistein from bile-to-blood (AUC_{bile}/AUC_{blood}) and brain-to-blood (AUC_{brain}/AUC_{blood}) were 1.85 ± 0.42 and 0.04 ± 0.01, respectively. After treatment with cyclosporine, the distribution ratios of genistein from bile-to-blood and brain-to-blood were 1.64 ± 0.07 and 0.05 ± 0.01, respectively. These results shown that genistein may not relate to the P-gp transport system in the distribution of bile-to-blood and brain-to-blood for the dosage of genistein (30 mg/kg, i.v.).

Castro and Altenberg observed that genistein interacts with P-gp and inhibits P-gp-mediated drug transport in P-gp-expressing cell lines [10]. In contrast to the effects of P-gp expressing cell lines, genistein did not affect the P-gp multidrug resistance cell lines (SW-1573/2R160, MCF7/DOX40 and KB8-5) [11]. However, both of these in vitro observations

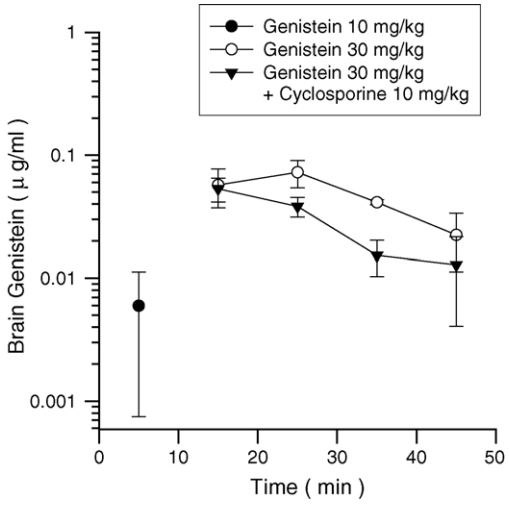

Fig. 6. Concentration–time profiles for genistein in brain after genistein i.v. administration at dosages of 10 and 30 mg/kg, with and without cyclosporine (10 mg/kg) administration. Each group of data is represented as means ± S.E.M. from six individual microdialysis experiments.

took place in the high genistein concentration of 200 μM which could not possibly occur in an in vivo system. Our data indicate that the maximum unbound levels of genistein in rat blood, brain and bile were around 1.1 ± 0.1 (3.7 μM), 0.07 ± 0.02 (0.3 μM) and 2.91 ± 0.42 μg/ml (10.8 μM), respectively after genistein administration (30 mg/kg, i.v.).

In conclusion, this liquid chromatographic method was developed and validated for the determination of protein unbound genistein in blood, brain and bile of anesthetized rat and its pharmacokinetic application. This paper also offers in vivo evidence for the properties of genistein in P-gp transporter that the P-gp may not regulate the BBB transportation and hepatobiliary excretion of genistein. The assay system

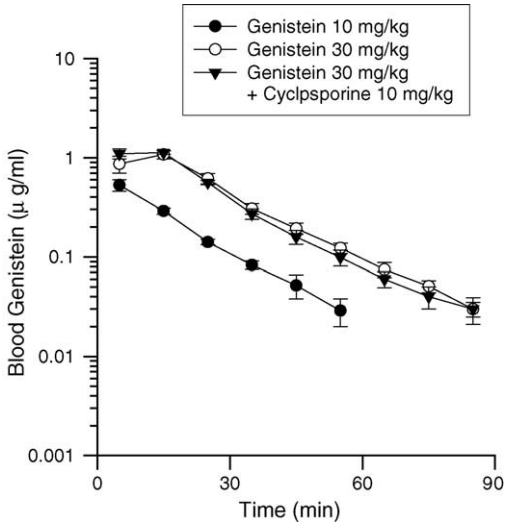

Fig. 5. Concentration–time profiles for genistein in blood after genistein i.v. administration at dosages of 10 and 30 mg/kg, with and without cyclosporine (10 mg/kg) administration. Each group of data is represented as means ± S.E.M. from six individual microdialysis experiments.

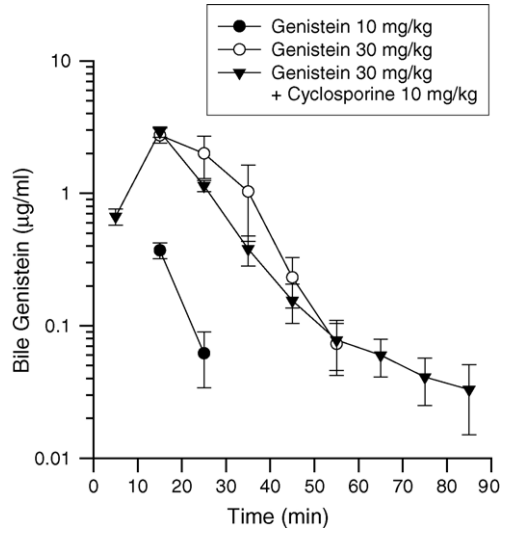

Fig. 7. Concentration–time profiles for genistein in bile after genistein i.v. administration at dosages of 10 and 30 mg/kg, with and without cyclosporine (10 mg/kg) administration. Each group of data is represented as means ± S.E.M. from six individual microdialysis experiments.

Table 4
Pharmacokinetic parameters of genistein administration (10 and 30 mg/kg) and its interaction with cyclosporine administration (10 mg/kg)

Parameters	Genistein (10 mg/kg)	Genistein (30 mg/kg)	Genistein (30 mg/kg) + cyclosporine (10 mg/kg)
Blood			
C_{max} (μg/ml)	1.0 ± 0.3	1.1 ± 0.1	1.2 ± 0.1
$t_{1/2}$ (min)	11.9 ± 1.6	15.9 ± 1.6	16.7 ± 3.4
AUC (μg min/ml)	16.2 ± 2.2	33.3 ± 3.8	34.3 ± 2.4
Cl (ml/min/kg)	642.5 ± 75.2	980.7 ± 179.8	882.8 ± 61.9
MRT (min)	16.4 ± 1.9	29.0 ± 1.6	26.5 ± 1.5
Brain			
C_{max} (μg/ml)	n.d.	0.07 ± 0.02	0.06 ± 0.01
$t_{1/2}$ (min)	n.d.	14.4 ± 2.7	7.3 ± 1.1*
AUC (μg min/ml)	n.d.	1.9 ± 0.5	1.3 ± 0.2
MRT (min)	n.d.	37.4 ± 3.0	28.7 ± 2.5
Bile			
C_{max} (μg/ml)	0.37 ± 0.05	2.91 ± 0.42	2.91 ± 0.26
$t_{1/2}$ (min)	12.7 ± 5.6	5.6 ± 0.6	10.1 ± 1.3*
AUC (μg min/ml)	7.1 ± 1.6	62.0 ± 16.8	54.5 ± 3.5
MRT (min)	23.8 ± 3.8	26.2 ± 1.5	25.2 ± 1.7
AUC_{brain}/AUC_{blood}	n.d.	0.04 ± 0.01	0.05 ± 0.01
AUC_{bile}/AUC_{blood}	0.48 ± 0.11	1.85 ± 0.42	1.64 ± 0.07

Data expressed as means ± S.E.M. ($n = 6$). n.d.: non-detectable; $t_{1/2}$: elimination half-life; AUC: area under the concentration vs. time curve; Cl: clearance; MRT: mean of residence time.
* $p < 0.05$ compare with genistein (30 mg/kg).

provides the advantages of (1) concurrent measurement of genistein in blood, brain and bile samples, (2) short run time for a single injection and (3) no requirement for further sample clean-up.

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