Pharmacokinetics of the Ginkgo B following Intravenous Administration of Ginkgo B Emulsion in Rats

Wei-Dong Chen, a,b Yan Liang, a Lin Xie, a Tong Lu, a Xiao-Dong Liu, a, b and Guang-Ji Wang a

a Key Lab of Drug Metabolism & Pharmacokinetics, China Pharmaceutical University; 1 Shennong Road, Nanjing City, 210038, China; and b The Hospital of the 17th Metallurgical Construction Company; 36 Hunan Road, Maanshan City, 243000, China. Received February 7, 2006; accepted September 12, 2006

Ginkgo B (GB) is an extract from the leaves of Ginkgo biloba, used in the treatment of dementia, cerebral insufficiency or related cognitive decline. In this paper, the main features of the pharmacokinetics of GB emulsion in rats were reviewed and the binding rate of GB to rat plasma and human plasma protein were investigated meanwhile. The concentrations of GB in plasma, tissue, and excretion of rats after i.v. administration of GB were measured using HPLC-ESI-MS. The metabolite was qualified by LC-MS/MS. Intravenously administered GB was eliminated in a biphasic manner with a prominent initial phase (half-life of 0.3 h) followed by a slower terminal phase (half-life of 1.5 h). After i.v. 4, 12 and 36 mg/kg GB emulsion, the pharmacokinetic parameters from a two compartment model analysis of plasma samples were $AUC_{0-\infty}$ (μg·min/ml): 53.7, 165.5 and 649.7; CL (l/min/kg): 0.07, 0.07 and 0.05; $V_c$ (l/kg): 2.27, 3.27 and 2.76, respectively. Peak concentrations generally occurred at 10 min except brain and fat. Tissue concentration then declined by several-fold during 6 h although still present in most tissues at 6 h. Single intravenous dose was mainly excreted in the urine (40—50%), fece contained less than 30%. The binding rate to rat plasma was little higher than to human plasma, but the difference was negligible. Some metabolites were found in urine and bile through qualitative analysis on the urine and bile by LC-MS/MS.

Key words: ginkgolide B; HPLC-ESI-MS; pharmacokinetics; metabolism

Ginkgo B (GB) has been used worldwide as a herbal medicine.1) GBE possesses many constituents, and is mainly composed of flavonoids such as quercetin and rutin, and terpenoids such as bilobalide and ginkgolides A, B and C.2) In German pharmaceutical analysis, the quality of GBE has been standardized as containing 22—27% flavonoid glycosides and 6% terpenoids.3)

In recent clinical and experimental experiments, GBE has been reported to be effective against ischemic brain injury,4,5) cerebrovascular insufficiency,6) cognitive speed,7) dementia and Alzheimer’s disease,8) peripheral vascular disease such as arterial occlusive disease9) and aging damages.10) Actually, these effects have been recognized clinically using many double-blind studies with GBE versus placebo.11—13) The mechanisms for the beneficial effects of GBE are considered to be due to the improvements of (a) haemodynamic disorders14,15); (b) PAF-associated abnormalities;16—18); and (c) decrease in ATP level during anoxia.19)

GBE modulates cytochrome (CYP) P450. GBE induced concentrations in biological samples after intravenous administration of GB were measured using LC-MS/MS. The metabolite was qualitated by LC-MS/MS. Intravenously administered GB was eliminated in a biphasic manner with a prominent initial phase (half-life of 0.3 h) followed by a slower terminal phase (half-life of 1.5 h). After i.v. 4, 12 and 36 mg/kg GB emulsion, the pharmacokinetic parameters from a two compartment model analysis of plasma samples were $AUC_{0-\infty}$ (μg·min/ml): 53.7, 165.5 and 649.7; CL (l/min/kg): 0.07, 0.07 and 0.05; $V_c$ (l/kg): 2.27, 3.27 and 2.76, respectively. Peak concentrations generally occurred at 10 min except brain and fat. Tissue concentration then declined by several-fold during 6 h although still present in most tissues at 6 h. Single intravenous dose was mainly excreted in the urine (40—50%), fece contained less than 30%. The binding rate to rat plasma was little higher than to human plasma, but the difference was negligible. Some metabolites were found in urine and bile through qualitative analysis on the urine and bile by LC-MS/MS.

Key words: ginkgolide B; HPLC-ESI-MS, pharmacokinetics, metabolism

Ginkgo B (GB) is an extract from the leaves of Ginkgo biloba, used in the treatment of dementia, cerebral insufficiency or related cognitive decline. In this paper, the main features of the pharmacokinetics of GB emulsion in rats were reviewed and the binding rate of GB to rat plasma and human plasma protein were investigated meanwhile. The concentrations of GB in plasma, tissue, and excretion of rats after i.v. administration of GB were measured using HPLC-ESI-MS. The metabolite was qualified by LC-MS/MS. Intravenously administered GB was eliminated in a biphasic manner with a prominent initial phase (half-life of 0.3 h) followed by a slower terminal phase (half-life of 1.5 h). After i.v. 4, 12 and 36 mg/kg GB emulsion, the pharmacokinetic parameters from a two compartment model analysis of plasma samples were $AUC_{0-\infty}$ (μg·min/ml): 53.7, 165.5 and 649.7; CL (l/min/kg): 0.07, 0.07 and 0.05; $V_c$ (l/kg): 2.27, 3.27 and 2.76, respectively. Peak concentrations generally occurred at 10 min except brain and fat. Tissue concentration then declined by several-fold during 6 h although still present in most tissues at 6 h. Single intravenous dose was mainly excreted in the urine (40—50%), fece contained less than 30%. The binding rate to rat plasma was little higher than to human plasma, but the difference was negligible. Some metabolites were found in urine and bile through qualitative analysis on the urine and bile by LC-MS/MS.

Key words: ginkgolide B; HPLC-ESI-MS, pharmacokinetics, metabolism

Ginkgo B (GB) is an extract from the leaves of Ginkgo biloba, used in the treatment of dementia, cerebral insufficiency or related cognitive decline. In this paper, the main features of the pharmacokinetics of GB emulsion in rats were reviewed and the binding rate of GB to rat plasma and human plasma protein were investigated meanwhile. The concentrations of GB in plasma, tissue, and excretion of rats after i.v. administration of GB were measured using HPLC-ESI-MS. The metabolite was qualified by LC-MS/MS. Intravenously administered GB was eliminated in a biphasic manner with a prominent initial phase (half-life of 0.3 h) followed by a slower terminal phase (half-life of 1.5 h). After i.v. 4, 12 and 36 mg/kg GB emulsion, the pharmacokinetic parameters from a two compartment model analysis of plasma samples were $AUC_{0-\infty}$ (μg·min/ml): 53.7, 165.5 and 649.7; CL (l/min/kg): 0.07, 0.07 and 0.05; $V_c$ (l/kg): 2.27, 3.27 and 2.76, respectively. Peak concentrations generally occurred at 10 min except brain and fat. Tissue concentration then declined by several-fold during 6 h although still present in most tissues at 6 h. Single intravenous dose was mainly excreted in the urine (40—50%), fece contained less than 30%. The binding rate to rat plasma was little higher than to human plasma, but the difference was negligible. Some metabolites were found in urine and bile through qualitative analysis on the urine and bile by LC-MS/MS.

Key words: ginkgolide B; HPLC-ESI-MS, pharmacokinetics, metabolism

Fig. 1. Structures of Ginkgolides

Ginkgolide A: R1=OH, R2=R3=H; ginkgolide B: R1=R2=OH, R3=H; ginkgolide C: R1=R3=OH, R2=H; ginkgolide J: R1=R2=OH, R3=H; and ginkgolide M: R1=H, R2=R3=OH.

* To whom correspondence should be addressed. e-mail: liangyan0679@hotmail.com

© 2007 Pharmaceutical Society of Japan
raphy was performed using a Shimadzu LC-10AD HPLC system equipped with an autosampler (SIL-HTc). The HPLC was coupled to a Shimadzu LCMS-2010A quadrupole mass spectrometer with an ESI interface. Data acquisition and processing were accomplished using Shimadzu LCMS solution Software for the LCMS-2010A system.

Chromatographic separation was achieved on a Shim-pack stainless-steel column (C_{18}, 5 μm, 150 mm×2.0 mm I.D., Shimadzu) at 40 °C. Mobile phase A consisted of 1 μmol/l ammonium acetate and 0.04% triethylamine in water and mobile phase B was methanol. All mobile phases were filtered through a 0.25 μm membrane and degassed under reduced pressure. Linear gradient elution was employed within a 10 min running time and its sequence was as follows: beginning with 40% of mobile phase A and 60% of mobile phase B, which was held up for 2.5 min, then the mobile phase A to B ratio was gradually changed to 10:90 at 3.5 min and held up to 5.0 min, followed by returning to the initial composition. Analyses were conducted at a flow rate of 0.2 ml/min. The ESI source was used in negative ionization mode. The [M−H]− ions of GB (m/z 423.15) and of 23-OH betulinic acid (m/z 471.00) were selected as ions for SIM detection. The quantification was performed using peak areas. The MS operating conditions were optimized and the optimized parameters were as follows: drying gas 1.5 l/min, CDL temperature 250 °C, block temperature 200 °C, probe voltage +4.5 kV.

**Plasma Sample Preparation** QC samples, calibration standards, and clinical plasma samples, were extracted employing a liquid–liquid extraction technique. To each tube containing 20 μl plasma, 20 μl of the 10 μg/ml solution of internal standard and 1.0 ml of diethyl ether were added, and the mixture was then vortexed for 2 min. The samples were then centrifuged for 10 min at 40000×g. The organic layer was removed and evaporated under a stream of nitrogen at 40 °C. The residue was re-dissolved in 300 μl methanol. An aliquot of 2 μl was injected into the LC-MS system.

**Single Dosing Study Design** A total of 30 (15 males, 15 females) SD rats, weighing 200±20 g, were fed on standard commercial rat pellets and were allowed access to fresh drinking water for 4 d before experiment. The study protocols were in accordance with the instructions for the care and use provided by the institution. Then, 30 rats were randomly assigned to three groups of 5 male and 5 female rats in each, weighed and injected with GB in 4, 12 and 36 mg/kg through the tail vein at a dose of 12 mg/kg. The rats were housed in restraining cages and allowed to free access to food and water. Their tissues were excised and dried with filter paper. Tissues were excised as follows: heart, liver, spleen, lung, kidney, stomach, duodenum, brain, fat (around testis or ovary), skin, skeletal muscle (femoral region), ovary and testis. One-tenth gram of each tissue was precisely weighed, placed into 1 ml of physiological saline, homogenized, and centrifuged at 10000×g for 5 min. Supernatant was processed and determined as plasma described above.

**Excretion in Bile after Single Administration** Five male and female SD rats were anaesthetized with sodium pentobarbitone. A polyethylene tube (PE-19; Clay Adams, U.S.A.) was then inserted into the common bile duct. After confirming the flow of bile, GB was injected to the rats in the tail vein at a dose of 12 mg/kg. The rats were housed in restraining cages and allowed to free access to food and water. Their bile was individually collected at 0—2, 2—4, 4—6, 6—8, and 8—12 h after administration. The amount of bile was measured and 20 μl supernatant was processed and determined as plasma described above. 2 μl extracted sample was directly injected onto the HPLC column.

**Plasma Protein Binding Determination** The plasma protein binding of GB was determined by equilibrium dialysis.12) Dialysis was carried out in a 25 ml vial with dialysis membrane (molecular weight cutoff, 10000) at 4 °C. Twenty vials filled with 10 ml phosphate buffer (0.133 mol/l phosphate, 0.9% sodium chloride, pH 7.4), were divided into four groups, and 0.05, 0.2, 1.0 and 5.0 mg/1 GB were added into the vials, respectively. Twenty dialysis membranes with two ends tied, each 5 cm-long, containing 0.5 ml blank plasma, were placed into the twenty-five vials. In a preliminary experiment, the optimum equilibrium time for GB binding to plasma protein was determined to be 90 h. Therefore, blank plasma was dialyzed for 90 h against phosphate buffer spiked with GB at 4 °C. At equilibrium, plasma and buffer samples were collected, extracted and determined as plasma samples described above. The plasma protein binding of GB was calculated according to the equation: the binding rate of GB to plasma protein (%)=(C_{in}−C_{out})/C_{in}×100%, where C_{in} is the concentration of GB inside the membrane, and Cout the concentration of GB outside the membrane.

**Metabolite Profiling—Sample Preparation** Urines and biles were pooled by route of administration and time period. To 20 μl rat urine and bile were added 1.0 ml methanol in a 2.0 ml centrifuged tube. The tubes were mixed 5 min and centrifuged for 5 min at 10000×g. Two microliters supernatant was injected into the LC-MS system.

**Preparation of Calibration Curves and Determination of Intra- and Inter-assay Precision** A stock solution containing 1.0 mg/ml GB was prepared in HPLC-grade
methanol, stored at 4 °C and prepared monthly. The required standard solutions were obtained by serial dilutions of the concentrated standard solution in methanol. Calibration curves were prepared daily by spiking duplicate drug-free rat plasma, tissue or excretion with GB, followed by extraction as described above. Quality control (QC) samples were prepared independently from the calibration curves by spiking drug-free plasma with known amounts of GB. Four different levels, corresponding to low (5 ng/ml GB in plasma), medium (20 ng/ml and 200 ng/ml in plasma) and high (2.0 µg/ml in plasma) concentrations of the QC samples were prepared. The intra-assay (within-day) precision and accuracy were assessed by analyzing six sets of quality control samples in a batch. The inter-assay reproducibility was assessed by analyzing six sets of quality control samples on three different batches.

Pharmacokinetic and Statistical Analysis Pharmacokinetic parameters were calculated for each individual experiment. The curves were imitated using BAPP program of pharmacokinetic (Key Lab of Drug Metabolism & Pharmacokinetics, China Pharmaceutical University, Nanjing, China). Pharmacokinetic parameters of GB were calculated and presented as mean values. The concentration of GB in tissues and excretion of GB were expressed as x±s.

RESULTS

Chromatography The ultrabase C18 column and the simple mobile phase used were found to be appropriate for the analysis of ginkgolide B. Under the assay conditions described, retention times for ginkgolide B and the internal standard were found 4.9 and 8.9 min, respectively. In Fig. 2, the chromatograms obtained for drug-free rat samples (plasma and liver) and post-dose rat samples obtained after intravenous administration of ginkgolide B. Results obtained showed that no endogenous interferences were observed in the blanks. Chromatograms of heart, spleen, lung, kidney, brain, muscle, stomach, duodenum, rectum, skin, fat and genitalia were similar to those of liver samples.

The concentrations of GB in plasma, tissue, and excretion samples were calculated from relative calibration lines. The calibration lines were computed using peak-area to the concentrations of GB spiked with plasma, tissue, or excretion. We decided to keep the calibration range from 5 to 1000 ng/ml and dilute the samples which concentration beyond 1000 ng/ml using corresponding blank sample. The analytical method has a limit of quantification (LOQ) for GB, defined as the lowest concentration measured with acceptable precision and accuracy, was 1 ng/ml and was linear between 5 and 1000 ng/ml. The coefficient of determination for the calibration curve (peak-area) was more than 0.999. The mean absolute percentage recoveries of GB were more than 90%, from spiked plasma, tissue, and excretion samples determined at four different concentrations of 5, 20, 200, and 1000 ng/ml (n=5). At four different concentrations of 5, 20, 200, and 1000 ng/ml, the within-run relative standard derivations (RSD) were 5.2%, 5.7%, 2.4% and 1.2% (n=6), respectively. Between 3 days, they were 10.6%, 9.7%, 5.0% and 2.1% (n=6), respectively. These data showed the method to be precise, reproducible, and suitable for the analysis of biosamples.

Pharmacokinetic Parameters of Single Dosing Figure 3 shows the decay in the concentration of GB in rat plasma as a function of time after i.v. administration of 4, 12, and 36 mg/kg. The concentration–time curves were adequately described by a two-compartment model. Pharmacokinetic parameters from a two-compartment model analysis of plasma samples for intravenous GB are summarized in Table 1. Under these experimental conditions, the elimination half-life of GB ranged from 60.3 to 95.6 min, its total body clearance from 0.05 to 0.07 l·kg⁻¹·min⁻¹ and AUC₀→∞ from

Table 1. Pharmacokinetic Parameters of GB in Rats after i.v. Administration of GB at Single Doses of GB

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Vc (l/kg)</th>
<th>T1/2α (min)</th>
<th>T1/2β (min)</th>
<th>CL (l/min/kg)</th>
<th>AUC₀→t (µg·min/ml)</th>
<th>AUC₀→∞ (µg·min/ml)</th>
<th>MRT (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>2.27±1.27</td>
<td>2.9±1.2</td>
<td>80.4±25.3</td>
<td>0.07±0.02</td>
<td>53.7±12.8</td>
<td>59.7±15.9</td>
<td>171.32±76.93</td>
</tr>
<tr>
<td>12</td>
<td>3.27±1.34</td>
<td>6.1±3.9</td>
<td>83.5±48.6</td>
<td>0.07±0.02</td>
<td>165.5±29.9</td>
<td>170.5±33.2</td>
<td>101.61±23.39</td>
</tr>
<tr>
<td>36</td>
<td>2.76±1.05</td>
<td>4.9±5.5</td>
<td>85.6±27.9</td>
<td>0.05±0.01</td>
<td>649.7±91.8</td>
<td>676.5±99.4</td>
<td>127.29±14.36</td>
</tr>
</tbody>
</table>
59.72 to 676.47 μg·min⁻¹·l⁻¹.

**Pharmacokinetic Parameters of Multiple Dosing** The minimum serum concentrations from days 5 to 7 were analyzed (see Table 2) and similar concentrations were found when 12 mg/kg of GB were administrated, therefore we can say that steady state was achieved before 5th day. Pharmacokinetic parameters from a two-compartment model analysis of plasma samples are summarized in Table 3. The mean degree of accumulation of GB was consistent with the degree of accumulation of GB was consistent with the dose frequency and was not considered relevant.

**Distribution of GB in Rats** Table 4 shows the concentrations of GB in rat tissues after i.v. administration of GB at a dose of 12 mg/kg. The experimental results demonstrated that after i.v. administration GB, greatest concentrations of GB in all tissues except brain and fat reached a peak at 10 min. The concentration levels in many tissues such as heart, liver, kidney, stomach and duodenum were approximately 1.5—5 times greater than in plasma for all the time, and the highest concentration was observed in liver. As the time increased, the concentrations of GB rapidly decreased from each tissue by several folds (by 20—50 times), in which the drug distribution rate decreased gradually.

**Excretion in Urine, Feces and Bile** The cumulative excretion ratios of GB in the urine and feces after i.v. administration of GB at a dose of 12 mg/kg were 45.41% and 25.95%, respectively, of the total dose by 48 h after dosing. Excretion of GB into bile was a little fraction, and almost completed within 4 h after administration, with cumulative excretion ratio of 0.47% of the dosed GB. The majority of GB was excreted within 24 h after administration.

**Plasma Protein Binding of GB** Using equilibrium dialysis method, the binding rates of GB to rat and human plasma protein were found to be 21.09—26.70% and 17.99—21.49%, respectively, varying with the concentration of GB from 0.05 to 5.0 mg/l (Table 6). The binding rate to rat plasma was little higher than to human plasma, but the difference was negligible (p>0.05).

**Metabolite Profiles** Urine: After intravenous doses, many new components were scanned by LC-MS/MS. The mass spectrum of the major new components had molecular ions (m/z 441 t_R = 3.0 min, m/z 457 t_R = 3.6 min), of which accounted for more than 15% (see Fig. 4).

Bile: In the 0—4 and 4—12 h biles of i.v. dosed rats, the components (m/z 441 t_R = 3.0 min, m/z 457 t_R = 2.7 min) were detected by LC-MS/MS. The mass chromatograms of the new component were shown in Fig. 5. The metabolism study of GB is being undertaken in our Lab and the experimental results will be reported afterwards.
DISCUSSION

The proposed analytical method provided a rapid, sensitive and specific assay for GB determination in biological samples. A simple liquid–liquid extraction procedure and a short run time limited the total analysis time, and this is important for large sample batches. It was shown that this method is suitable for the analysis of GB in pre-clinical and clinical studies.

The pharmacokinetic studies have shown that, after i.v. administration to rats, elimination of GB dose range from 4 to 36 mg/kg. The linear and dose-independent pharmacokinetics of GB indicate that the processed leading to elimination of GB are not saturated. When comparing the \( AUC \), \( C_{\text{max}} \) between single dosing and multiple dosing, no significant difference was found. Furthermore, GB was well tolerated when administered at doses of 12 mg/kg in this group of rats. No serious adverse events were observed.

GB in plasma was thought to be eliminated mainly by uptake into liver. After i.v. administration of GB to rats, the highest concentration of GB appear in the liver all the time. Sixty minutes after i.v. administration of GB to rats, the concentration of GB in brain was 148.9±45.6 ng/ml, we can conclude that GB can quickly transport the blood brain barrier, which conform to the treatment of cerebrovascular pharmacodynamic action of GB.

The dosed GB was mainly excreted into urine in unchanged form, and about 2.5% of the dosed GB into feces. This indicated that more than 50% of GB was finally eliminated in metabolite form. The metabolism study of GB is being undertaken in our lab and the experimental results will be reported afterwards.

The protein binding ratio of GB in the plasma is about 20%, in other words, the unbound fraction is around 80%. That is the reason why the urinary excretion pathway is the main elimination route.

In summary, pharmacokinetic research results show that GB has a great potential clinic value. The distributional properties of GB in the present rats study are beneficial to its cerebrovascular pharmacodynamic action.

REFERENCES