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Transport characteristics of ginkgolide B by Caco-2 cells and examination of ginkgolide B oral absorption potential using rat in situ intestinal loop method

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Abstract

The intestinal absorptive characteristics of ginkgolide B were investigated. The Caco-2 cells and the in situ closed loop were used as models of the intestinal mucosa to assess transepithelial transport of ginkgolide B. The determination of ginkgolide B was performed by HPLC–MS. In the Caco-2 cells, the absorptive transepithelial transport of ginkgolide B was pH dependent and the transport was enhanced at weakly acidic pH on the apical side. No concentration dependence and saturation were observed for the absorptive transport of ginkgolide B at concentrations up to 50 μM. In the in situ closed loop, the absorption of ginkgolide B was intestinal segment-selective. The results indicate that the intestinal absorption of ginkgolide B in the upper intestine was significantly higher than that in the lower intestine.

Keywords: Ginkgolide B; Intestinal absorption; Caco-2 cells; In situ closed intestinal loop

1. Introduction

Ginkgo biloba (Ginkgoaceae) is an ancient Chinese tree, which has been cultivated and held sacred for its health-promoting properties (Maclennan et al., 2002). Over the last decade, substantial scientific evidence has been accumulated which suggests that concentrated and partially purified extracts of *Ginkgo biloba* leaves afford protection against some kinds of neural and vascular damage and treat cognitive deficits and other age-associated impairments (Le Bars et al., 1997; Kanowski et al., 1996). Ginkgolides are a unique group of diterpenes that exist naturally in the leaves of the *Ginkgo biloba* tree. The Insitiute Henri Beaufour uses the nomenclature BN-52020, BN-52021 and BN-52022 to refer to ginkgolides A, B and C, respectively; ginkgolide B is by far the most potent platelet activating factor (PAF) receptor antagonist (Biber and Koch, 1999). And there are many possible clinical applications of ginkgolide B. In particular, ginkgolide B has been shown to protect against neural damage in a variety of circumstances. In addition, it has been shown to have beneficial effects on circulatory and inflammatory conditions (Maclennan et al., 2002).

Due to the presence of three lactone rings (Fig. 1), ginkgolides could ionize easily at physiological pH or above (Van Beek, 2005). Recently, in order to increase the stability of lactone ring, the lactone rings of ginkgolide A were converted into corresponding tetrahydrofuran moieties via DIBAL-H reduction followed by deoxygenation of the formed lactols (Ishii et al., 2005).

Frequently, drug molecules with ionizable groups have only a narrow absorption window, and are absorbed only in that segment of the gastrointestinal tract where the unionized form constitutes a significant fraction of the drug molecules. In such cases, it is difficult to determine from in vivo studies that the poor absorption of a drug is due to ionization or due to poor intrinsic permeability of the uncharged drug, or both (Liang-Shang et al., 1997; Sha and Fang, 2004).

Oral sustained release formulations could guarantee therapeutic plasma drug levels for at least 24 h, thereby, improving the patients' compliance by allowing once-daily oral administra-

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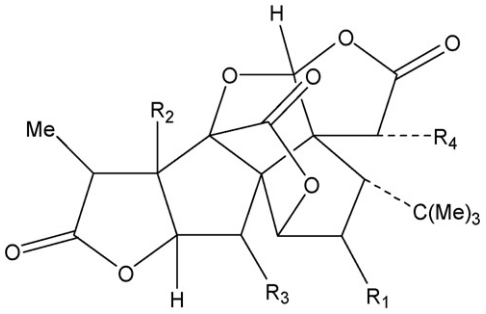

Fig. 1. The chemical structures of the compounds studied.

tion. An absorption enhancement strategy to design a sustained release preparation of ginkgolide B has to be developed based on its transport mechanism. Therefore, intestinal absorption and the good absorption position are of specific importance and should be investigated to direct the design of dosage form of ginkgolides.

To develop a theoretic basis for understanding the oral absorption of ginkgolide B, we designed to investigate the intestinal absorption of ginkgolide B using Caco-2 cells and in situ closed loop (Mikihisa et al., 2004; Saffar et al., 2005).

2. Materials and methods

2.1. Materials

Ginkgolide B was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). [^{14}C] Mannitol (0.2 μCi/μL) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), defined fetal bovine serum was purchased from Hyclone (UT, USA), Hank's balanced salt solution (HBSS), *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), trypsin ethylene diamine tetraacetic acid (EDTA), Dulbecco's modified Eagle's medium (DMEM), nonessential amino acid solution, L-glutamine and penicillin–streptomycin were purchased from Gibco BRL(Grand Island, NY, USA). Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Distilled, deionized water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA).

2.2. Animal studies

Male Sprague–Dawley rats (220–300 g) were obtained from Sino-British Sippr/BKLab Animal Ltd. (Shanghai, China) and were fasted overnight with free access to water. Rats were anaesthetized with pentobarbital (30 mg/kg) intraperitoneally. The rats were placed on a surgery table in a supine position and kept at 37 °C. Animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of China Pharmaceutical University (Nanjing, China) and protocol was approved by the Animal Ethics Committee of this institution.

2.3. Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown routinely in 75 cm^2 plastic culture flasks. The culture medium consisted of DMEM containing 4.5 g/L D-glucose and 3.7 g/L $NaHCO_3$, supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids and 1% penicillin–streptomycin at 37 °C under an atmosphere of 5% CO_2 and 90% relative humidity. The medium was replaced every 2–3 days after incubation. Cells were passaged approximately every 5 days (at 80% confluence) using trypsin–EDTA at a split ratio of 1:5. For the transport experiments, cells were seeded at a density of 1×10^5 cells/well on permeable polycarbonate inserts (Transwell cell culture inserts, Millipore, USA) in 24-well tissue culture plates (Costar). The inserts were fed every 2 days for the first week and then daily until they were used for experiments 21–24 days after seeding. Cell passages between 24 and 30 days were used in the experiment. The integrity of the cell monolayers was evaluated by measuring transepithelial electrical resistance (TEER) values with an EVOM^{TM} epithelial volt ohmmeter (World Precision Instrument, Sarasota, FL) and [^{14}C] mannitol transport. The cell inserts were used in the experiments when the resistance exceeded 600 Ω cm^2. The permeability of mannitol was determined to be <0.3% of the dose/h, corresponding to a P_{app} value of 4×10^{-7} cm/s, which indicated that cell monolayers were tight.

2.4. Ginkgolide B transport studies

Transport of ginkgolide B across the Caco-2 cell monolayers was studied using monolayers 21–24 days post-seeding. Before the experiments, the monolayers were washed twice with HBSS containing 25 mM HEPES (pH 7.4). After washing, the monolayers were preincubated at 37 °C for 20 min, and TEER was measured. HBSS solution on both sides of the cell monolayers was then removed by aspiration. For the measurement of the apical (AP) to basolateral (BL) transport, 0.4 mL of HBSS (pH 6.5) containing ginkgolide B (3.125–50 μM) was added to the AP side, and 0.6 mL blank HBSS (pH 7.4) was added to the BL side. The monolayers were placed in an incubator at 37 °C, and shaken at 50 rpm during transport process to minimize the influence of the aqueous boundary layer. Samples were taken from the receiving chamber at 15, 30, 60, 90 and 120 min followed by an immediate replacement of the same volume of prewarmed fresh HBSS.

The effect of apical pH (5.0–8.0) on the transport of 25 μM ginkgolide B from the apical to the basolateral side was examined under constant pH of the basolateral side (pH 7.4).

2.5. Intestinal absorption of ginkgolide B from in situ closed loop

A closed loop in situ experiment was performed to study the absorption of ginkgolide B from the duodenum to colon. The desired segment (the first 10 cm distal to the pylorus was regarded as the duodenum, the next 10 cm was regarded as the jejunum, the 10 cm proximal to the caecum was regarded as the ileum and the segment distal to caecum was regarded as the colon) was pulled out by measuring with a thread,

and a small incision was made at the distal and proximal ends to allow washing of the intestinal luminal contents with normal saline warmed to 37 °C. The segment was carefully ligated both above and below the incisions to form a closed loop. The 10 μM ginkgolide B was administered to the loop, and the segment was returned to its original place inside the peritoneal cavity and sutured. Blood samples (200 μL) were collected by orbital sinus bleeding at 0, 5, 10, 20, 30, 45, 60, 90 and 120 min. The plasma was immediately separated by centrifugation (7000 rpm for 5 min) and frozen until analysis.

2.6. Analysis of the samples

2.6.1. HPLC–MS instrumentation

Shimadzu LC–MS 2010A liquid chromatography–mass spectrometry equipment (including two LC-10ADvp pumps, a constant temperature automatic sampler, a quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface source and LC–MS solution chromatography workstation), and a six-port switching valve were used. Chromatographic separation was achieved on a Shimadzu C_{18} column. The column temperature was maintained at 40 °C. The mobile phase consisted of methanol–0.01% of ammonia water (with a gradient elution mode) at a flow-rate of 0.2 mL/min. The mass spectrometer was operated in the negative mode. Quantitation was performed using a selected ion monitoring (SIM) mode of *m*/*z* 423 (Lv et al., 2006).

2.6.2. Sample preparation

The transport sample or the plasma sample (100 μL) was added to a 1 mL plastic test tube together with 10 μL of IS solution. After vortex mixing for 30 s, 500 μL ether was added. The analyte and IS were extracted from transport sample or plasma by vortexing for 5 min. Then the sample was centrifuged at 2000 × *g* for 10 min. The organic layer was quantitatively transferred to a 1 mL plastic test tube and evaporated to dryness using evaporator at 45 °C. Then the dried extract was reconstituted in 100 μL methanol and 10 μL aliquot was injected into chromatographic system.

2.7. Calculations

Apparent permeability coefficients (P_{app}) of ginkgolide B was calculated in both AP to BL and BL to AP directions according to the equation: $P_{app} = (dQ/dt)/A \times C_0$ where the dQ/dt (μM/min) is the drug permeation rate, A is the cross-sectional area (0.6 cm^2) and C_0 (μM) is the initial ginkgolide B concentration in the donor compartment at $t = 0$ min. Permeability rates (dQ/dt) were calculated by plotting the amounts of drug transported to the BL side versus time and determining the slope of these plots. The permeability rates were then plotted versus the initial concentrations (C_0) to obtain the value of the slope, $(dQ/dt)/C_0$. The correlation coefficients (r^2) obtained from the least-squares linear regression analysis were in the range of 0.97–1.00.

2.8. Statistical analysis

Results are given as mean ± S.D. Statistical significance was tested by two-tailed Student's *t*-test or one-way ANOVA. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Absorptive transport of ginkgolide B across Caco-2 cell monolayers

The influence of ginkgolide B concentration on the AP to BL transport of ginkgolide B across the Caco-2 cell monolayers was measured. No concentration dependence or saturation was observed for the absorptive transport of ginkgolide B in the concentration range of 3.125–50 μM. The absorption P_{app} (AP–BL) of ginkgolide B had no change over all concentration range (Fig. 2A). The absorptive flux increased linearly with increasing of ginkgolide B concentration (3.125–50 μM) indicated a simple passive diffusion pathway for the transport of ginkgolide B across Caco-2 cell monolayers (Fig. 2B).

3.2. Effect of pH on the transport of ginkgolide B across Caco-2 cell monolayers

The transepithelial transport of ginkgolide B across Caco-2 cell monolayers was examined at pH 5.0, 6.5, 7.0, 7.4, 8.0 on the

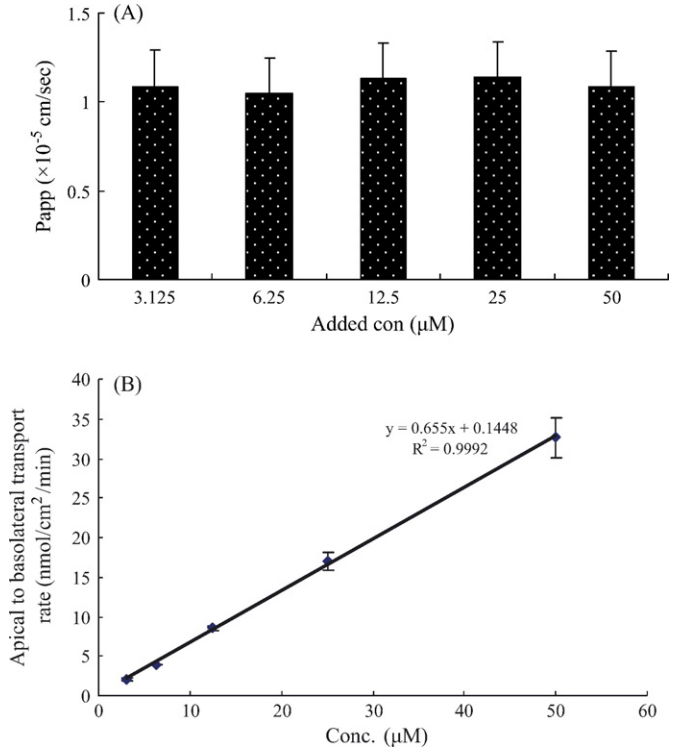

Fig. 2. (A) Concentration in dependence of the apical to basolateral apparent permeability (P_{app}) of ginkgolide B across Caco-2 cell monolayers ($n = 4$). (B) Transport rate of apical to basolateral of ginkgolide B across Caco-2 cell monolayers ($n = 4$).

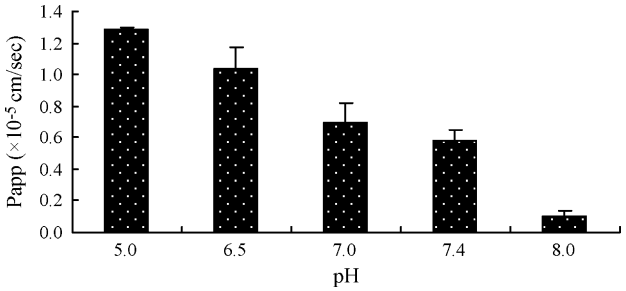

Fig. 3. Effect of pH on P_{app} of ginkgolide B ($n = 4$).

apical side, and at pH 7.4 on the basolateral side. As the result of preliminary study, the apical to the basolateral transport of ginkgolide B showed linearity within the range of 3.125–50 μM concentration. The following studies were performed at 25 μM of ginkgolide B concentration. Effect of apical pH on the transepithelial transport is shown in Fig. 3. When ginkgolide B was added to the apical compartment at pH 5.0, the accumulation transport amount and P_{app} of the apical to the basolateral were significantly higher than other pH on the apical side (about 10-fold greater than those at apical pH 8.0). The transepithelial transport of gingkoglide B is pH dependent. We, therefore, performed the following experiments using the apical side medium at pH 5.0. (Table 1).

3.3. Intestinal absorption of ginkgolide B evaluated by in situ loop method

The intestinal absorption of ginkgolide B was examined by the in situ closed loop method. A solution containing ginkgolide B (10 μM) was administered into four different intestinal segments, and concentration of ginkgolide B in the plasma was measured by LC–MS over time. As shown in Fig. 4A, ginkgolide B concentration in the plasma increased gradually with time and reached an almost constant level at about 1 h. The plasma concentration of ginkgolide B and the area under the concentration–time curve ($AUC_{0–120\,min}$) after administration into the upper intestine were much higher than those after administration into the lower intestine ($AUC_{0–120\,min}$: duodenum, 10.43 ± 2.80 μmol min/mL; colon, 1.20 ± 0.10 μmol min/mL, $^{**}P < 0.01$) (Fig. 4B).

4. Discussion

In this study, we have characterized the transport of ginkgolide B across Caco-2 cell monolayers. High apparent permeability coefficient, which remained unchanged throughout the concentration range studied, indicates that transport of ginkgolide B across Caco-2 monolayers in the AP to BL direction occurs predominantly via passive route.

Table 1
Effect of apical pH on P_{app} of ginkgolide B across Caco-2 cell monolayers ($n = 4$)

Added concentration (μM)	P_{app} ($\times 10^{-5}$ cm/s)	
	$\bar{X}$	S.D.
5	1.29	0.01
6.5	1.04	0.14
7	0.69	0.14
7.4	0.59	0.06
8	0.11	0.03

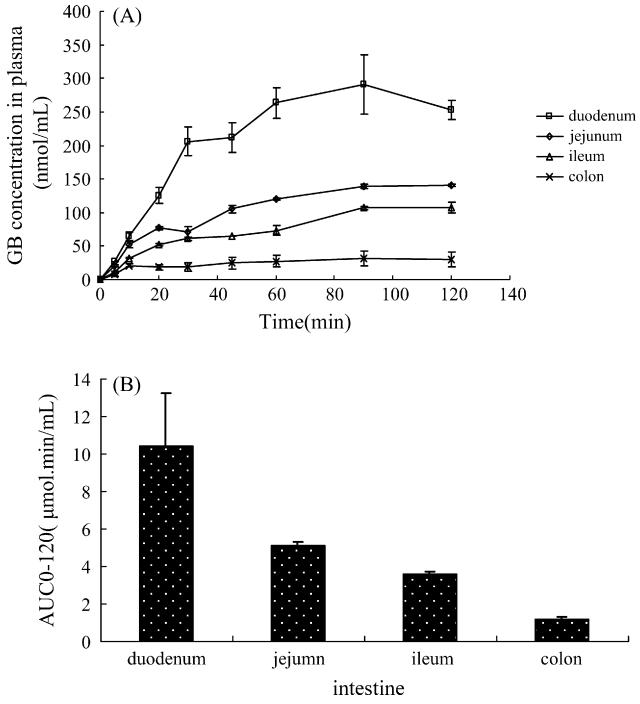

Fig. 4. (A) Absorption of GB from four different intestines evaluated by in situ closed loop method. (B) The area under the concentration–time curve ($AUC_{0–120\,min}$) in four different intestines evaluated by in situ closed loop method.

A good correlation between the transport across Caco-2 cell monolayers and absorption in humans has been obtained for several compounds. The high P_{app} across Caco-2 cell monolayers of ginkgolide B (more than 1×10^{-5} cm/s) suggests that it may be absorbed well from the small intestine in the human.

In the present study, the transepithelial transport of ginkgolide B across Caco-2 cell monolayers showed pH dependence and was enhanced at weakly acidic pH on the apical side. The explanation for the result is that ginkgolide B is hydrolyzed under physiological condition, i.e., at pH 7 or above, with the lactone ring readily opened to yield the inactive carboxylate form of ginkgolide B. First, the carboxylate form displays decreased membrane associations. Secondly, ring opening results in a charged drug species, which exhibits limited diffusibility through cell lipid bilayer domains of low dielectric constant.

To further study the transport mechanism of ginkgolide B, the absorptive transport of ginkgolide B was investigated in in situ closed loop model. In situ closed loop technique is a simple and effective method to study the drug absorptive transport in the different intestine. Intestinal pH rises along the small intestine to large intestine. The duodenum and upper intestine is more acidic than the lower intestine. In in situ closed loop, absorption of ginkgolide B from the upper intestine was much higher than from the lower intestine. The plasma concentration of ginkgolide B and the area under the concentration–time curve ($AUC_{0–120\,min}$)

after administration into the upper intestine were much higher than after administration into the lower intestine ($AUC_{0-120\,min}$: upper (duodenum), 10.43 ± 2.80 μmol min/mL; lower (colon), 1.20 ± 0.10 μmol min/mL, $^{**}P < 0.01$). These results suggest that the lower absorption of ginkgolide B in the lower intestine may be due to the poor transport rate of the dianionic form of the drug at high pH values.

In conclusion, the results presented here show that passive membrane diffusion dominates the absorptive transport behavior of ginkgolide B and the pH of the intestine is the critical factor for the absorption of ginkgolide B. In the upper intestine, which has low pH, the absorption of ginkgolide B was significantly higher than that in the lower intestine, which has high pH. Therefore, attempts have been made to develop a sustained release preparation of ginkgolide B to enhance the absorption of ginkgolide B by releasing the drug into the upper intestine with reduction in the ionization of the drug molecules in the lower intestine.

Acknowledgements

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