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Proliposomes containing a bile salt for oral delivery of *Ginkgo biloba* extract: Formulation optimization, characterization, oral bioavailability and tissue distribution in rats

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ABSTRACT

Proliposomes containing a bile salt were developed to improve the oral bioavailability of *Ginkgo biloba* extract (GbE). GbE loaded proliposomes (P-GbE) were successfully prepared by spray drying method. The formulation was optimized using the response surface methodology. FE-SEM, DSC, and FT-IR were used to study the surface morphology and molecular state of proliposomes, and demonstrated key interactions between the formulation ingredients. *In vitro* studies showed delayed release and enhanced dissolution of *Ginkgo* flavonoids and terpene lactones from GbE proliposomes. Proliposomes significantly enhanced GbE absorption in the gastrointestinal tract and decreased its elimination. The bioavailabilities of quercetin, kaempferol, isorhmnetin, ginkgolide A, ginkgolide B and ginkgolide C from proliposomes relative to the control were 245%, 211%, 264%, 203%, 333%, and 294%, respectively. Proliposomes were shown to selectively deliver GbE to critical target tissues. In conclusion, development of proliposomes formulation for GbE solved the problem of its poor oral bioavailability, prolonged its duration of action, and increased drug distribution in critical tissues, especially in the brain, therefore, warrant further investigation.

1. Introduction

Ginkgo biloba, a medicinal plant, has been used for the treatment of bronchial asthma and pulmonary conditions in China since ancient times (Gong et al., 2008). *G. biloba* extract (GbE), isolated from the dried leaves of *G. biloba*, has been reported to have many beneficial activities, including free radical scavenging (Wang et al., 2010), antioxidative (Boghdady, 2013), anti-inflammatory (Chen et al., 2013b), anti-tumor activities (Tsai et al., 2014), and neuroprotective effects (Abd-Elhady et al., 2013). GbE has become one of the best-selling botanical supplements in many European countries and in the USA for the prevention and adjunctive treatment of vascular and cognitive diseases, such as Alzheimer's disease (Oken et al., 1998; Tchantchou et al., 2007), vertigo (Hamann, 2007; Sokolova et al., 2014), tinnitus (Hilton et al., 2013; Tziridis et al., 2014), and peripheral arterial occlusive diseases (Unger, 2013; Wang et al., 2007). The flavonoids and terpene lactones are believed to be key active ingredients that are responsible for the pharmacological functions of GbE (Singh et al., 2008). Standardized EGb761 contains 22–27% flavonoids (glycosides of quercetin, kaempferol, and isorhamnetin) and 5–7% terpene lactones (bilobalide, ginkgolide A, ginkgolide B and ginkgolide C) (Mesbah et al., 2005). However, with regard to the oral administration of marketed GbE products, there remains a critical challenge in the low oral bioavailability of the active components of GbE, *Ginkgo* flavonoids and terpene lactones, especially glycosides of quercetin, kaempferol and isorhamnetin (Drago et al., 2002). Low lipid and water solubility, and high first pass metabolism of flavonoids (Barve et al., 2009) may have been the main reasons.

Several novel formulations have been developed to enhance the oral bioavailability of GbE, including GbE hybrid liposomes (Yamamoto et al., 2002), GbE50 proliposomes (Li et al., 2007), GbE phospholipid complexes (Chen et al., 2010), GbE niosomes (Jin et al., 2013), GbE solid dispersion (Wang et al., 2014), etc. Among them, formulations based on liposomes have shown the greatest potential because they can entrap both lipophilic and hydrophilic components of GbE, and they have excellent biocompatibility. Liposomes are lipid bilayers vesicles, composed of phospholipid and cholesterol. They can be prepared by many

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techniques including thin film hydration, organic solvent injection, reverse phase evaporation and so on. Although liposomes have been studied as an oral drug delivery system for many years, some problems remains to be addressed. Liposomes are unstable under the harsh conditions found in the gastrointestinal tract. These include low pH, high phospholipases and high concentrations of bile salts. Bile salts play a key role on the destruction of liposomes (Andrieux et al., 2009; Hermida et al., 2009). Interestingly, studies have shown that the insertion of bile salts into lipid bilayers could actually stabilize the membrane structure in the gastrointestinal environment (Conacher et al., 2001; Mann et al., 2006). Therefore, liposomes containing bile salts have been developed. The primary mechanisms by which this formulation enhance the delivery of insoluble drugs in the gastrointestinal tract are as follows: (1) lipid vesicles can adhere to the mucosal membrane, increasing absorption; (2) drugs entrapped in lipid vesicles can be absorbed by endocytosis; (3) addition of physiological bile salts into lipid bilayers can further enhance absorption by changing the phase transition behavior of lipid vesicles (Chen et al., 2009; Jain et al., 2014). However, the application of liposomes containing bile salts in oral delivery has been limited by a number of factors including drug leakage, vesicle aggregation and sedimentation, and phospholipid hydrolysis and oxidation. A proliposome formulation is adopted in the current study because it provides a key breakthrough in addressing these issues (Janga et al., 2012; Song et al., 2005; Xiao et al., 2006; Xu et al., 2009).

In this study, GbE proliposomes (P-GbE) containing sodium deoxycholate (NaDC) were successfully prepared by spray drying method and were characterized. The composition of P-GbE was optimized using the Box-Behnken design (BBD) and response surface methodology (RSM). Field emission-scanning electron microscope (FE-SEM), differential scanning colorimetric (DSC) and fourier transform infrared (FT-IR) spectroscopy were used to evaluate the solid state characteristics of P-GbE. Oral bioavailability and tissue distribution were studied in rats.

2. Materials and methods

2.1. Materials

G. biloba extract (GbE) was a gift from Yangtze River Pharmaceutical Group (Jiangsu, China) and its contents of quercetin, kaempferol, isorhamnetin, bilobalide, ginkgolide A, ginkgolide B and ginkgolide C in GbE were ~5.4%, 3.2%, 2%, 3.2%, 2.6%, 1.6% and 1.4%, respectively. Egg yolk phosphatidylcholine (ePC), the purity of which was over 80%, was purchased from the Guangzhou Hanfang Modern Chinese Medicine Research and Development Co. Ltd. Ethyl acetate (EtOAc, 99.9%), acetonitrile (HPLC-grade), methanol (HPLC-grade), formic acid (HCOOH, 99.9%), phosphoric acid (HPLC-grade) were obtained from Sigma–Aldrich Co. (MO, USA). Sodium deoxycholate (NaDC), mannitol, anhydrous ethanol, dichloromethane, hydrochloric acid, and other chemical reagents of analytical grade or better were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China).

The reference standards for *Ginkgo* flavonoids (quercetin, kaempferol and isorhamnetin), terpene lactones (bilobalide, ginkgolide A, ginkgolide B and ginkgolide C), and taxifolin (IS) were acquired from the National Institute for Food and Drug Control of China.

2.2. Preparation of GbE proliposomes

A spray drying method was used in this study for the preparation of proliposomes. Briefly, egg yolk phosphatidylcholine (ePC) was dissolved in anhydrous ethanol to obtain a lipid solution. *G. biloba* extract (GbE) and sodium deoxycholate (NaDC) were dissolved in ethanol/water (v/v, 1:10) with sonication to obtain a drug solution. The lipid solution was injected quickly using a peristaltic pump into the drug solution under stirring at 60 °C to obtain a liposome suspension. Then, manntiol was added as a carrier and the suspension was stirred for 5 min to dissolve it completely before the spray drying procedure. The suspension was stirred continuously to ensure its homogeneity during spray drying. The inlet temperature was set at 130 °C with a feed rate of 450 ml/h. The proliposomes product was transferred from the collecting chamber into a desiccator until use.

2.3. Optimization of proliposomes formulation

Based on the single-factor test, three factors that affected the drug entrapment efficiency (EE), namely the amount of ePC (X_1), the amount of NaDC (X_2), and the amount of manntiol (X_3) were selected to further optimize the composition of GbE proliposomes (P-GbE). The Box-Behnken design (BBD) and response surface methodology (RSM) were employed to analyze the three factors. The design scheme was composed of 15 experimental groups selected by three-factor, three-coded level BBD (Zhao et al., 2012). The experimental variables are shown in Supplementary Table 1. Furthermore, the relationship among the three factors was described by plotting the response surface by fitting the data to an equation. Finally, a number of experiments were carried out to validate the optimal composition.

2.4. Determination of entrapment efficiency

Entrapment efficiency (EE) is a key parameter of P-GbE (Yang et al., 2012). The EE of total *Ginkgo* flavonoids was selected to represent P-GbE, and the detection method used was as follows. Firstly, 0.2 g of P-GbE was added into 50 ml of dichloromethane and the resultant suspension was sonicated for 5 min before vacuum filtration. Then, the filtrate was dried in a water bath at 80 °C. Then, 20 ml methanol/25% hydrochloric acid (4:1) was added and the solution was heated to reflux for 30 min. After cooling to room temperature, the solution was transferred into a 25 ml volumetric flask and diluted with methanol to the appropriate volume and mixed. Finally, 20 μl of the resulting solution was injected into an HPLC system. Based on its low solubility in dichloromethane, the unentrapped *Ginkgo* flavonoids could be removed by vacuum filtration. The amounts of *Ginkgo* flavonoids in P-GbE before (B) and after vacuum filtration (A) were used to calculate the *EE* (%) using the following equation:

$$EE\ (\%) = (A/B) \times 100$$

This method was validated by applying filtration to a physical mixture of GbE, ePC, NaDC and mannitol. The concentrations of quercetin, kaempferol and isorhamnetin in the filtrate were undetectable. This validates the filtration method for the removal the unentrapped *Ginkgo* flavonoids.

2.5. Measurement of particle size and zeta potential of the reconstituted liposomes

The liposomes suspension was reconstituted by mixing appropriate a proliposome powder with deionized water followed by vortexing for 2 min. A Nicomp Zeta Potential/Particle Sizer (Model 380 ZLS, Santa Barbara, California, USA) was used to measure particle size and zeta potential (Yang et al., 2014). The measurement was made at 23 °C with a viscosity setting of 1.333 cP. Particle size values were obtained in the mode of intensity-weighted Gaussian size distribution.