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Xingnaojing mPEG$_{2000}$-PLA modified microemulsion for transnasal delivery: pharmacokinetic and brain-targeting evaluation

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Abstract
Xingnaojing microemulsion (XNJ-M) administered intranasally is used for stroke treatment. In order to decrease the XNJ-M-induced mucosal irritation, XNJ-M modified by mPEG$_{2000}$-PLA (XNJ-MM) were prepared in a previous work. The present work aimed to assess the impact of mPEG$_{2000}$-PLA on pharmacokinetic features and brain-targeting ability of XNJ-M. The bioavailability and brain-target effects of borneol and geniposide in XNJ-M and XNJ-MM were compared in mice after intravenous (i.v.) and intranasal (i.n.) administrations. Gas chromatography, high-performance liquid chromatography, and ultra-performance liquid chromatography/tandem mass spectrometry methods were developed for the quantification of borneol and geniposide. Blood and brain samples were collected from mice at different time points after i.v. and i.n. treatments with borneol at 8.0 mg/kg, geniposide at 4.12 mg/kg. In addition, near-infrared fluorescence dye, 1,1-dioctadecyl-3,3,3,3-tetramethyl indotricarbocyanine iodide was loaded into microemulsions to evaluate the brain-targeting ability of XNJ-M and XNJ-MM by near-infrared fluorescence imaging in vivo and ex vivo. For XNJ-M and XNJ-MM, the relative brain target coefficients (Re) were 134.59% and 198.09% (borneol), 89.70% and 188.33% (geniposide), respectively. Besides, significant near-infrared fluorescent signal was detected in the brain after i.n. administration of microemulsions, compared with that of groups for i.v. administration. These findings indicated that mPEG$_{2000}$-PLA modified microemulsion improved drug entry into blood and brain compared with normal microemulsion: the introduction of mPEG$_{2000}$-PLA in microemulsion resulted in brain-targeting enhancement of both fat-soluble and water-soluble drugs. These findings provide a basis for the significance of mPEG$_{2000}$-PLA addition in microemulsion, defining its effects on the drugs in microemulsion.

Introduction
Stroke is an acute-onset clinical syndrome that develops following a vascular insult to the brain and is one of the leading causes of death and disability worldwide$^{1,2}$. With an aging population, the incidence and prevalence of stroke are predicted to rise$^3$. Despite the decades of research, however, treatment options remain limited. In recent decades, advances in the pharmaceutical industry have led to the development of traditional Chinese patent medicine, which has been routinely used for treating stroke patients in hospitals$^{4-6}$.

Xingnaojing (XNJ) is an effective traditional Chinese patent medicine, which is based on the famous Chinese medicine prescription "An Gong Niu Huang Wan" and contains borneol, muscone, gardenia extracts, and curcumae volatile oil. Studies have reported that XNJ can reduce brain injury and enhance functional recovery after stroke in different clinical trials and animal models of injury$^7$. Xingnaojing injection (XNJ-I) is commonly used for stroke treatment in clinical application. However, due to its high content in liposoluble constituents, high amounts of Tween-80 are used in XNJ-I, which may cause adverse reactions$^8$. Therefore, it is necessary to find safer routes for traditional Chinese medical compounds, with high bioavailability, quick absorption rate, and improved brain targeting. The intranasal (i.n.) route is noninvasive, essentially painless, does not require sterile preparation and is easily and readily administered by the patient or a physician in an emergency setting, making it a good alternative route. Most importantly, drugs would be absorbed sufficiently and rapidly into blood when administered intranasally$^{9-11}$ and transported from the nasal cavity directly into the central nervous system$^{12,13}$.

In order to enhance the convenience and safety of XNJ, a nasal administration system is under development in our laboratory. We used a solvent containing ethanol and propylene glycol at a ratio of 4:6 to prepare XNJ nasal drops (XNJ-D). However, organic solvents may be associated with serious mucosal irritation. Therefore, the microemulsion technology was applied to XNJ which is an effective method to improve bioavailability and targeted delivery of drugs$^{14-16}$. Consequently, XNJ microemulsion (XNJ-M) was prepared, dissolving compounds with different polarity, which resulted in improved drug loading and helped avoid excessive solvent addition$^{17}$. 

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Microemulsion is composed of oil phase, aqueous phase, emulsifier, and co-emulsifier. Tween18, poloxamer19, polyoxethylene-hydrogenated castor oil20, and lecithin20 are often used as emulsifiers for microemulsion preparation. However, high emulsifier and co-emulsifier concentrations have negative effects on organisms22,23 and may cause mucosal irritation and toxicity. The commonly used emulsifier polyoxethylene castor oil (EL) causes adverse reactions such as complement activation-related pseudoallergy24, peripheral neurotoxicity25, and cell toxicity26. EL-35 was used as emulsifier in XNJ-M, and caused nasal mucosa irritation, partially affecting the physiological characteristics of nasal mucosa27. Therefore, less toxic emulsifiers are desirable.

Polylactic acid (PLA), a FDA-approved biodegradable and non-cytotoxic material with a good track record in offering great potential for controlled release, has stood out and is extensively used in drug delivery applications. Its intermediate metabolite lactic acid is the normal metabolic product, with CO₂ and H₂O as the final degradation products in vivo28. However, PLA contains multiple ester bonds and exhibits poor hydrophilicity and biocompatibility29. Interestingly, methoxy poly(ethyleneglycol) (mPEG) is associated with good hydrophilicity and biocompatibility, and has been widely used as a long-circulating agent to improve the biocompatibility and increase the colloidal stability through steric hindrance, mPEG has been often incorporated in drug carriers for delivery in humans30. mPEG2000-PLA is a block copolymer of PLA and mPEG that exhibits surfactant-like properties, combining hydrophilic and hydrophobic segments; mPEG2000-PLA is promising in the field of drug delivery due to its good biodegradability and reliable biological safety31,32. Moreover, mPEG-PLA could make long-lasting circulation possible for pharmaceuticals, opening new perspectives for controlled drug delivery in particular33–35. Therefore, mPEG-PLA was considered to be applied for modifying XNJ-M on the purpose of reducing mucosal irritation and toxicity of emulsifier.

Our previous study explored mPEG-PLA potential for use as emulsifier in microemulsion to reduce mucosal irritation induced by XNJ-M. XNJ microemulsion was prepared using mPEG2000-PLA and the resulting drug was named XNJ-MM. We have previously shown that the irritating effects of XNJ-MM on nasal mucosa were lower compared with XNJ-M after 7 days of treatment36, indicating that the mPEG2000-PLA-modified microemulsion is a promising dosage form of XNJ. However, the pharmacokinetics and capacity to cross the blood–brain barrier of effective components in XNJ-M and XNJ-MM have not been explored. Specifically, it is unclear what the characteristics and advantages of distribution and metabolism of effective components in XNJ-MM are. Indeed, it is unclear whether mPEG2000-PLA can improve long-lasting circulation and brain targeting of drug loaded microemulsion.

Herein, we assessed the pharmacokinetic features after both intravenous (i.v.) and i.n. administrations to determine the impact of mPEG2000-PLA on microemulsion, comparing the bioavailability and brain-target effects of liposoluble constituent borneol and water-soluble constituent geniposide, both of which are effective components with relatively high quantity in XNJ-M and XNJ-MM. In addition, in vivo imaging system was applied to explore the characteristics of drug delivery to brain and tissue distribution of XNJ-M and XNJ-MM.

Materials and methods

**Instruments**

The ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) system was composed of an ACQUITY UPLC system (Waters Corp., Milford, MA) and a TQS triple-quadrupole tandem mass spectrometer (Waters Corp., Milford, MA) equipped with an electrospray ionization (ESI) source. Data were acquired and processed using MassLynx 4.1 software (Waters Corp., Milford, MA). A gas chromatography (GC) system with D7980 station (Tianmei Scientific Instrument Company, Shanghai, China) and an Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) equipped with UV detector were used for quantitative analysis. Other instruments used in this research included in vivo imaging system (FX Pro, Carestream Health, Rochester, NY), vortex mixer (MX-S, Beijing Dragon Laboratory Limited, Beijing, China), centrifuge (G16, Beijing Baiyang Centrifuge Co., Ltd., Beijing, China), homogenizer (IKA T10BS25, Germany), and analytical balances (BT25S, BS110S, Beijing Sartorius Instrument Company, Beijing, China).

**Chemicals and reagents**

Borneol and geniposide reference standards were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China). Octadecane was purchased from Alfa Aesar China Co., Ltd (Shanghai, China). 1,1’-dioctadecyl-3,3,3’,3’-tetramethyl indotricarbocyanine iodide (DiR) was purchased from Amyjet Scientific Inc (AAI Bioquest, Wuhan, China). LC/MS-grade of methanol and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ), high-performance liquid chromatography (HPLC)-grade formic acid was supplied by ROE Scientific (Newcastle, DE), and ultrapure water was generated from the Synergy UV water purification system (Millipore Corp., Billerica, MA). Pure water (Wahaha, Hangzhou, China) was obtained from the market. Ethyl acetate was of HPLC grade (Fisher Scientific, Pittsburgh, PA) and all other reagents were of analytical grade.

**Animals**

Male ICR mice and CD-1 nude mice were obtained from Vital River Laboratories (Beijing, China). All animals were clinically healthy and appeared normal throughout the experimental period. The animals were fasted for 12 h with free access to water prior to treatment. All experimental procedures were conducted in compliance with the guidelines evaluated and approved by the Beijing Municipal Science and Technology Commission.

**Preparation of XNJ-M and XNJ-MM**

XNJ-M and XNJ-MM were prepared by the dropping aqueous phase method, based on preliminary studies in our laboratory36,37. In brief, the required amounts of borneol, muscone, curcumae volatile oil, IPM, EL-35, and ethanol were placed in a 10 mL volumetric flask, and stirred at a rate of 300 r/min with a magnetic stirrer at room temperature. Geniposide solution was later added slowly, diluting with water to volume. XNJ-MM was obtained by replacing 10% emulsifier in the XNJ-M with mPEG2000-PLA.

**In vitro release studies**

The release experiments were conducted for borneol and geniposide separately using release media comprising normal saline containing 0.5% Tween-80 for borneol and normal saline for geniposide. The media were selected based on the solubility of each drug in the respective solvents to ensure that the selected media provides a sink condition. The release study for each batch of formulation was run using dialysis bags (Beijing BioDee Biotechnology Co. Ltd, Beijing, China, molecular weight cutoff of 7 kDa). The bag was placed in a glass container containing release media (200 mL, prewarmed to 37 ± 0.5 °C) and a magnetic bar. The whole assembly was placed in a magnetic
stirrer-cum-hot plate to maintain the temperature of release media at $37 \pm 0.5 \, ^\circ\text{C}$ and slow stirring speed (300 rpm). 0.5 mL of media was withdrawn at selected time points (0.25, 0.5, 1, 2, 4, 6, 8, 12 h), and the withdrawn volume was replaced with an equal amount of fresh media. The quantity of borneol in each sample was determined using the GC method after being extracted with ethyl acetate, and geniposide was determined by HPLC after filtering with 0.22 μm millipore filter. Based on the drug quantity in the sample and total volume of release media, percentage drug release was calculated.

Pharmacokinetic study

The study was based on a single-dose, randomized design. One hundred and sixty male ICR mice were randomly divided into four groups (I, II, III, and IV). Groups I and II were injected (i.v) with 0.2 mL drug preparations which were diluted 50 times by water of XNJ-M and XNJ-MM respectively, containing 8.0 mg/kg borneol and 4.12 mg/kg geniposide. Groups III and IV were treated with 4 mL XNJ-M and XNJ-MM at the same dose in unilateral nostril under anesthesia (ether). Blood samples were collected into heparinized tubes by retro-orbital puncture at 1, 3, 5, 10, 30, 60, 90, and 120 min after drug administration, and plasma was obtained after centrifugation. After euthanasia, animal brains were collected and washed by normal saline to remove excess blood and dried by filter paper and then accurately weighed. Five animals were studied at each time point.

Sample analysis

Sample preparation

Plasma samples were processed as follows: 100 μL plasma were mixed in a centrifuge tube with 10 μL octadecane solution (46.3 μg/mL in ethyl acetate, internal standard solution). Then, 90 μL of ethyl acetate were added and vortexed for 1 min, and the mixture was centrifuged at 10 000 r/min for 10 min. Finally 1 μL supernatant aliquots were injected into the GC system for analysis of borneol.

Another 200 μL plasma samples were placed into centrifuge tubes, mixed with 600 μL acetonitrile–methanol (1:3) mixture. The mixture was vortexed for 1 min and centrifuged at 10 000 r/min for 10 min. Supernatant was removed and evaporated under airstream in a water bath at 60°C. The residue was dissolved in 100 μL of methanol and shook for 1 min, centrifuged at 12 000 r/min for 5 min. Finally, 20 μL of supernatant was injected into the HPLC system for analysis of geniposide.

The clean brains were homogenized in normal saline solution (1.5 times the weight). 100 μL homogenate were mixed in a centrifuge tube with 10 μL octadecane solution (46.3 μg/mL in ethyl acetate, internal standard solution). Then, 90 μL of ethyl acetate were added and vortexed for 1 min, and the mixture was centrifuged at 10 000 r/min for 10 min. Finally 1 μL supernatant aliquots were injected into the GC system for analysis of borneol.

Another 500 μL brain homogenate were mixed in a centrifuge tube with 50 μL peoniflorin solution (485 ng/mL, internal standard solution). The mixture was vortexed for 30 s and 10 μL 20% formic acid was added. After mixing for 30 s, the mixture was treated by 1400 μL acetonitrile to precipitate proteins, vortexed for 1 min and centrifuged at 10 000 r/min for 10 min. Supernatant was removed and evaporated under airstream in a water bath at 60°C. The residue was dissolved in 200 μL of methanol and 700 μL of acetonitrile and vortexed for 1 min respectively, centrifuged at 12 000 r/min for 10 min. Again the supernatant was taken into new centrifuge tubes and evaporated under airstream in a water bath at 60°C. The residue was dissolved in 250 μL of methanol and shook for 2 min, centrifuged at 12 000 r/min for 10 min. Finally, 5 μL of supernatant was injected into the UPLC-MS/MS system for analysis of geniposide.

Chromatographic conditions

GC was performed on a Tianmei D7980 GC equipped with flame ionization detection (FID). Chemical separation was carried out on a TM-1701 capillary column (30 m × 0.32 mm inner diameter, 0.25 μm film, Tianmei, China). The oven temperature was programmed to rise from 100°C (remaining 5.5 min) to 200°C at a rate of 30°C/min (hold at 200°C for 5 min) followed by an increase to 250°C at a rate of 50°C/min (hold for 3 min). Nitrogen was used as carrier gas at a flow rate of 28 mL/min and the injection volume was 1 μL. The injector and detector temperatures were set at 250°C and 300°C, respectively.

The chromatographic separation of HPLC analysis was accomplished on a Merck C18 (4.6 × 250 mm inner diameter, 5.0 μm) analytical column under the following conditions: acetonitrile–water (13:87) at a flow rate of 1.0 mL/min; sample injection, 20 μL; column temperature, 25°C and detection wavelength, 238 nm.

Chromatographic separation of UPLC-MS/MS was performed on an ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm inner diameter, 1.7 μm; Waters Corp., Milford, MA). The column temperature was maintained at 40°C and the autosampler was conditioned at 4°C. The mobile phase was composed of 0.1% formic acid aqueous solution (A) and acetonitrile (B) at a flow rate of 0.4 mL/min in a run time of 4.5 min. Gradient condition of the mobile phase was as follows: 5% B at 0~0.5 min; 5%→15% B at 0.5~1.0 min; 15%→25% B at 1.0~2.0 min; 25%→80% B at 2.0~2.5 min; 80% B at 2.5~3.0 min; 80%→95% B at 3.0~3.5 min, then to the system was equilibrated using the initial condition (acetonitrile–water, 5.95, v/v) for 1.0 min. The injection volume was 5 μL and the partial loop with needle overfill mode was used for sample injection. Mass spectrometer was operated in the negative ion mode by multiple reaction monitoring (MRM) of the transition of m/z 449.2→299.1 for geniposide. The optimal MS parameters were as follows: capillary voltage 2.5 kV, cone voltage 30 V, the temperature of the source and desolvation were set at 150 and 400°C, respectively. Nitrogen was used as the desolvation gas and cone gas with the flow rate at 800 and 150 L/h, respectively. Argon was used as collision gas at a pressure of approximately $3.4 \times 10^{-3}$ mbar.

Method validation

To evaluate linearity, calibration standards at different concentrations were prepared and assayed in triplicate on 3 consecutive days. Linear regression techniques were used to assess the calibration curves and the determination of the correlation coefficients. To evaluate accuracy, precision, recovery and stability, quality control (QC) samples were prepared at low, medium, and high concentrations in the same manner as the calibration standards. Accuracy was defined as the relative error (RE%) and the precision was calculated as the relative standard deviation (RSD%). The recovery rates were calculated as the ratio of peak area from plasma or brain samples spiked before extraction and those from samples spiked after extraction. Stability (at room temperature for 24h) was assessed by comparing the concentrations of QC at different time and the theoretical concentrations.

Statistical analysis

The pharmacokinetic parameters were calculated by non-compartmental methods using Kinetica Version 4.4 (InnaPhase, Boston, MA). SPSS Version 17.0 was used for statistical analysis.
The data were presented as mean ± standard deviation (SD). Differences between group means were evaluated by t-test. A significance level at $p < 0.05$ was applied.

**Preparation of DiR-loaded XNJ-M and XNJ-MM**

To investigate the brain-targeting ability of XNJ-M and XNJ-MM, DiR was chosen as fluorescence probe to be loaded into microemulsions. 5 mg DiR was accurately weighed in a 5 mL volumetric flask, and the required amounts of liposoluble drugs, IPM, EL-35, and ethanol were placed in the flask, stirred at a rate of 300 r/min with a magnetic stirrer at room temperature. Geniposide solution was later added slowly, diluting with water to volume, afterwards the DiR-loaded XNJ-M (DXM) was obtained. The preparation of DiR-loaded XNJ-MM (DXMM) was similar to DXM, with mPEG-PLA replacing 10% of EL-35.

**Characterization of DXM and DXMM**

The mean particle size distribution and zeta potential of DXM and DXMM samples were analyzed using a laser particle size measurer (Zetasizer Nano, Malvern, UK). Measurements were performed in at least three different batches to obtain an average value and standard deviation for the particle diameter and zeta potential.

The *in vitro* release study of DiR in DXM and DXMM was investigated by dialysis method. 0.5 mL of DXM and DXMM was transferred into the dialysis bag, which was then put into 50 mL of 0.5% Tween-80 normal saline at 37 °C with stirring at 300 r/min. Aliquots of release medium were withdrawn at designated time points and the withdrawn volume was replaced with an equal amount of fresh media. The DiR concentration was then determined at 780 nm with a Multiskan Go microplate reader (Thermo Scientific, Waltham, MA).

**In vivo imaging**

For *in vivo* optical imaging, 36 CD-1 nude mice were randomly divided into six groups. Three groups were intranasally administered (i.n.) with 4 μL of DXM, DXMM, and DiR-loaded XNJ nose drops (DXD) respectively, and the other three groups were injected intravenously via tail vein with 0.2 mL of DXM, DXMM, and DiR solution (0.5% Tween-80 normal saline with the DiR concentration of 0.2 mg/mL) respectively. The drugs for i.v. were diluted 5 times by water with corresponding microemulsions for i.n. Mice were anesthetized by intraperitoneal injection of 20% urethan at 1 mL/100 g body weight. At preset time points (3, 10, 30, 60, 120, and 240 min), the anesthetized mice were put into the chamber and the fluorescent images were detected using an *in vivo* imaging system equipped with filter sets (excitation/emission, 738/780 nm). Fluorescence images and X-ray images were fused together with Carestream Molecular Imaging software. After images collection, the mice were sacrificed by cervical dislocation and brain, heart, liver, spleen, lung, and kidney were dissected. The *ex vivo* fluorescent images of the organs were also detected.

**Results and discussion**

**In vitro release studies**

*In vitro* release profiles of borneol and geniposide are shown in Figures 1 and 2. More than 50% of borneol was released over the period of 12 h from XNJ-M, and XNJ-MM showed maximum 70% release of borneol up to 12 h. The release of geniposide both in XNJ-M and XNJ-MM exhibited similar character that was significantly faster with 90% of total drug releasing in fourth hour as geniposide is predominantly present in the external aqueous phase. By comparison, borneol release was relatively slow as this lipophilic drug remains located within the oily phase and undergoes slow partitioning into the aqueous phase. Therefore, mPEG-PLA had little impact on the release of hydrophilic drug, but accelerated the release of borneol, which was probably because the compatibility between mPEG-PLA and borneol is weaker than that of EL-35 and borneol. In addition, we explored *in vitro* release of muscone during this experiment, however muscone in the withdrawn samples was not detected over the period of 12 h. The amount difference between borneol and muscone (7:1) in microemulsions may be the main factor resulting in the difference that borneol and muscone performed in release and stability in microemulsions, even though they are both highly lipophilic drugs.

**Method validation for pharmacokinetic samples**

This study used GC to determine the concentration of borneol in biological samples. Liquid–liquid extraction was selected for plasma and brain sample preparation and small sample amounts were required. In addition, pretreatment was simple and fast. Compared with blank biological samples, an excellent separation of borneol and octadecane in biological samples was obtained, without interference from other metabolites and endogenous substances. After verification of the methodology for linearity, precision, and recovery rate we concluded that the requirements for pharmacokinetic studies of borneol in plasma and brain samples were met. As for plasma samples, the limit of detection of borneol was 41.75 ng/mL in plasma (S/N ≥ 3) and a linear range between 0.08 and 33.4 μg/mL was obtained. There was a good linearity between peak area (A) and concentration (C)
The recovery of borneol in plasma at low, medium, and high concentrations was 98.7 ± 1.76%, 96.3 ± 3.11%, and 96.50 ± 1.40%, respectively. The RSD of intra-day precision at low, middle and high borneol concentrations was 1.64%, 1.28%, and 1.55%, respectively, while inter-day precision of 2.93%, 0.65%, and 1.65%, respectively, was obtained. As for brain samples, the limit of detection of borneol was 52.5 ng/g in brain (S/N≥3); the calibration curves were linear between 0.21 and 66.80 ng/g. There was a good linearity between A and C (A = 0.0807C + 0.0340, r = 0.9997). The recovery of borneol in brain at low, middle, and high concentrations was 93.68 ± 2.47%, 99.7 ± 3.22%, and 98.19 ± 3.03%, respectively. The RSD of intra-day precision at low, middle, and high borneol concentrations was 3.71%, 3.26%, and 3.39%, respectively, while inter-day precision was 4.81%, 4.10%, and 2.97%, respectively.

The HPLC used to determine geniposide in plasma samples was specific and efficient. No significant endogenous interference of the analyte was observed. The calibration curves of geniposide were plotted. They showed a good linearity between A and C over the range of 0.0105–5.250 μg/mL. The regression equation was A = 303.1C + 9.6485 (r = 0.9999), and the detection limit was 0.005 μg/mL (S/N≥3). The RSD of intra-day precision at low, middle, and high concentrations was 0.79%, 5.14%, and 1.37%, respectively, while inter-day precision was 7.33%, 3.40%, and 1.91%, respectively. The recovery rates of geniposide from plasma samples were 100.0 ± 1.58%, 100.0 ± 0.74%, and 100.0 ± 1.01% at high, medium, and low concentrations, respectively. The limit of detection of geniposide was 0.128 ng/g in brain (S/N≥3); the calibration curves were linear between 1.275 and 318.75 ng/g. There was a good linearity between Y (Y = peak area of IS × geniposide concentration/IS concentration) and C (Y = 20.646C + 770.55, r = 0.9993). The recovery of geniposide in brain at low, middle, and high concentrations was 99.5 ± 14.32%, 109.94 ± 25.09%, and 101.21 ± 10.36%, respectively. The RSD of intra-day precision at low, middle, and high geniposide concentrations was 4.09%, 3.63%, and 3.90%, while inter-day precision was 6.76%, 8.34%, and 5.96%, respectively. The matrix effects derived from QC samples were in the range of 94.3%–114.4% and IS was 95.4%. The analytes were found to be stable in the brain samples when stored at –20°C for 1 month (RSD 2.26 ± 3.66%) and through three freeze-thaw cycles (RSD 3.74 ± 5.52%). Post-preparative samples were also stable when kept in the auto-sampler at 4°C for 5 h (RSD 3.58 ± 5.02%). Therefore, plasma and brain samples assay methods were deemed fit for our study.

**Pharmacokinetic studies**

In this study, we found that after i.v. administration the concentration of borneol was greatly reduced in 1 min. In order to make the data more objective and conducive to study, we supplementarily explored the borneol concentration in blood and brain in 0.1 min after administration. The concentration–time profiles of borneol in plasma after i.n. administration are shown in Figure 3 and the pharmacokinetic data are presented in Table 1. We found C_{max} values in plasma to be 9.02 ± 3.62 (III) and 9.68 ± 2.14 (IV) μg/mL, AUC_{0–120} were 154.8 ± 56.07 (III) and 236.6 ± 36.11 (IV) μg/mL-min, respectively. For XNJ-M and XNJ-MM, after i.n. administration, T_{max} were 1.80 ± 1.79 min and 0.2 ± 0.40 min, respectively. The plasma pharmacokinetics results showed that after i.n. administration, XNJ-M and XNJ-MM were quickly absorbed into the blood (T_{max}<2 min). Meanwhile, we found that AUC values obtained in both cases were greater than those obtained with i.v. administration, and the absolute bioavailability F values were both more than 100%. This is likely due to the rapid tissue distribution after i.v. administration of the microemulsion, which resulted in quick decreased blood drug concentration, leading to important but difficult evaluation of plasma concentration at the starting time point; i.n. administration follows an absorption process, and drugs that are not quickly taken up by tissues decreased slowly in concentration after reaching the peak. Though the AUC_{blood} value was obtained through i.v., it could not reflect the real concentration of drugs in blood, as the reasons described above. The solution or injection of a drug should be applied intravenously for exploring the absolute bioavailability, not the microemulsion. Therefore, the data for i.v. of microemulsions were not exhibited in this paper, and the exact concentration of drugs in blood needs further exploration.

The concentration–time profiles of borneol in brain after i.v. and i.n. administrations are shown in Figure 4 and...
pharmacokinetic data are presented in Table 2. The relative brain targeted coefficient $Re$ was calculated according as follows: $Re = \frac{AUC_{brain \text{ (i.n.)}}}{AUC_{brain \text{ (i.v.)}}} \times 100\%$; the brain/blood drug ratio $Te$ was determined by the equation $Te = \frac{AUC_{brain}}{AUC_{blood}} \times 100\%$. Average values of $C_{max}$ obtained in brain were $11.7 \pm 0.91$ (I), $10.00 \pm 0.79$ (II), $2.82 \pm 0.58$ (III), and $3.63 \pm 0.49$ (IV) $\mu g/g$, while $T_{max}$ were $0.1$ (I), $0.1$ (II), $2.24 \pm 2.28$ (III), and $0.1$ (IV) min. $AUC_{0-120}$ were $121.38 \pm 31.84$ (I), $87.50 \pm 51.25$ (II), $163.36 \pm 66.61$ (III), and $173.32 \pm 98.30$ (IV) $\mu g/g$-min. Therefore, $Re$ of 134.59% and 198.09% was obtained for XNJ-M and XNJ-MM, respectively. The results indicated that borneol in XNJ could be promptly and thoroughly transported into brain by i.n. administration. The amount and distribution speed in brain of borneol by i.n. administration was higher than i.v. administration. The brain pharmacokinetics showed that after i.v. administration of XNJ-M and XNJ-MM, the $C_{max}$ values were higher than those obtained for blood samples and brain samples after i.n. administration, which indicated that the microemulsion administrated intravenously distributed into tissues rapidly. Interestingly, the blood $C_{max}$ value for XNJ-MM was higher than that of XNJ-M after i.v. administration, while brain $C_{max}$ value was lower than that of XNJ-M. These findings indicated that the introduction of mPEG-PLA reduced tissue binding with microemulsion and decreased the phagocytosis and capture of microemulsion oil phase by tissues, which resulted in long-lasting circulation of drugs in oil phase. Both microemulsions could quickly enter the phase of microemulsion after mPEG-PLA modified, and easier more compact connection between water-soluble drugs and oil phase of microemulsion made geniposide stronger than that of EL-35 and geniposide, making more compact connection between water-soluble drugs and oil phase of microemulsion after mPEG-PLA modified, and easier distribution into tissues of geniposide along with the oil phase.

The concentration–time profiles of geniposide in plasma after i.n. administrations are shown in Figure 5 and pharmacokinetic data are presented in Table 1. $C_{max}$ values in plasma were $1.31 \pm 0.14$ (III) and $1.57 \pm 0.81$ (IV) $\mu g/mL$, while $AUC_{0-120}$ were $24.99 \pm 3.96$ (III) and $30.79 \pm 3.13$ (IV) $\mu g/mL$-min, respectively, indicating that XNJ-MM enhances the bioavailability of geniposide via i.n. route. After i.v. administration, $C_{max}$ value and $AUC$ of XNJ-MM were lower than the features of XNJ-M, thus we inferred that the trend and degree in distributing into tissues of geniposide in XNJ-MM was higher than that in XNJ-M via i.v. route. It was probably the affinity between mPEG-PLA and geniposide stronger than that of EL-35 and geniposide, making more compact connection between water-soluble drugs and oil phase of microemulsion after mPEG-PLA modified, and easier distribution into tissues of geniposide along with the oil phase.

The concentration–time profiles of geniposide in brain after i.v. and i.n. administrations are shown in Figure 6 and pharmacokinetic data are presented in Table 3. The values of $C_{max}$ obtained in brain were $136.75 \pm 43.56$ (I), $133.60 \pm 48.48$ (II), $114.89 \pm 23.41$ (IV) $\mu g/g$, while $AUC_{0-120}$ were $3654.70 \pm 784.18$ (I), $3411.22 \pm 366.13$ (II), $3278.19 \pm 181.68$ (III), and $6424.51 \pm 1071.89$ (IV) $\mu g/g$-min. Therefore, $Re$ of 89.70% and 188.33% was obtained, respectively. The $AUC$ and $Re$ value of XNJ-MM were significantly greater than for XNJ-M, suggesting apparent increase of XNJ-MM on the targeting efficiency to the brain of mice. The concentration–time curve of XNJ-MM after i.n. administration showed that the

![Image](319x200 to 554x344)

**Figure 4.** Mean brain concentration-time curve of borneol via i.n. and i.v. administration in mice (mean ± SD, $n = 5$).

**Figure 5.** Mean plasma concentration-time curve of geniposide via i.n. administration in mice (mean ± SD, $n = 5$).

<table>
<thead>
<tr>
<th>Group</th>
<th>$C_{max}$ (µg/g)</th>
<th>$T_{max}$ (min)</th>
<th>$AUC_{0-120}$ (µg/g min)</th>
<th>$MRT_{0-120}$ (min)</th>
<th>$Re$ (%)</th>
<th>$Te$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>11.73 ± 0.91</td>
<td>0.10</td>
<td>121.38 ± 31.84</td>
<td>45.42 ± 5.93</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>II</td>
<td>10.00 ± 0.79</td>
<td>0.10</td>
<td>87.50 ± 51.25</td>
<td>33.82 ± 15.21</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>III</td>
<td>2.82 ± 0.58</td>
<td>2.24 ± 2.28</td>
<td>163.36 ± 66.61</td>
<td>55.46 ± 5.02</td>
<td>134.59</td>
<td>105.47</td>
</tr>
<tr>
<td>IV</td>
<td>3.63 ± 0.49</td>
<td>0.10</td>
<td>173.32 ± 98.30</td>
<td>53.47 ± 9.38</td>
<td>198.09</td>
<td>73.25</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD ($n = 5$).

$\Delta p<0.05$. 

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geniposide concentration of brain had a gradual rise over the period from 60 min to 120 min, which differed from XNJ-M. This finding may be related with the drug transported into brain from blood, and we also speculated that the pathway and mechanism underlying i.n. delivery to the brain of microemulsion was changed to some extent due to the introduction of mPEG-PLA. Most of geniposide in XNJ-MM may reach brain via extracellular mechanism of transport along olfactory nerve like XNJ-M which requires only several minutes to 30 min for a drug to reach the olfactory bulbs and other areas of the brain after i.n. administration, while other geniposide in XNJ-MM is likely to be delivered to the brain via intracellular transport mechanism which takes several hours for a drug to appear in the brain. However, this conjecture and other factors affecting the brain target of XNJ-MM require further elucidation in our future work.

Preparation and characterization of DXM and DXMM

The mean diameters of DXM and DXMM were 32.96 (PDI = 0.175) and 33.93 (PDI = 0.195) nm, respectively, and the zeta potential were −4.87 and −2.55 mV. The DXM and DXMM showed similar release kinetics with 12 h that no DiR was observed, in other words there was no DiR leakage from DXM and DXMM until 12 h. Therefore, the DXM and DXMM exhibited required property and good stability and could be used to investigate the brain-targeting ability of XNJ-M and XNJ-MM.

In vivo imaging

The direct and real-time of brain-targeting efficacy, in vivo biodistribution could be easily monitored by imaging near-infrared fluorescence images. In order to investigate whether XNJ-M and XNJ-MM could actively target to brain, we performed in vivo and ex vivo imaging. As shown in Figure 7, fluorescent signals were mainly observed in nasal cavity and head from the mice treated with DXM and DXMM from 3 min to 240 min post i.n. administration. The nude mice of DXD group presented difficult breath and were finally died after 60 min of drug treatment, which was possibly because DXD was mainly inhaled into the lungs by mice as shown in Figure 8(A). Moreover, Figure 8(A) shows the representative ex vivo fluorescence images of mice tissues after 60 min administration. Strong fluorescence intensity were observed in the mice brains, and no obvious fluorescence were obtained in other tissues with DXM and DXMM treated, which showed direct evidence of successful transport of drugs into brain and in vivo accumulation of microemulsions in mice brains. From the results shown in Figure 8(B), it can be seen that the fluorescent signals in brains occurred at just 3~10 min, gradually increased during the tested time and the 120 min groups exhibited the highest fluorescence intensity of both DXM and DXMM. In addition, the drug mainly distributed in the olfactory bulb and the amount of distribution was gradually reduced from olfactory bulb to the inner parts of brain, which can come to an initial speculation that DXM and DXMM were transported to the olfactory bulb from nose via olfactory nerve pathway. These results demonstrated that active

Table 3. Main brain pharmacokinetic parameters of geniposide in mice after i.n. and i.v. administration.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group</th>
<th>Cmax (ng/g)</th>
<th>Tmax (min)</th>
<th>AUC0–120 (ng·g−1·min)</th>
<th>MRT0–120 (min)</th>
<th>Re (%)</th>
<th>Te (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>136.75 ± 43.56</td>
<td>3.40 ± 1.67</td>
<td>3654.70 ± 784.18</td>
<td>41.01 ± 13.56</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>133.60 ± 48.48</td>
<td>3.00 ± 0.00</td>
<td>3411.22 ± 366.13</td>
<td>42.07 ± 10.85</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>96.47 ± 13.09</td>
<td>3.80 ± 1.10</td>
<td>3278.19 ± 181.68 *</td>
<td>40.61 ± 2.39 *</td>
<td>89.70</td>
<td>13.18</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>114.89 ± 23.41</td>
<td>6.20 ± 3.56</td>
<td>6424.51 ± 1071.89</td>
<td>50.82 ± 5.75</td>
<td>188.33</td>
<td>20.87</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD (n = 5).

*p < 0.01 versus Group IV.
brain-targeting ability of microemulsion based on mPEG-PLA modification, which are expected to increase the brain delivery and therapeutic efficiency of lipophilic drugs for brain diseases treatment.

From the results of preliminary experiment, it was found that the in vivo and ex vivo fluorescence was not easily observed if the mice were injected with the same amount of DiR administrated intranasally. Therefore, the microemulsions for injection were diluted 5 times with the drugs for i.n., namely the DiR amount in microemulsions for i.v. was 10 times as much as that for i.n. administration. The biodistribution of DXM and DXMM via i.v. administration in mice are shown in Figures 9 and 10. High and quick uptake of DXM and DXMM into skin and tissues after i.v. administration was observed, which was slightly decreased at 4 h postinjection. As shown in Figures 9 and 10(A), the reduction of DXMM was quicker than that of DXM, which probably due to the weaker combination between tissues and oil phase in DXMM, and this result was consistent with the conclusion drawn at the pharmacokinetic studies. Besides, there was little DiR solution (DS) distributed into skin and tissues until 2 h, and the liver showed the strongest fluorescence intensity at 2- and 4-h post administration. The biodistribution of DS was very different to that found for microemulsions, and this result suggested that the fluorescent signals in vivo of DXM and DXMM could represent the distribution of XNJ-M and XNJ-MM, respectively, but not the distribution of DiR itself. As presented in Figure 10(B), strong fluorescent signals emitted from mice brains were observed at 3 min after i.v. administration of both DXM and DXMM, the highest uptake in brain was obtained at 10 min postinjection and the fluorescence intensity was decreased gradually from 10 min to 4 h. Overall, the results above indicated that i.n. administration of DXM and DXMM displayed significant brain-targeting efficiency by comparison with the drugs administrated for i.v.

Conclusion
Compared to nasal drops, XNJ microemulsion had significant advantage in brain-targeting, and mPEG<sub>2000</sub>-PLA modified microemulsion improved drug entry into blood and brain compared with normal microemulsion; the introduction of mPEG<sub>2000</sub>-PLA in microemulsion contributed to brain-targeting enhancement of fat-soluble and water-soluble drugs. In addition, XNJ-MM was transported into brain from nose via the olfactory nerve pathway. Overall, our findings indicate that mPEG<sub>2000</sub>-PLA
improves brain-targeting of drug-loaded microemulsion and provide a basis for the application of mPEG$_{2000}$-PLA in microemulsion.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of this article. This work was supported by the National Natural Science Foundation of China (No. 81102816) and Independent Research Project of Beijing University of Chinese Medicine (No. 2013-JYBZZ-XS-083).

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