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Development and in vitro–in vivo evaluation of a water-in-oil microemulsion formulation for the oral delivery of troxerutin

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Abstract

Objective: The main objective of this study was to develop and evaluate a W/O microemulsion formulation of troxerutin to improve its oral bioavailability.

Methods: The W/O microemulsion was optimized using a pseudo-ternary phase diagram and evaluated for physical properties. In vitro MDCK cell permeability studies were carried out to evaluate the permeability enhancement effect of microemulsion, and in vivo absorption of troxerutin microemulsion in the intestine was compared with that of solution after single-dose administration (56.7 mg/kg) in male Wistar rats.

Results: The optimal formulation consisted of lecithin, ethanol, isopropyl myristate and water (23.30/11.67/52.45/12.59 w/w) was physicochemical stable and the mean droplet size was about 50.20 nm. In vitro study, the troxerutin-loaded microemulsion showed higher intestinal membrane permeability across MDCK monolayer when compared with the control solution. The W/O microemulsion can significantly promote the intestinal absorption of troxerutin in rats in vivo, and the relative bioavailability of the microemulsion was about 205.55% compared to control solution.

Conclusion: These results suggest that novel W/O microemulsion could be used as an effective formulation for improving the oral bioavailability of troxerutin.

Introduction

Troxerutin (TX, vitamin P4), a semi-synthetic derivative of the natural flavonoid rutin, is the main component of venoruton. It has aroused considerable interest due to its broad pharmacological and biological properties such as anti-oxidative, anti-inflammatory, anti-fibrinolytic, anti-thrombotic effects1–3. Troxerutin is clinically used for the management of cardiovascular and cerebrovascular diseases, including chronic venous insufficiency (CVI), hematomas and blood flow disorders2. And this hydrophobic drug is available as 120–180 mg oral tablets given every 8 h and 240–480 mg intravenous injection given every 24 h. Currently, troxerutin is mainly administrated orally, but low plasma concentration in humans is achieved, which may lead to the use of an increased dosage or frequency and increase the risk of side effects. In a pharmacokinetic study of troxerutin, it was reported that the maximum plasma concentration (Cmax) was 2931 ± 1018 pg·mL⁻¹ after oral administration of 300 mg troxerutin to 18 volunteers4, and a similar study has been done with the same dose5, the maximum plasma concentration (Cmax) was 0.75 ± 0.31 ng·mL⁻¹. Both pharmacokinetic studies showed that the oral bioavailability of troxerutin is very low. However, so far, most investigations of troxerutin have been focused on its pharmacological and side effects, while new formulations to increase the absorption of troxerutin have been less studied. Therefore, there was a need for a new formulation to increase the absorption of troxerutin in the gastrointestinal tract and improve its bioavailability.

Microemulsion, with droplet diameters less than 100 nm, is characterized as clear, isotropic, thermodynamically stable, heterogeneous system of two immiscible liquids, can be used to improve its bioavailability in the gastrointestinal tract6–8. Microemulsions have been intensively utilized as drug delivery systems because of its prominent advantages including better drug solubilization, ease of preparation and protection against enzymatic hydrolysis, as well as the potential for promoted absorption through lymphatic pathways which makes a significant contribution to absorption from the intestine bypassing the hepatic first-pass effect9,10. In the past few years, many reports have revealed that microemulsion is a suitable delivery system for the oral delivery of both hydrophilic and lipophilic drugs, especially for enhanced oral absorption11. Oil-in-water microemulsions are promising in improving the bioavailability of poorly water-soluble compounds12. Water-in-oil microemulsions, on the other hand, have shown potential to increase the permeability of hydrophilic drugs across the intestinal mucosa, thus enhancing their bioavailability. For example, employing the microemulsion formulation to fexofenadine improved its oral bioavailability of 376.76% compared with the commercial syrup formulation in rabbits13.
Similarly, hydroxysafflor yellow A was orally administered as a W/O microemulsion, the relative bioavailability was about 1937% compared with a control solution in bile duct-nonligated rats.

Based on the above mentioned considerations, we thought it may be plausible to improve the bioavailability of troxerutin by the use of a microemulsion. Thus, in this study, with the aim of improving the oral absorption of troxerutin, a stable W/O microemulsion formulation was developed and characterized by morphology, droplet size, pH value, conductivity, stability and content. Furthermore, MDCK cell permeability studies were carried out to evaluate the enhancement effect of microemulsion, and the pharmacokinetics of troxerutin microemulsion were determined to evaluate the potential of the microemulsion to be an effective oral delivery carrier for drugs.

Materials and methods

Materials

Troxerutin was purchased from Aladdin Industrial Corporation (Shanghai, People’s Republic of China). Lecithin with a phosphatidylcholine (PC) content of (70–97%) was obtained from Shanghai Tai-wei Pharmaceutical Co., Ltd. (Shanghai, People’s Republic of China). Methanol, ethanol, glacial acetic acid, formic acid and isopropyl myristate (IPM) were provided by Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, People’s Republic of China). MDCK cells were obtained from Zhejiang University of Technology. Modified eagle’s medium (MEM), Hank’s balanced salt solution (HBSS) and fetal bovine serum (FBS) were all obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents were of analytical grade and used as received.

Construction of pseudo-ternary phase diagrams

Safe and biocompatible non-ionic hydrophilic surfactants, lecithin, and a cosurfactant, ethanol, were selected to form microemulsions. In order to find out the concentration ranges of components for the existing range of microemulsions, the pseudo-ternary phase diagrams were constructed by the aqueous titration method at room temperature (25 °C). Briefly, the surfactants (lecithin) were melted and blended with the cosurfactant (ethanol) at different weight ratios (Km) of 1:2, 1:1, 2:1 and 3:1 (w/w) in each tube, and were vortexed vigorously for 30 s to make the surfactant mixture (Sm). For each phase diagram, the oil phase (isopropyl myristate) and the surfactant mixture (Sm) were mixed, where the ratios of oil to Sm in the mixtures were varied from 9:1 to 1:9 (w/w). Distilled water was then added drop by drop under moderate stirring to allow equilibration. Subsequently the mixtures were visually examined for transparency. The points from clear to turbid and turbid to clear were noted as the critical points and the respective mass fraction of surfactant, cosurfactant, oils and distilled water at these critical points were then used to construct the phase diagrams for the determination of microemulsion domain.

Preparation of microemulsion

The pseudo-ternary phase diagram, which allowed the obtainment of the largest existence area of the microemulsion, was selected for identifying the concentration range of components in the microemulsions and the composition of the final formulation was selected from the microemulsion region. To prepare the troxerutin-loaded microemulsion, the drug was previously dissolved in distilled water, then the drug solution was dropped into the mixture under moderate stirring at room temperature. Thus, a clear, transparent, homogeneous and easily flowable microemulsion of troxerutin was obtained.

Characterization of troxerutin microemulsion

Macroscopic appearance

The prepared microemulsion is inspected for the colour, homogeneity, isotropy, phase separation and flowability after visual examination at room temperature.

Morphology of microemulsion

The morphology of microemulsion was examined by transmission electron microscopy (TEM, Tecnai, TF20). The samples were prepared by dropping one drop of diluted microemulsion onto a carbon coated copper grid and negatively stained at room temperature with 2% phosphotungstic acid (PTA).

Droplet size and polydispersity index

The average droplet size and polydispersity index (PDI) of microemulsions were measured by dynamic light scattering (DLS), using a Zetasizer Nano ZS90 (Malvern Instrument, Worcestershire, UK). The samples were loaded into cuvettes in a thermostated scattering chamber and measured 24 h after their preparation at 25 °C. The measurements were performed in triplicate.

pH measurements

The pH values of the samples were measured by a pH meter (Leici Instrument Co., Shanghai, China) at room temperature and triplicate measurements were made.

Conductivity measurements

The electrical conductivity (σ) of both formulations was determined by a DDS-11AW conductivity meter coupled with a platinum electrode (Bante Instrument Co., Shanghai, China). Before conductivity measurement, standard KCl solutions were used to calibrate the conductivity cell. Each sample was measured in triplicate at ambient temperature.

Content of troxerutin

An HPLC assay was used for the loading content. Samples were determined by diluting 100 times with methanol and injected directly into the HPLC system without further treatment. A Shim-pack ODS C18 column (Shimadzu Corporation, Kyoto, Japan), 5μm particle size, 250mm×4.6mm, was used as stationary phase. Methanol and 1.7% glacial acetic acid were used as the mobile phase at a flow rate of 1.0mL·min⁻¹. The detection wavelength was set as 350 nm. The chromatographic column temperatures was 40 °C.

Stability of microemulsions

Five milliliter of drug-loaded microemulsion samples was stored at three temperatures (4, 25 and 40 °C) for three months. During this period, samples were periodically checked for any physical change, including clarity, transparency, phase separation, precipitation of drug, color change and drug content. Furthermore, to evaluate the accelerated physical stability of microemulsions, samples were centrifuged at 10000 rpm for 30 min and observed physical instability such as phase separation and aggregation visually.

Preclinical pharmacokinetic studies

Experimental design

A pharmacokinetic study of troxerutin microemulsion and solution were compared in twelve healthy male Sprague–Dawley rats (230 ± 10 g, provided by the Central Animal
Laboratory of Anhui Medical University), with the permission from the institutional ethical committee of the Anhui Medical University. Prior to the experiment, the rats were divided into two groups and starved overnight for 12 h with water ad libitum. For each group, troxerutin solution or microemulsion formulations at the same doses of 56.7 mg/kg were orally administered. After administration, serial blood samples were withdrawn into tubes containing heparin at the intervals of 0.25, 0.50, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0 and 24.0 h. And then, the blood samples were placed on wet ice until centrifugation at 3500 rpm (4 °C) for 15 min. The plasma was separated and immediately stored at −20 °C until analysis by a coupled ultra-high-performance liquid chromatography-tandem mass spectrometry system (UPLC-MS/MS).

**LC-MS/MS assay of plasma samples**

At the time of analysis, 200 μL of each plasma sample was mixed with 200 μL of internal standard (IS) solution (quercitrin 80 ng/mL) and 600 μL of methanol. After centrifuging for 5 min at 13,000 rpm, the supernatant was evaporated to dryness under a stream of nitrogen gas at 40 °C. The residue was reconstituted with 200 μL of mobile phase and analyzed using UPLC-MS/MS. The liquid chromatography separation was performed on a Waters ACQUITY UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm, Waters, Milford, MA), and the column temperature was maintained at 30 °C. The mobile phase consisted of a mixture of methanol and 0.1% formic acid (27:73, v/v) was kept at a constant flow rate of 0.3 mL/min. Detection was achieved using a Waters Xevo TQ-S mass spectrometer equipped with an electrospray ionization (ESI) in the positive ion mode. The working parameters of the mass spectrometer were set as follows: electrospray capillary voltage, 3.49 kV; source temperature, 150 °C; desolvation gas flow, 550 L/hr; desolvation temperature, 450 °C; collision energy, 40 eV; cone voltage, 25 V; cone gas flow, 150 L/hr. Quantification was performed in multiple reaction monitoring (MRM) mode with the following transitions: m/z 743.2 → m/z 449.3 for troxerutin and m/z 303.132 for quercitrin, respectively. The concentrations of troxerutin were measured by the calibration curve in the concentration range of 5–250 ng/mL.

**Pharmacokinetic analysis**

The main pharmacokinetics parameters of troxerutin in both groups were calculated by DAS 3.0 (Drug and Statistics, Shanghai University of Traditional Chinese Medicine, Shanghai, China). Data were analyzed for the maximum plasma concentration (C_max), time of peak (T_max), the area under the curve (AUC_0–∞), the half-life (t_1/2) and relative bioavailability. The relative bioavailability of the oral drug delivery system was calculated as follows:

Relative bioavailability = \( \frac{(AUC) \text{ microemulsion}}{(AUC) \text{ solution}} \times 100\%

**Permeability studies in MDCK cell monolayer**

**MDCK cell cultures**

MDCK cells were generously provided by Pro. Biwei Song (Zhejiang University of Technology, China) and used from passage 70 to 85. To maintain expression, MDCK cells were cultivated in 75 cm² culture flasks containing modified Eagle’s medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin. Cultures were maintained at 37 °C in an atmosphere of 5% CO₂ and 95% relative humidity.

For transport assay, the cell monolayers were prepared by seeding 3 × 10⁵ cells/well on Millicell Hanging cell culture inserts (12 mm diameter, 1.13 cm² area, 1.0 μm pore diameter; Millipore Corporation, Billerica, MA) in 12 Transwell plates and used for the assays on days 7–10. The culture medium was refreshed every other day and the day before the experiment. The integrity of each cell monolayer was verified by evaluation of the transepithelial electrical resistance (TEER) values before and after the experiments with a Millicell ERS meter (Millipore). Cell monolayers were used for the transport studies when the TEER values were high and stable (250 Ω cm²).

**Bidirectional transport studies across MDCK**

The permeability of the formulations was measured in apical to basolateral (A to B) and basolateral to apical (B to A) directions at 37 °C with moderate shaking (120 rpm) for 2 h. After the verification by TEER values, cell monolayers were washed twice with prewarmed Hank’s buffered salt solution (HBSS, pH 7.4) and equilibrated in HBSS for 30 min at 37 °C. Then the troxerutin microemulsion diluted with HBSS, in which the concentration of troxerutin was 200 μmol/mL, was added to the A side (0.6 mL) or B side (1.2 mL), while the acceptor chamber contained 1.2 mL of HBSS (B side) and 0.6 mL of HBSS (A side), respectively. Each direction was performed with triplicate inserts. During the transport studies, 200 μL of medium were removed from the the acceptor chamber at pre-established time points (30, 60, 90, 120 min). The acceptor chamber samples were replaced with the same volume (200 μL) of fresh prewarmed HBSS, and samples were analyzed quantitatively by HPLC. The same procedure was used for the troxerutin solution. The apparent permeability values (P_app, cm/s) was calculated according to the following equation:

\[ P_{\text{app}} = \frac{dQ/dt}{A \times C_0} \]

where \( dQ/dt \) is the drug permeation rate (mmol/s), \( A \) is the filter surface area (1.13 cm²), and \( C_0 \) is the initial concentration in the donor chamber (μg/mL). The efflux ratio (ER) was calculated from the following Equation 18:

\[ ER = \frac{P_{\text{app}} (B \text{ to } A)}{P_{\text{app}} (A \text{ to } B)} \]

A compound with the ER values greater than 2.0 was qualified as a substrate of the efflux mechanism.

**Statistical analysis**

All data were statistically analyzed by one-way analysis of variance (ANOVA). The differences were considered statistically significant when \( p < 0.05 \).

**Results and discussion**

Pseudo-ternary phase diagram study and preparation of microemulsion

Microemulsions are thermodynamically stable, clear, transparent system and developed using several commercially available and pharmaceutically acceptable lipid-based excipients. Lecithin, a non-toxic permeation enhancer with phosphatidylcholine (PC) content of (70–97%), has received much attention as food-acceptable components and it can enhance transdermal drug permeability and transmembrane passage across the gastrointestinal tract, thus improving the bioavailability of the guest molecules. Ethanol was also selected for the preparation of microemulsion due to its low and non-toxic doses can induce the opening of tight junctions, resulting in increased drug permeability across the gastrointestinal epithelium. Furthermore, studies have found that a mixture of lecithin and ethanol can form a W/O microemulsion with a higher amount of water. Thus, in this study, the W/O microemulsion formulation consisting of lecithin, ethanol, isopropyl myristate and water was selected, which are all pharmaceutically acceptable and have been employed to improve the intestinal drug absorption without damaging the intestinal mucosa.
Four pseudo-ternary phase diagrams were constructed, as described in the methods section, by titration with water. Figure 1 depicts the pseudo-ternary phase diagrams with various weight ratios \((K_m)\) of lecithin to ethanol. The shaded areas in these diagrams represent the existence field of stable, clear and transparent W/O microemulsions. As shown in Figure 1, the area of the microemulsion region increased along with the \(K_m\) from 1:2 to 2:1 and the lecithin/ethanol 2:1 v/v ratio allowed the obtainment of the largest area of existence of the microemulsions among the tested weight ratios. When \(K_m\) was fixed at 3:1, the area of the microemulsion region is almost equal to that of 2:1, and the high amount of surfactants might lead to a series of problems such as increased viscosity and decreased safety of the microemulsion. On the basis of all these considerations, \(K_m\) 2:1 was then selected for the preparation of drug-loaded W/O microemulsions.

As shown in Figure 1(c), a clear and transparent microemulsion was identified in the microemulsion zone. The composition of the final formulation was selected from this area, and composed of 23.30% lecithin, 11.67% ethanol, 52.45% isopropyl myristate and 12.59% water according to the principle of high drug-loading efficiency and low proportion of surfactants that contributed to mucosal toxicity.

**Characterization of microemulsion**

Different physico-chemical parameters of the microemulsions were characterized and the results are given in Table 1. Figure 2 shows the microemulsion formulations were homogeneous, transparent without any precipitates, good fluidity, optically isotropic and yellow colored at room temperature. The morphology of microemulsion observed by TEM is shown in Figure 3. The micrograph depicted that the microemulsion droplets possessed a spherical appearance and uniform size. A small droplet size of microemulsion provides increased stability against creaming, sedimentation, flocculation, and coalescence. As shown in Figure 4, the mean droplet size of microemulsions measured by the particle detector was 50.20 nm and the polydispersity index values always less than 0.5. The low value of PDI confirmed the actual formation of homogeneous microemulsions. In addition, the pH values observed are considered physiologically acceptable and incorporation of the drug caused no significant change. In the case of conductance, the results indicated that the microemulsions were water-in-oil type due to the fact that oil has low conductance capacity and the electrical conductivity was almost 0 \(\mu\text{s/cm}\). The concentrations of troxerutin in the microemulsions remained almost constant, and no degradation was observed. It is clear from the physico-chemical data that...
no remarkable variation for any parameter was observed for the addition to the formulation of troxerutin. After storage at 4°C, 25°C and 40°C for three months, the optimized troxerutin microemulsion exhibited transparency, clarity and no drug precipitation or color change was observed. The drug content was measured (data not shown) and no significant change was found, suggesting that the microemulsion formulations were stable under the above conditions. In addition, there was no sign of phase separation or breaking or drug precipitation observed for the formulations after the centrifugation, indicating the formulations were physically stable against centrifugation.

Pharmacokinetic studies in rats

Representative chromatograms of troxerutin in rat plasma are shown in Figure 5. Obviously, no significant interference was observed at retention time of troxerutin or the IS. Figure 6 shows the plasma concentration–time profiles of troxerutin microemulsion and solution after oral administration to rats at a dose of 56.7 mg/kg body weight. The concentrations of troxerutin in the microemulsion group at each time interval were found to be higher than that in the solution group. The relevant pharmacokinetic parameters for troxerutin are presented in Table 2. The result shows that AUC0–1 values of solution was 375.65 ± 43.75 h ng/mL and that of the microemulsion was 772.16 ± 113.63 h ng/mL, yielding a relative bioavailability of 205.55%. Troxerutin microemulsion was rapidly absorbed and reached a Cmax of 107.78 ± 21.28 ng/mL at a Tmax of 2.33 ± 0.42 h, whereas, the Cmax for the solution was 61.66 ± 10.46 ng/mL at a Tmax of 1.83 ± 0.28 h after oral administration. In addition, troxerutin microemulsion showed longer t1/2 than solution. Without a doubt, all of these studies demonstrated that the adsorption of troxerutin could be significantly improved by loading the drug into the microemulsion formulations.

As a promising oral delivery system, microemulsions can significantly improve the oral bioavailability of lipophilic drugs by promoting drug solubility, forming mixed micelles, opening tight junctions, and enhancing lymphatic delivery25. However, the mechanism by which W/O microemulsion promotes the absorption of hydrophilic drug is not yet well understood. In our study, the W/O microemulsion significantly increased the oral bioavailability of troxerutin and it may attribute to its promotion of lymphatic transport through transcellular pathway26,27. Furthermore, the lipids and surfactants in the microemulsion can improve the permeability of hydrophilic drug molecules by increasing membrane fluidity to facilitate transcellular absorption and open the tight junctions between cells to allow paracellular transport28,29. Thus, the microemulsion could actually improve the oral bioavailability of troxerutin even if microemulsion could make no contribution on elevating drug solubility.

Permeability of troxerutin in MDCK cell monolayer

The cell culture models, such as Caco-2 and MDCK, are now recognized as an efficient method for assessing membrane permeability properties due to its similarity to human intestinal epithelium30,31. It is already demonstrated that the MDCK cells are similar with Caco-2 cells and have lower TEER values and shorter culture times than Caco-2 cells32,33. Thus, we choose MDCK cell line as the model of epithelial barrier in this study to assess the permeability of microemulsion and solution.
Figure 7 shows the amount of troxerutin concentration from microemulsion and solution across the MDCK cell monolayers. As shown in the figure, the W/O microemulsion significantly increased both A to B and B to A transport of troxerutin across the MDCK cell monolayer compare to the control solution. The Papp values for both formulations were compared and results are shown in Table 3. It shows that the Papp of troxerutin in solution was $1.27 \pm 0.07 \times 10^{-7}$ cm/s (permeability from A to B direction) and $1.44 \pm 0.06 \times 10^{-7}$ cm/s (permeability from B to A direction), and that of troxerutin loaded in microemulsion increased up to $3.64 \pm 0.31 \pm 0.36 \times 10^{-7}$ cm/s (permeability from A to B direction) and $3.93 \pm 0.49 \times 10^{-7}$ cm/s (permeability from B to A direction). For both formulations, $P_{app}$ (B to A) was larger than $P_{app}$ (A to B), indicating that troxerutin may be a substrate of P-gp.

The transepithelial electrical resistance (TEER), as an index of integrity for the cell monolayers, were monitored before and after the transport experiment to confirm the opening of the intracellular tight junctions. As Table 4 showed, experiments performed with the solution caused a similar 10% decrease in TEER values in both directions. In the microemulsion group, TEER values showed a reduction of 14.3 and 16.1% in the apical
to basolateral and basolateral to apical directions, respectively. Compared to the solution, there were statistically significant changes on the TEER values after treatment with the microemulsion. The decrease on TEER values was associated with an increase in permeability of troxerutin and microemulsion can trigger the increase of monolayer permeability. These findings suggest that troxerutin in the microemulsion has higher permeability than when in the solution. This also indicates that the microemulsion is an enhancer of troxerutin permeability.

**Conclusion**

In an attempt to improve the absorption of troxerutin, water-in-oil microemulsion formulations were investigated as possible bioavailability enhancement. A stable W/O microemulsion formulation consists of lecithin, ethanol, isopropyl myristate and water (23.30/11.67/52.45/12.59 w/w) was successfully developed and the mean droplet size was about 50.20 nm. The in vitro and in vivo studies together suggested that the W/O microemulsions developed in this study may serve as a potential delivery vehicle for intestinal delivery of troxerutin after oral administration.

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**Declaration of interest**

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