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An oil-free microemulsion for intravenous delivery of diallyl trisulfide: Formulation and evaluation

Xinru Li[a,1], Yuanting Yue[a,1], Yanxia Zhou[a], Yating Fan[a], Chao Fan[a], Yanqing Huang[b], Fei Wu[a], Yan Liu[a,*]

[a] Department of Pharmaceutics, School of Pharmaceutical Sciences, Peking University, Xueyuan Road 38, Beijing 100191, China
[b] Pharmaceutical Teaching Experiment Center, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China

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ABSTRACT

The aim of the present study was to develop an oil-free o/w microemulsion, Cremophor EL:ethanol–propylene glycol:saline, for diallyl trisulfide (DATS) for intravenous (i.v.) administration to modify the safety and pharmacokinetics of DATS. The ternary diagram was constructed to identify the regions of dilutable microemulsions, and the optimal composition of microemulsion was determined by evaluation of injection safety such as hemolysis, intravenous stimulation and injection anaphylaxis compared to commercial formulation Chentian®. Promising microemulsion with modified injection safety was developed that could incorporate 100 mg/g of DATS. The droplet size of the microemulsion was about 26 nm in diameter with narrow distribution (polydispersity index: 0.14). Acute toxicity test showed that median lethal dose (LD_{50}) of DATS microemulsion was 1.69-fold higher than that of Chentian®. Pharmacokinetics was assessed by comparing with the commercial injection after intravenous administration to rats at a dose of 30 mg/kg. The developed microemulsion showed significant higher area under the drug concentration–time curve and lower clearance and distribution volume than those of Chentian® ($p < 0.05$). This helped DATS to reach higher level in vessel, and circulate in the blood stream for a longer time resulting in better therapeutic effect. In conclusion, microemulsion would be a promising intravenous delivery system for DATS.

1. Introduction

Diallyl trisulfide (DATS) is an oil-soluble sulfur compound that is one of decomposition products of allicin, extracted from garlic and produced by the interaction of the enzyme alliinase with its substrate alliin (S-allyl-L-cysteine sulfoxide) (Stoll and Seebeck, 1951). Its chemical structure and bioconversion pathways of DATS were shown in Fig. 1. About 60% of garlic oil was reported to be DATS, indicating that it is the most prevalent oil soluble garlic constituent. It demonstrated several potential pharmacological activities such as antifungal action (Davis, 2005), antihypertensive action (Mousa and Mousa, 2007), antioxidation (Wu et al., 2001), cardioprotection (Predmore et al., 2010), induced apoptosis in tumor cells (Herman-Antosiewicz et al., 2007; Hong et al., 2000; Kim et al., 2007; Milner, 2001; Shukla and Kalra, 2007; Xiao et al., 2004, 2006), lowing blood cholesterol (Steiner et al., 1996), hypoglycemic effect (Liu et al., 2005), liver protection (Zeng et al., 2008), platelet inhibition (Chan et al., 2003), prevention of endotoxin-induced intestinal damage (Chiang et al., 2006) and anti-HCMV activity (Zhen et al., 2006). However, the optimum method of administering the drug has not been fully established, since DATS has poor aqueous solubility. The currently clinically intravenous dosage form is injection containing high concentration of Tween 80 as solubilization agent. It exhibited severe side effects such as venous irritation which made patients painful and hypersensitivity. Several intravenous dosage forms, such as β-cyclodextrin inclusion complex (Qi et al., 2004), liposome (Wang et al., 2005), submicron emulsion (Guo et al., 2005), have been developed in an attempt to overcome these drawbacks. However, there were two main problems associated with their use. On one hand, none of these could effectively reduce the side effects of Chentian®. On the other hand, the liposome and submicron emulsion formulations had physical instability, and the drug-loading efficiency of inclusion complex was extremely low.

To ensure both higher solubility, lower venous irritation of DATS and better stability of formulations, a suitable carrier was needed. Microemulsion has recently attracted much attention in pharmaceutical research areas (Gan et al., 2009; Tsai et al., 2010; Yin et al., 2009). High thermodynamic and kinetic stability, low viscosity and optical transparency make them very attractive in the pharmaceutical applications. Microemulsions form spontaneously and are composed of surfactant, co-surfactant, oil and water with a particle size of less than 100 nm in diameter. The combination of surfactants with oils to form microemulsions offers an advantage

* Corresponding author. Tel.: +86 10 82801508; fax: +86 10 62015584.
E-mail address: yanliu@bjmu.edu.cn (Y. Liu).
[1] The authors who contributed equally to this work.

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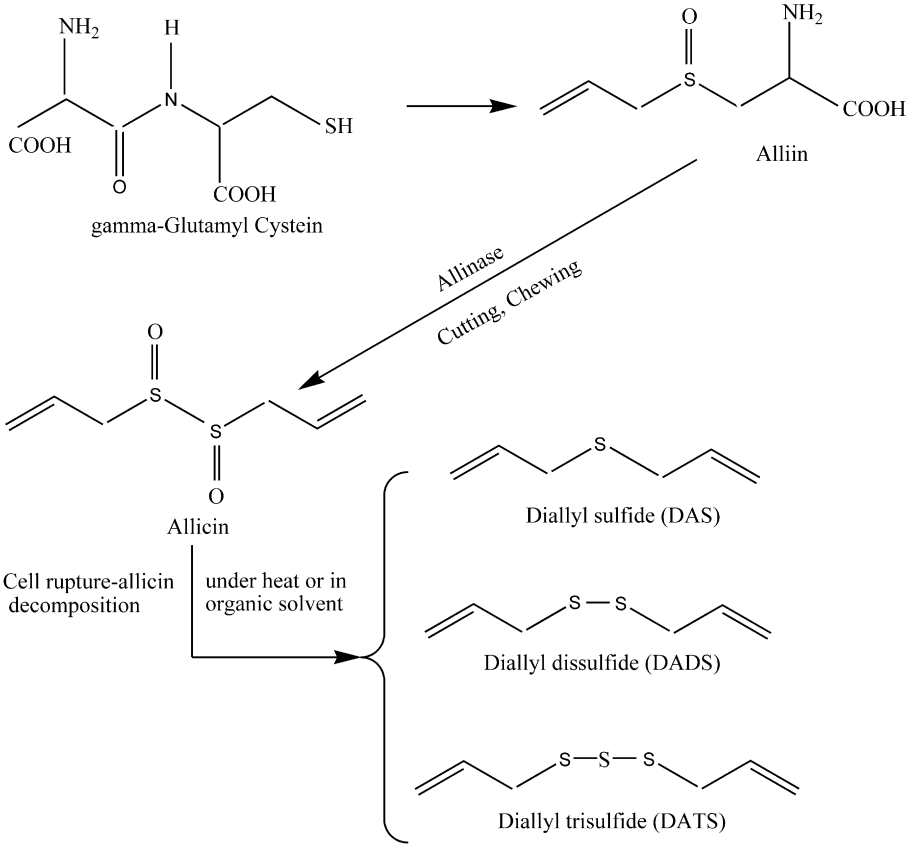

Fig. 1. Bioconversion or chemical action pathway of diallyl trisulfide (DATS).

over a micelle or co-solvent system in terms of drug solubilization capacities for lipophilic compounds, because of the extra locus for solubilization provided by the oil phase (Lawrence and Rees, 2000). This means that the drugs with poor aqueous solubility can be easily loaded and some side effects of drugs can be minimized. Also, because of the small droplet diameter, microemulsions can be sterilized by filtration (Darole et al., 2008), as the non-ionic surfactants contained in the formulation are unable to withstand high temperatures used in autoclaving. All these excellent characterizations of microemulsion may contribute to investigation of intravenous DATS and make the microemulsion be an appropriate carrier for DATS. However, microemulsion has been extensively studied for transdermal (El Maghraby, 2010), intranasal (Li et al., 2002; Zhang et al., 2004), and oral delivery of drugs (Hirunpanich and Sato, 2009; Kawakami et al., 2002; Yin et al., 2009). While, fewer studies have reported the use of microemulsion for the intravenous drug delivery due to the fact that upon dilution in saline, a hazy dispersion will be observed and precipitation of incorporated drugs with clinically unacceptable sizes eventually occurs if microemulsions are not diluted infinitely with saline (Nornoo et al., 2008). Therefore, the application of microemulsions to intravenous administration will be a challenge.

The purposes of the present work were to develop and evaluate an oil-free intravenous microemulsion for oily DATS. Optimal microemulsion formulations composed of DATS/Cremophor EL/ethyl alcohol–propylene glycol/saline were selected regarding the ability of resistance to dilution, reduced amount of surfactant, physical stability and safety such as hemolysis, venous irritation and injection anaphylaxis. The physicochemical characteristics of microemulsion system were investigated for the optimization of microemulsion composition. In addition, acute toxicity and pharmacokinetic characteristic thereof were also evaluated in a hope that side effects would be reduced and physical stability of the formulation would be improved.

2. Materials and methods

2.1. Materials

DATS was obtained from Qingjiang Pharmaceuticals (Jiangsu, China). Commercial DATS injection Chentian® was purchased from Lukang Cisen Pharmaceutical Co., Ltd. (Shangdong, China). Cremophor EL was purchased from BASF (Germany). Propylene glycol (PG) was supplied by Haiwang Fine Chemical Co., Ltd. (Tianjin, China). All the reagents were of the highest purity available and used as supplied.

New Zealand albino rabbits, guinea pigs, Kunming mice and Sprague–Dawley (SD) rats were obtained from the Peking University Health Science Center. All the animals were pathogen free and allowed to access food and water freely. All care and handling of animals were performed with the approval of Institutional Authority for Laboratory Animal Care of Peking University.

2.2. Pseudo-ternary phase diagrams preparation and microemulsion formulation

In the case of our experiment, oily DATS was selected as the oil phase of o/w microemulsion. In our preliminary test, the best solubilization and microemulsifying effect and resistance to infinite dilution were found for DATS/Cremophor EL/ethyl alcohol–PG/saline combinations. The pseudo-ternary phase diagrams of oil (DATS), surfactant (Cremophor EL), cosurfactants (alcohol and PG), and saline were therefore developed using saline titration method to obtain the concentration ranges that can result

in large existence area of microemulsion. Briefly, surfactant was mixed with cosurfactants (alcohol/PG at the mass ratio of 1:1, 2:1 and 3:1, respectively) in fixed mass ratios (K_m, mass ratio of surfactant to cosurfactant) of 1:2 and 1:3. For each phase diagrams at a specific mass ratio of surfactant/cosurfactants, aliquots of each surfactant–cosurfactant mixture (S_m) were mixed with DATS. Then, each mixture was titrated with saline under magnetic stirring or vortexing. The equilibrated samples were assessed visually and determined as being clear and transparent microemulsions, or crude emulsions. The physical states were represented on a pseudo-ternary phase diagram with one axis representing saline, one representing oil and the third representing the S_m. The influence of mass ratio of surfactant to cosurfactant and alcohol to PG on the area of o/w microemulsion region was investigated on the pseudo-ternary phase diagram.

Once the microemulsion region was identified, the microemulsion formulations at desired component ratios were prepared. In order to form the microemulsion, a series of microemulsion were prepared simply through mixing the components with varying ratio of oil, surfactant, and cosurfactants. DATS, ethyl alcohol, PG and Cremophor EL were accurately weighed into glass vials, adding the required mass of saline. The components were then mixed by gentle stirring and vortex mixing until homogeneous mixture formed. The mixture was sealed in a glass vial and stored at room temperature until used. Their physical stability was evaluated by observing the occurrence of phase separation and determining the particle size of microemulsions after stored at 40 °C for 10 days.

2.3. Characterization of o/w microemulsion

2.3.1. Size and size distribution

A certain volume of microemulsion was diluted with saline to a definite volume in a flask and shaken gently to mix thoroughly. Samples were then passed through 0.22-μm pore-size filter before size measurement to remove dust particles. The particle size and polydispersity index (PDI) of so-formed microemulsion were determined by dynamic light scattering (DLS) (Zetasizer ZEN 3600, Malvern, UK) with a scattering angle of 90° at 25 °C. The correlation decay functions were analyzed by the cumulant method to determine the Z-average size. The constrained regularized CONTIN method was used to obtain the particle size distributions. The results were the mean values of three experiments for the same sample.

2.3.2. Transmission electron microscope

The morphology of microemulsion was also observed by transmission electron microscopy (TEM) (JEM-1230, JEOL, Japan). A droplet of microemulsion diluted with saline and stained by a drop of 1 wt% phosphotungstic acid solution was placed on a copper grid with carbon film, followed by removal of the excess fluid with filter paper, and dried for 48 h before examination on TEM at an acceleration voltage of 80 kV.

2.4. Intravenous injection safety assessment

2.4.1. Hemolysis test

The effect of microemulsions on the integrity of erythrocyte membranes was investigated by in vitro hemolysis assay (Zhu et al., 2007). The release of hemoglobin from the erythrocytes (RBC) was used as a measure of toxicity of microemulsions. Briefly, rabbit RBC were separated from 20 ml fresh rabbit blood by centrifugation at 2000 × *g* for 15 min and then washed three times with 20 ml of normal saline. The purified RBC was resuspended in normal saline to obtain 2% (v/v) of RBC suspension. Immediately thereafter, 2.5 ml of the RBC suspension was incubated with 2.5 ml of tested samples at 37 °C for 0.5, 1, 2 and 3 h in an incubator shaker and then centrifuged at 3000 × *g* for 10 min. The percentage of hemolysis was measured by UV–vis analysis of the supernatant at 545 nm absorbance. Normal saline was used as the negative control with 0% hemolysis, and distilled water was used as the positive control with 100% hemolysis. All hemolysis data points were presented as the percentage of the complete hemolysis. Hemolysis percent was calculated according to the following equation:

$$\text{Hemolysis percent } (\%) = \frac{\text{ABS}_{\text{sample}} - \text{ABS}_{\text{saline}}}{\text{ABS}_{\text{distilled water}} - \text{ABS}_{\text{saline}}} \times 100.$$

The developed microemulsions and Chentian® were diluted to 0.24–0.60 mg/ml with normal saline as tested samples and control, respectively.

2.4.2. Intravenous irritation test

Eighteen male New Zealand rabbits weighting 2.0–2.5 kg were divided into six groups. Groups of three rabbits received an infusion of 15 mg/ml DATS microemulsion (formulations I–IV), 15 mg/ml DATS commercial formulation Chentian® (positive control), and an equivalent volume of saline solution into their marginal ear vain (negative control) (Lovell et al., 1994). The rate of infusion was maintained at 1 ml/min and the total drug dose was 8.4 mg/(kg day) for 3 days. Following infusion, visual observations of the site of infusion were made every day. Twenty-four hours after the last administration, three rabbits from each group were killed and a piece of vascular tissue at the site of injection was removed and histological sections were prepared for histopathological examination using an optical microscope (Olympus IX71, Japan) (Lu et al., 2008).

2.4.3. Injection anaphylaxis

Healthy male guinea pigs (250–400 g) were randomly assigned to equal groups: microemulsions (formulations I–IV); negative control (0.9%, w/v, aseptic saline); positive control (10%, w/v, bovine serum albumin solution); Chentian®; and Cremophor EL. The concentration of DATS for microemulsions and Chentian® was 0.12 mg/ml by appropriate dilution with aseptic saline. 0.5 ml of tested solutions was injected intraperitoneally to animals every other day for five times. The body weight of animals was monitored during the test. Ten days after the last administration, animals in each group were injected with a challenge dose of the corresponding solution (1.5 ml) into the vein at the lateral of crus curvilineum (challenge dose was 3-fold of the administration dosage). The anaphylactic response was observed and recorded in 3 h after the challenge injection.

2.5. Acute toxicity assessment

To assess the acute toxicity of DATS microemulsion, the median lethal oral dose (LD_{50}) was determined. Kunming mice (half male and half female, 18–22 g) were housed under normal conditions with free access to food and water. Fifty mice were randomly divided into five groups. Groups of mice received a single intravenous injection of DATS microemulsion via the tail vein at doses of 100, 126, 158, 200, and 252 mg/kg (the volume of each administration was 20 ml/kg), respectively. Clinical symptoms including mortality, clinical signs, and gross findings were observed once a day for 15 days. On day 15, the mice were sacrificed and examined by necropsy. Gross histological examinations of the major organs were carried out after dissection. The LD_{50} was calculated using the Bliss method. As control, the LD_{50} of Chentian® was tested as described above, the doses of DATS were 79, 96, 120, 152 and 192 mg/kg, respectively.

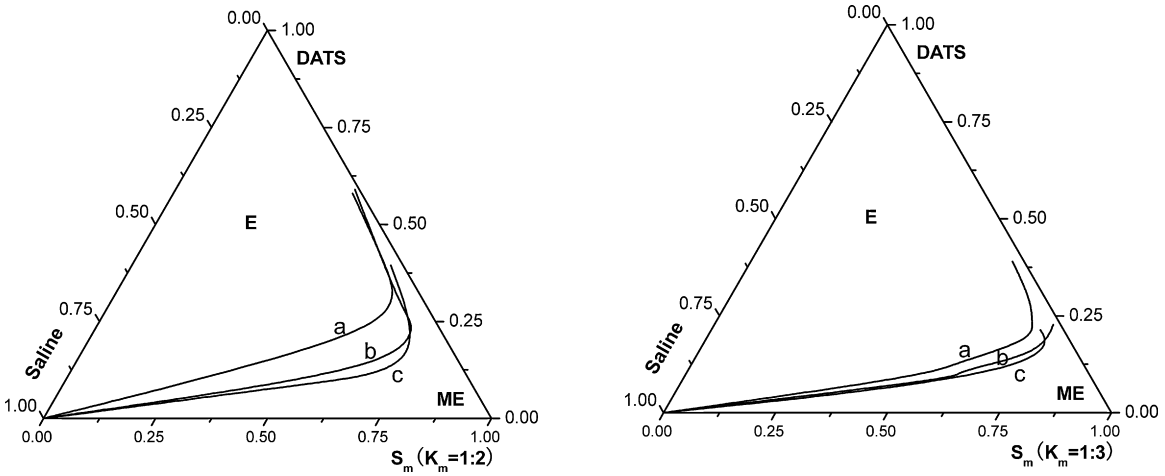

Fig. 2. Pseudo-ternary phase diagrams composed of diallyl trisulfide (DATS), Cremophor EL, alcohol and propylene glycol (PG), and saline. The ratio of alcohol to PG: a, 3:1; b, 2:1; c, 1:1 (w/w). K_m, mass ratio of Cremophor EL (surfactant) to alcohol/PG (cosurfactant). S_m, the mixture of surfactant and cosurfactant. E, emulsion; ME, o/w microemulsion.

2.6. Pharmacokinetics evaluation

Eight male SD rats weighing 205 ± 15 g, fasted for 12 h prior to the experiments but allowed free access to water, were divided into two equal groups. Groups of rats received a single intravenous injection of either Chentian® or microemulsion at a dose of 30 mg/kg (calculated according to the skin surface area conversion table based on the clinical dose for human) through the tail vein. 0.5 ml of blood samples were taken into heparin treated tubes at predetermined time intervals after drug administration. The plasma was immediately collected by centrifugation at 8000 rpm for 10 min and stored at −20 °C until analysis.

Liquid–liquid extraction procedure for plasma samples was as follows: in a 5 ml polypropylene screw-capped conical tube was added 200 μl of plasma followed by 60 μl of 10% (w/v) trichloroacetic acid aqueous solution. The mixture was vortexed for 1 min to remove the precipitated protein and then 400 μl of n-hexane was added. After vigorous vortex mixing for 3 min followed by centrifugation at 4000 rpm for 10 min, an organic layer was collected and a 20 μl was injected the HPLC system for subsequent analysis.

A modified HPLC/UV method was employed to determine DATS level in rat plasma. The Waters series HPLC system (Waters 1525 pump, Waters, USA) was equipped with a UV detector (Waters 2487) and reversed phase column (ODS C18, 5 μm, 4.6 mm × 250 mm, Dikma, China). The UV absorbance was detected at 218 nm. The mobile phase was composed of acetonitrile:deionized water:tetrahydrofuran (70:27:3, v/v/v). The mobile phase was pumped at a flow rate of 1.0 ml/min. Acceptable results with respect to precision and accuracy were obtained for the analyte. The limit of detection (LOD) was 35.3 ng/ml.

The pharmacokinetic parameters including area under the drug concentration–time curve up to last time (AUC), mean residence time up to last time (MRT), apparent volume of distribution at steady state (V_{ss}) and total clearance (CL) were assessed by using non-compartmental analysis method.

2.7. Statistical analysis

All the data are presented as mean ± standard deviation (SD). The statistical significance of differences was determined by one-way ANOVA using SPSS for Windows versions 13.0 (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Phase diagram studies

Drug-containing microemulsions should be dilutable with water without causing precipitation of the drug incorporated during clinical use (Kiepert, 1989; Lawrence and Rees, 2000). Hence the selection of appropriate surfactants is critical for oil-free o/w microemulsion to load oily drugs, which was based on their suitability for intravenous microemulsions, their ability to incorporate large amounts of oily drugs into microemulsions. Based on these criteria, Cremophor EL was selected as surfactant.

In the second stage of formulation development, it was found that the surfactant/cosurfactant blends Cremophor EL:ethanol/PG (3/1, 2/1, 1/1) in ratio of 1:2 and 1:3 were able to incorporate the largest amounts of water when combined with DATS.

The goal in the formulation of an o/w microemulsion was to have the lowest possible surfactant content with an optimal solubilization of the lipophilic components. The pseudo-ternary phase diagrams were therefore utilized in the third stage of development to determine regions of microemulsion formation, from which a large number of potential microemulsions were identified. Phase diagrams were constructed for Cremophor EL:ethanol/PG (1:2 and 1:3) (Fig. 2), when combined with DATS to further characterize these microemulsion systems. In the present study, only the boundary between the single and multi-phase region were identified. The regions to the right of the boundary lines were transparent o/w microemulsion regions, and to the left turbid emulsion regions. The area of microemulsion region increased with increasing ratio of alcohol to PG when K_m was constant, indicating that the increase of ethanol content was beneficial to the incorporation of DATS in microemulsions, and the viscosity of the microemulsion formulation visually decreased. When the short chain alcohol, ethanol is introduced, the polar solvent (water) becomes less hydrophilic. Also, because the ethanol will partially be incorporated in the polar parts of the interface, there is an increase in the area of each polar head group of surfactant molecules. As a result, the spontaneous curvature of the interface towards the oil changes, thereby decreasing the stability of the lamellar liquid crystalline phase and increasing the isotropic single phase region (Gradzielski, 1998) that was seen with the studied system. In addition, the area of microemulsion region became larger with increasing K_m at each ratio of alcohol to PG, which was consistent with previous report (Zhang et al., 2004). In conclusion, promising oil-free microemul-

Table 1
Formulation compositions of the investigated microemulsions except saline.

Formulation	Ethanol:PG	K_m	S_m:DATS
I	3:1	1:2	9:1
II	3:1	1:3	9:1
III	2:1	1:2	10.1:1
IV	2:1	1:3	10.1:1

sions with resistance to infinite dilution by saline were developed in this study into which DATS could be incorporated.

Based on these results, several dilutable microemulsions with favorable physical stability (data not shown) and higher incorporated drug content could be identified in these regions. These microemulsions were to be used as drug delivery systems for DATS to be administered intravenously. Hence, an optimal composition necessary to form a microemulsion with limited toxicity was chosen. As a result, the ratios of the components that were used in microemulsions to determine the intravenous injection safety potential were identified as formulations I, II, III and IV (Table 1), in which DATS was incorporated 100, 100, 90, 90 mg/g, respectively.

3.2. Intravenous injection safety assessment and optimization of microemulsion formulation

3.2.1. Hemolysis test

The ability of any nanoproduct to cause hemolysis after parenteral delivery is one of the most restrictive properties in all pharmaceutical applications. The hemolytic potential of the injectable forms has generally been found to correlate with the severity of lesions (Bjerregaard et al., 2001). Therefore, the disruption of erythrocytes is a major barrier to in vivo application and the hemolytic activity has been suggested as a toxicity screen in vitro, serving as a simple and reliable measure for estimating the membrane damage caused by formulations with surfactants or solvents. In the case of our experiments, the complete hemolysis was clearly observed for positive control at 15 min presenting as the red clear-diaphanous, and no erythrocyte survived at the bottom of the tube. While the erythrocyte precipitated at the bottom of the tube for negative control and tested samples with different concentration of DATS and redispersed after shaking within the 3 h observation, indicating that increasing the incubation time led to a negligible change in the membrane damage induced by microemulsions. The hemolysis percent of microemulsions and Chentian® was shown in Fig. 3. The hemolysis percent was below 1% and altered insignificantly with the incubation time ($p > 0.05$). In addition, relatively insignificant differences in hemolysis between nanoparticles obtained from the various microemulsion templates were

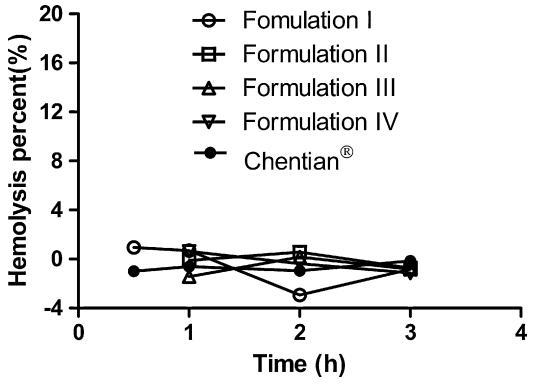

Fig. 3. Variation of hemolysis percent of the prepared DATS microemulsions and commercial formulation Chentian® with incubation time in the erythrocytes at 37 °C ($n = 3$).

observed, so was between microemulsions and Chentian®. Considering that microemulsions would be injected intravenously and, therefore, their concentration in the serum would be smaller than in vitro experiments, it could assume that the obtained microemulsions could be relatively safe carriers of DATS in the circulation.

3.2.2. Intravenous irritation assessment

The DATS microemulsions were evaluated in the rabbit vein irritation test relative to Chentian® as the positive control and aseptic saline as the negative control. After a 3-day administration of microemulsions, Chentian® and 0.9% saline solution, there was a slight vascular injury in all groups including aseptic saline and microemulsion-treated rabbits at the injection site related to trauma associated with venipuncture (Wang et al., 1999). Nevertheless, there was no obvious visible erythema, edema and tissue necrosis along marginal ear vein for microemulsion-treated or negative group, while slight reddish discoloration was observed in the positive control group. It was evident from the histopathological examination results presented in Fig. 4 that no thrombus, swelling or hyperplasia of endothelial cells was appeared in the blood vessel. In addition, no apparent pathological changes could be observed such as hemorrhage, thrombosis, necrosis and inflammatory cell infiltrate in the vessel wall and surrounding tissues for all microemulsion-treated groups compared with the saline control. The histopathology examination results of the rabbit ears administrated with microemulsions were similar to those of the positive control group. These results indicated that no intravenous irritation was induced in the ear vein of rabbit after intravenous administration of microemulsions, and Chentian® produced intravenous irritation to some extent.

3.2.3. Injection anaphylaxis

All the groups of the guinea pigs were eucrasia and their body weight increased continuously and significantly (data not shown) during and after the administration. After challenge, in positive control, significant anaphylaxis symptom such as dyspnea, convulsion and aconuresis was observed and all the animals died. The negative control group did not respond to the last challenge. However, Chentian® caused positive anaphylaxis with quick, frequent and sustained symptom which might be caused by free DATS itself. As anticipated, the microemulsions treated groups exhibited weakly positive anaphylaxis and formulation II had a favorable performance with slightest and negligible anaphylaxis among the total, which was attributed to the encapsulation of DATS into the inner core of microemulsions. These results showed that a significant hypersensitivity reduction was obtained with microemulsions compared with Chentian®.

Nevertheless, it was worth noting that the clinical acute hypersensitivity reaction is the severe side effect of paclitaxel formulation Taxol® caused by existed Cremophor EL, which was characterized by dyspnoea, flushing, rash, chest pain, tachycardia, hypotension, angio-oedema, and generalized urticaria. Despite premedication, consisting of high-dose corticosteroids, H1 and H2 antagonists, minor reactions (flushing and rash) still occur in 41–44% of all patients and major, potentially life threatening reactions in 1.5–3% (Eisenhauer et al., 1994; Rowinsky et al., 1993; Weiss et al., 1990). It was reported that the Cremophor-induced complement activation in human serum was clearly concentration-dependent with a minimum activating Cremophor EL level in the order of 2 ml/ml (i.e. about 2.1 g/ml), a concentration readily achieved clinically in the plasma following standard doses of paclitaxel (Gelderblom et al., 2001; van Zuylen et al., 2000). In the case of our experiment, the dose of Cremophor EL was 0.06 mg for each guinea pig, being equivalent to be only 1.94 mg for human, which was remarkably lower compared with that reported early

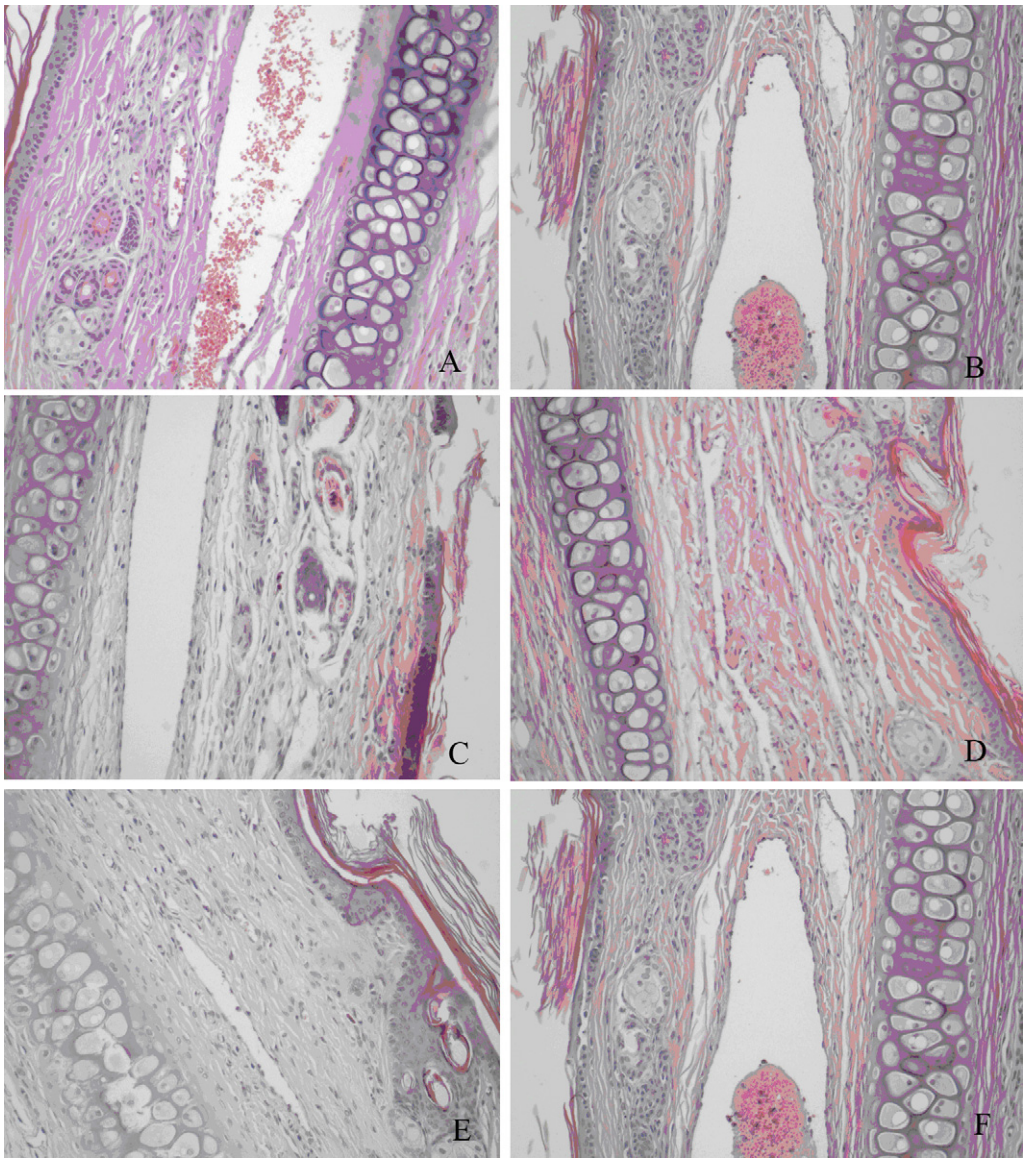

Fig. 4. Optical microscopic images of histopathology slides of rabbit ear-rim auricular vein following different infusions: (A) formulation I; (B) formulation II; (C) formulation III; (D) formulation IV; (E) commercial formulation Chentian®; (F) aseptic saline.

(Gelderblom et al., 2001). Therefore, the slight and negligible hypersensitivity reaction might be attributed to the lower concentration of Cremophor EL in microemulsions. This was also confirmed in our study that the same concentration of Cremophor EL contained in microemulsions did not cause hypersensitivity.

Based on these results, formulation II, consisted of DATS 10% (w/w), Cremophor EL 22.5% (w/w), ethanol 50.6% (w/w), PG 16.9% (w/w) and saline, was selected as the favorable microemulsion for the subsequent studies.

3.3. Characterization of o/w microemulsion

Biopharmaceutical characteristics and preparation stability are directly related to the physical properties of microemulsions, including the microstructure, droplet size, and location of the drug molecule in the microemulsion (Lawrence and Rees, 2000). As seen from Fig. 5, the mean droplet size of the optimized o/w microemulsions was 26.6 nm in diameter with narrow distribution (PDI = 0.14). The morphology of microemulsion was shown in Fig. 6. The images clearly indicated the presence of homogeneous and spherical shape of microemulsion droplets.

3.4. Acute toxicity assessment

Mortality, clinical signs of mice, was measured for 15 days. The acute toxic symptoms such as jump, tachypnea, spasm, severe prostration, apathy, respiratory distress, and catatonia were observed

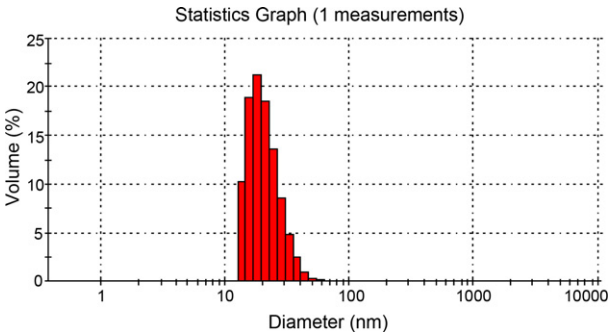

Fig. 5. Particle size distribution of the optimized DATS microemulsion (formulation II) determined by dynamic light scattering (DLS).

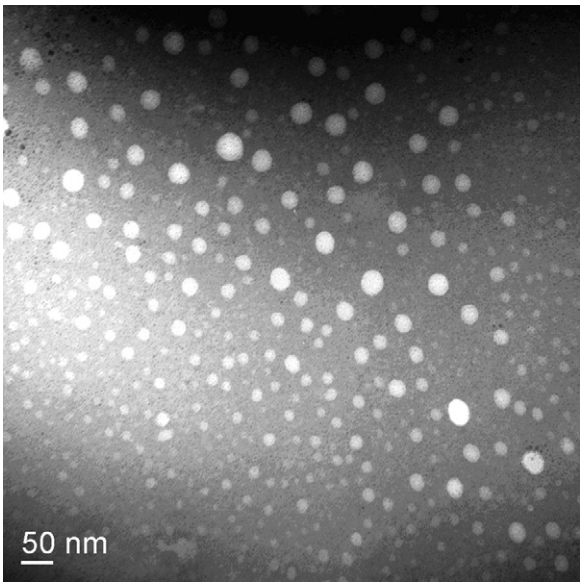

Fig. 6. Transmission electron microphotography (TEM) of the optimized DATS microemulsion (formulation II).

and several mice died after administration throughout the 15 days observation period. Moreover, histopathological analysis of various organs for survivals did not show any significant pathological changes. The dose-toxicity relationship for the two formulations was shown in Fig. 7. Based on these results, the LD_{50} of DATS microemulsion and Chentian® was calculated to be 185.6 and 110.9 mg/kg, respectively. The LD_{50} of DATS microemulsion was 1.69-fold higher than that of Chentian® ($p < 0.05$), suggesting that the DATS microemulsion had lower toxicity and higher safety compared with Chentian®. Noticeably, pulmonary blood clot was observed for died mice which showed dose-dependence (data no shown). This might be attributed to the rapid distribution of DATS after administration as reported earlier (Wang, 1988) and favorable to a certain extent in some conditions. Recent reports revealed that DATS prevented development of poorly differentiated prostate cancer and pulmonary metastasis multiplicity in mice and selectively caused Bax- and Bak-mediated apoptosis in human lung cancer cells (Singh et al., 2008; Xiao et al., 2009).

3.5. Pharmacokinetics evaluation

The pharmacokinetics of DATS microemulsion (formulation II) was investigated and compared with Chentian® after intravenous administration to rats. The mean plasma concentration–time profiles of microemulsion and Chentian® were shown in Fig. 8 which presented a relatively fast distribution phase (0–0.5 h) followed by a much slower elimination phase (3–36 h). Also, it could be seen from Fig. 8 that the elimination shown by the two formulations curves were similar and there was slower distribution for microemulsion compared with Chentian®. This was likely due to the fact that after intravenous administration, Chentian® is rapidly distributed and penetrated into tissues while the drug in microemulsion needed more time to be released from the oil phase into blood. Furthermore, the paradoxical plasma concentration–time profiles of the two formulations were obtained and in agreement with the previous report (Sun et al., 2006), which indicated the complicated disposition of DATS in vivo. It was reported that DATS was rapidly distributed and penetrated into lung after intravenous administration and then into blood (Sun et al., 2005), which might explain the phenomenon why pulmonary blood clot was seen in mice after intravenous administration in acute toxicity test.

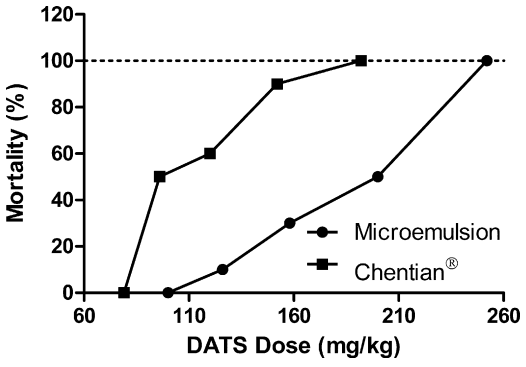

Fig. 7. Mortality of mice for the optimized DATS microemulsion (formulation II) (●) and commercial formulation Chentian® (■) by once intravenous administration in 15 days in acute toxicity test ($n = 10$).

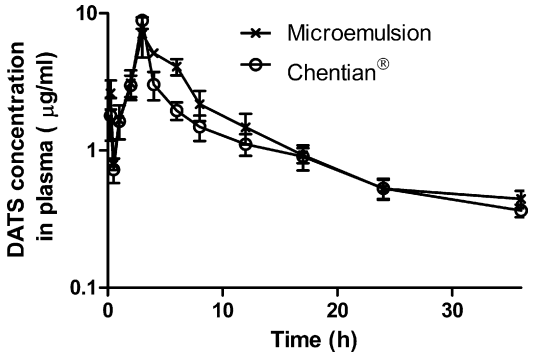

Fig. 8. DATS plasma concentration vs. time curve after intravenous administration of the optimized DATS microemulsion (formulation II) (×) and commercial formulation Chentian® (○) to fasted SD rats at a dose of 30 mg/kg ($n = 4$).

The main pharmacokinetic parameters were calculated based on non-compartment analysis method and summarized in Table 2. On one hand, half of the parameters showed no significant differences between microemulsion and commercial formulation such as T_{max}, C_{max} and MRT ($p > 0.05$). On the other hand, the microemulsion had a 1.24-fold increase in AUC compared to Chentian®, while apparent volume of distribution (V_{ss}) and clearance (CL) after administration of microemulsion were 1.37 and 1.20 times lower than those of Chentian®, respectively ($p < 0.05$). This result was consistent with the result of cinnarizine (Shi et al., 2010). This phenomenon could be explained by the fact that, after i.v. Chentian®, DATS underwent rapidly distribution and penetration into tissues, while in the case of microemulsion, since the drug is located in the inner phase, this structure would delay penetration and distribution into tissues for DATS. Therefore, microemulsion produced higher plasma concentrations together with significantly lower V_{ss} and CL than Chentian®, while the AUC of microemulsion was

Table 2
Pharmacokinetic parameters of DATS after intravenous administration of microemulsion and commercial preparation Chentian® to rats at a dose of 30 mg/kg ($n = 4$).

Parameter	Microemulsion	Chentian®
T_{max} (h)	3.00 ± 0.00	3.00 ± 0.00
C_{max} (μg/ml)	7.06 ± 2.30	8.85 ± 1.15
AUC_{0-36} (μg·h/ml)	55.68 ± 4.7*	45.01 ± 5.49
MRT (h)	9.89 ± 0.63	10.28 ± 0.55
CL (ml/h/kg)	16.09 ± 1.58*	19.35 ± 1.86
V_{ss} (ml/kg)	232.70 ± 10.10*	319.8 ± 51.6

AUC, area under the concentration–time curve; MRT, mean residence time; CL, clearance; V_{ss}, steady-state apparent volume of distribution.
* $p < 0.05$ vs. commercial preparation.

significantly higher than that of Chentian®, which might suggest that the availability of microemulsion was increased. These data demonstrated that the microemulsion might enhance the therapeutic efficiency along with decreasing the side effects of DATS. Also, microemulsion in this study could reduce vein irritation and hypersensitivity while increasing patient compliance. Therefore, microemulsion would have a great potential for clinical applications. Nevertheless, more detailed studies on microemulsion need to be further conducted such as chemical stability.

4. Conclusions

In summary, the main difficulty associated with the study was the venous irritation and poor stability of Chentian®. This problem could be solved by loading the drug in the oil-free o/w microemulsions. Formulations for intravenous DATS were developed by considering the better physical stability, higher incorporated drug content, the lowest possible surfactant content, infinite dilution with water and intravenous injection safety, and the optimum one consisted of DATS 10% (w/w), Cremophor EL 22.5% (w/w), ethanol 50.6% (w/w), PG 16.9% (w/w) and saline. Except for the excellent solubility, physicochemical stability, the developed microemulsion could offer improved pharmacokinetics by increasing the bioavailability and reducing toxicity compared with the Chentian®, suggesting that oil-free o/w microemulsion formulation in this study might have a great potential as an intravenous delivery system for DATS for clinical applications and could be produced on an industrial scale.

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