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Research Article

Emulsifiers' Composition Modulates Venous Irritation of the Nanoemulsions as a Lipophilic and Venous Irritant Drug Delivery System

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Abstract. In this study, a nanoemulsion (NE) system was investigated for intravenous delivery of lipophilic and venous irritant drugs. NEs were prepared to deliver diallyl trisulfide (DT) for systemic therapy of bacterial and fungal infection, egg phospholipid was chosen as the main emulsifier, and two co-emulsifiers were also incorporated, including Poloxamer 188 (P188) and Solutol HS 15 (S15). Soybean oil was used as the dispersed phases, forming stable DT NEs with small particle sizes. The venous irritation of DT NEs was evaluated by *in vitro* human umbilical cord endothelial cells (CRL 1730) compatibility model with the intracellular adenosine triphosphate (ATP) and guanosine triphosphate (GTP) concentrations as the indices. The intracellular ATP and GTP reduction changed with the incorporation of a variety of co-emulsifiers, which varied in a free DT concentration-dependent manner. It was deduced that the free DT concentrations of NEs containing co-emulsifiers were determined by the partition coefficient of DT between oil and surfactant buffer solution. In conclusion, NE was an appropriate delivery system for lipophilic and venous irritant drug, and optimization of the composition of emulsifiers was an effective method to alleviate the venous irritation of DT NEs.

KEY WORDS: diallyl trisulfide; emulsifier; nanoemulsion; venous irritation.

INTRODUCTION

Emulsions with droplet size in the nanometric scale (typically in the range 20–200 nm) are often referred as nanoemulsion (NE), which was only a kinetically stable non-equilibrium system (1,2). Lipid NE has been widely used in pharmaceutics because of the controllable particle size, powerful solubilization ability, and perfect biocompatibility *etc.* (3,4). During the past several decades, as the parenteral drug delivery system, lipid NE has significant advantages including reducing injection pain and venous irritation of many drugs (5–7) and less chance of drug precipitation upon administration at the injection site (4,8). Taking these advantages into account, lipid NE has always been popular in intravenous delivery of lipophilic and venous irritant drugs, such as propofol (9) and clarithromycin (10).

Diallyl trisulfide (DT) is an organo-sulfur compound from garlic (*Allium sativum*), and it is commercially available as a preparation in China, known as Dasuansu, which is prescribed for the treatment of bacterial infections (11–13) and systemic fungal infections (14). However, DT has extremely low aqueous solubility. The current commercial formulation of DT (DT IV solution) employs 15 mg/mL DT in a Tween 80 and propylene glycol solution, but this formulation has been often complained of venous irritation and occasional thrombophlebitis (15). In our preliminary experiments in rabbits, when DT is loaded in the standard emulsion (10% Intralipid®), however, the venous irritation of DT NE only lessens compared to the DT solution. Venous irritation is a common side effect of intravenous therapy, and its pathogenesis is not fully understood; the incidence and duration of venous irritation appear to be dependent on a variety of factors. Chemical factors such as low pH (<5), hypertonicity, and the inherent nature of the drug have been shown to influence venous irritation (5). Physical factors such as the existence of particulates and precipitation of the drug out of solution on dilution are also known to result in venous irritation (8). Clinical factors involving injection technique (extravasation, type of needle, duration of infusion, *etc.*) can also contribute to the occurrence of venous irritation (16). The most prevalent opinion is that chemical irritation of the endothelium leads to a sterile inflammation (5,17), and moreover, many studies disclosed that the venous irritation of drug-loaded oil-in-water NE should be attributed to the free drug in the aqueous phase (18–20). These findings suggest that modulation of the free DT concentration was an effective method to reduce the venous irritation of DT NE for intravenous delivery. The free drug concentration has been shown to be affected by the following factors: drug partition coefficient, interfacial barriers, droplet charge, average droplet size, and size distribution (21–23).

The lipid NE was very sensitive to the pH changes and the presence of electrolytes, which was stabilized using phospholipids as the only emulsifier (4). A combination of

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phospholipids with the nonionic copolymer surfactant (Poloxamer 188, Solutol HS 15, *etc.*) constructed sterically stabilized NEs (24). However, the concentrations of the surfactants presented in oil-in-water NEs are usually higher than that necessary to form monolayer coverage of the dispersed oil droplets. These excess surfactants, which would be in the form of monomers, micelles, and liquid crystals, may affect drug-partitioning coefficient through micellar solubilization and change the emulsion droplet interfacial film characteristics, thus affecting the free drug concentration (21,22).

The aim of this work is to study the effects of co-emulsifiers (Poloxamer 188 and Solutol HS 15) on the venous irritation of lipid NEs for intravenous delivery of DT with extremely lipophilic and serious venous irritant characteristics. The mechanism of venous irritation of DT NEs is also elucidated in this paper.

MATERIALS AND METHODS

Materials

DT (purity >99%) was provided by Qingjiang Pharmaceutical Corp., China. Soybean oil was purchased from Tieling Beiya Pharmaceutical Corp., China. Egg lecithin (Lipoid E 80®), and oleic acid were provided by Shanghai Dongshang Corp. (Shanghai, China). Glycerol, propylene glycol, and Tween 80 were obtained from Shanghai Chemical Reagent Corporation, China. Poloxamer 188 and polyoxyethylene-660-12-hydroxystearate (Solutol HS15®) for injection were kindly donated by BASF (BASF (China) Co., Ltd). Double-distilled water was used for all preparations. Other chemicals were of high-performance liquid chromatography (HPLC) or analytical grade.

Preparation of DT Nanoemulsion

A high-energy homogenization method was applied to prepare the drug-loaded NEs (3). The oil phase was obtained by dissolving DT (1.50 g), oleic acid (0.03 g), and egg lecithin (1.20 g) into the soybean oil (10.00 g), and the aqueous phase was prepared by dissolving 2.25 g glycerol and co-emulsifier into water (85.0 mL) under magnetic stirring. Then, the oil phase was added dropwise to the aqueous phase, followed by shear using a high-shear mixer (Model 1001, Shanghai Weiyu Corp., China) at 8,000 rpm for 15 min, and then the coarse emulsion was obtained. The coarse emulsion was further homogenized with a high pressure homogenizer (APV 2000, Denmark), and the NE containing DT were prepared at four cycles and 1,000 bars homogenization pressures. Subsequently, the pH values of the resulting NEs were adjusted to 7±0.05 using 0.1 mol/L sodium hydroxide solution because DT is only stable in a pH value scope between 5.00 and 7.00. The control DT NE without co-emulsifiers and the control NE without DT were prepared under the same condition mentioned above.

In order to get control DT IV solution, stock solution containing 1.50 g DT was prepared with the mixture of 9.00 g Tween 80 and 10.00 g propylene glycol; next, the stock solution was added to the water drop-by-drop under a magnetic stirring at ambient temperature, and then the solution was diluted to 100 ml.

The final DT NE and IV solution were packaged in 20 ml ampules and sealed after nitrogen purging, then sterilized by rotating water bath at 100°C for 30 min. All formulations were carried out in triplicate. In addition, the process of shear, homogenization, and vials filling and sealing were conducted in nitrogen atmosphere.

Vesicle Size and Zeta Potential

The mean particle size and zeta potential of DT NEs were measured by photon correlation spectroscopy (Nano ZS 90, Malvern Instruments, UK). The measurements were performed at 25°C using a He-Ne laser, and the scattering angle was settled at 90°. DT NEs were diluted 40 times with double-distilled water before measurement, and three samples were prepared for one NE.

Stability of Nanoemulsions

The physical stabilities of DT NEs were evaluated by observing the homogenicity and measuring of the particle size at 4°C for 3 months. The centrifuge test of 15 min at 4,000 rpm was also carried out to assess the physical stability of the DT NEs.

Endothelial Cell Compatibility of DT Nanoemulsions

Cell Cultures

Human umbilical cord endothelial cells (CRL 1730, ATCC) at passage 12 were grown following the standard protocol (25). The cells at passages 16 to 20 for experiments were cultured at a density of 2×10^4 cells/cm^2 in 35 mm diameter dishes.

Incubation with Nanoemulsion

Confluent cells for irritation tests were obtained after 4 days and counted using a hemocytometer. Test solutions were prepared by diluting the samples four times using phosphate-buffered saline (PBS). Medium was discarded from confluent cells, and the cells were washed twice with PBS (pH7.4); then, test solutions (1 mL) were added and cells incubated for 30 min at 37°C. Control cells were exposed to PBS alone. Next, the cells were washed twice with PBS after test, and control solutions were removed. Thereafter, cells were lysed by addition of 150 μL cold 0.42 mol/L perchloric acid (PCA) solution and stored at −20°C for 30 min. Finally, the cell lysate was neutralized with 150 μL cold 1 mol/L potassium phosphate dibasic solution (pH>7.2). After centrifugation, 250 μL supernatant was collected and immediately frozen and stored at −20°C until chromatographic analysis.

Determination of Intracellular ATP and GTP via HPLC

Separation and quantification of adenosine triphosphate (ATP) and guanosine triphosphate (GTP) in the PCA extracts were made by reverse-phase-HPLC essentially as previously described (26). The concentrations of ATP and GTP were quantified by determining the ratio of peak areas

in relation to corresponding standards. The linear range for all two nucleotides was between 0.75 and 50 μmol/L. Then, the percentage of intracellular ATP and GTP reduction were determined for each formulation with the following equation: $\%\,(\text{ATP or GTP reduction}) = (C_{\text{control}} - C)/C_{\text{control}} \times 100$, where C is the ATP or GTP concentration of cells incubation with the formulation, and C_{control} is the ATP or GTP concentration of cells incubation with PBS control.

Determination of the DT Concentration in the Aqueous Phase of Nanoemulsions

The DT concentrations in the aqueous phase of NEs were determined through reverse dialysis bag technique (20,23,27). Test solutions were prepared by diluting the DT NEs 1 mL in 4 mL with PBS. One hundred milliliter of test solutions were placed into Erlenmeyer flask (150 mL) with magnetic stirring; three dialysis bags (MWCO 3500, Biotech, China) containing 1 mL of 2.5% glycerol solution were immersed. These sacs were equilibrated with the glycerol solution for 12 h prior to experiment. After equilibration for 24 h at ambient temperature, the dialysis bags were withdrawn, and the concentration of DT in the contents was assayed by HPLC (Agilent, Model 1100, USA), equipped with a UV detector set at 240 nm and a reversed phase column (Elite-C_{18}, 5 μm, 4.6 mm ID×25 cm, China). The mobile phase was a mixture of water and methanol (20:80, *v*/*v*), and the flow rate was 1.0 mL/min. Preliminary experiments were conducted to determine the time to reach equilibrium. Samples were collected and analyzed at 3, 6, 12, 24, 36, and 48 h points. It showed that the equilibrium was achieved within 24 h. Each sample was measured in triplicate.

Determination of Partition Coefficients Between Oil and Surfactant Solutions of DT

Twenty milliliter of soybean oil containing DT was kept in contact with 20 mL of pH7.0 phosphate-buffered surfactant solution in a 100-mL vial at 25°C for 48 h. Preliminary experiments were conducted to determine the time to reach equilibrium. Samples were collected and analyzed at 24, 48, 72, and 96 h, and it showed that equilibrium was achieved within 48 h. After equilibrium, the two phases were separated by centrifugation, and the aqueous samples were assayed using HPLC. These experiments were repeated three times.

Data Analysis

Statistical analysis of differences between different treatments was performed using analysis of variance (ANOVA) test, using Statistical Package for the Social Sciences (SPSS) 12.0 for windows (SPSS software, LEAD technologies, USA). $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Physicochemical Characteristics and Stability of Nanoemulsion

The major components of NE contain 1.2% (*w*/*v*) of egg lecithin, co-emulsifiers, 10% (*w*/*v*) oil, 0.03% (*w*/*v*) oil acid, 2.25% (*w*/*v*) glycerol, and 86.5% (*v*/*v*) double-distilled water.

The effect of the co-emulsifiers (P188 and S15) on the particle size of the DT NEs is presented in Table I. DT NE and blank NE have similar average diameters and polydispersity index. The addition of P188 and S15 to phospholipids leads to a significant decrease in the particle size of DT NE ($p<0.05$), but the particle size slightly increase with the increase of the P188 and S15 concentration. The additional nonionic co-emulsifiers localized in the oil/water interface area of each oil droplet and compressed the egg phosphatidylcholine, leading to a more flexible phospholipids film. The increase of P188 and S15 concentration resulted in a slight increase of the particle size, which may be due to the formed hydrophilic chain layer that covers the phospholipids layer (28).

The absolute zeta potentials of the NEs with co-emulsifiers are shown in Table I. The addition of nonionic co-emulsifiers results in a slight decrease of the zeta potential of DT NE. In these NEs, the anionic fractions in egg lecithin such as phosphatidylserine, phosphatidic acid, phosphatidylgycerol, phosphatidylinositol, and 80% phosphatidylcholine are responsible for the negative surface charges under weak acidic environment (29). P188 and S15, which are nonionic polyoxyethylene surfactant with positive charge, might interact electro-statically with phosphoric head groups (30).

All DT NE systems are physically stable at 4°C during the 3 months. Phase separation has not been observed, and no change of particle sizes occurred during the period. The centrifugation tests also show that all DT NE systems have good physical stability. The good stability of DT NE systems may be due to the small particle size of droplets and the steric stabilization effects (28), and the electrostatic repulsive forces due to the high negative zeta potentials also contribute to the stability of DT NEs (29).

Endothelial Cells Compatibility of DT Nanoemulsions

There were a serial of methods to evaluate the venous irritation of parenteral drug formulations, such as *in vivo* rabbit ear model, *in vitro* cell toxicity test, and *in vitro* hemolysis test (8,31). The *in vitro* cell toxicity test was appreciated for its accurate assessment and no perplexity of animal ethics. The *in vitro* cell tolerance model (HUVEC CRL 1730. *etc.*) was appreciated for its accurate assessment and no perplexity of animal ethics (25,32,33). By determining the intracellular contents of ATP and GTP of the treated cells, intact endothelial metabolism was examined (32–34).

The effects of DT NEs made of egg lecithin and DT solution on intracellular ATP and GTP levels are presented in Fig. 1. One-way ANOVA shows that the control DT NEs and the DT solution significantly reduce the intracellular ATP and GTP ($p<0.01$), and the percents of the ATP and GTP reduction due to the DT solution are significantly higher than that of the control DT NEs ($p<0.01$). However, the blank NE only reduces the intracellular ATP and GTP slightly. This suggests DT is responsible for the endothelial cells damage, and encapsulation of DT in NE could shield the endothelial cells from the cytotoxicity of DT (35). The report that 50 μg/mL DT in the medium might adversely affect the viability of the hepatocytes also corroborated the venous irritant nature of DT (36).

Table I. The Particle Size and Zeta Potential Characterization of DT Nanoemulsions

Nanoemulsions	Size (nm)	Polydispersity index	Zeta potential (mV)
Control blank NE	245.7±7.4	0.07	−63.2±2.3
Control DT NE	248.5±8.3	0.06	−64.9±3.1
Additives of co-emulsifiers			
+Poloxamer 188 (0.2%) DT NE	174.4±1.6	0.09	−61.3±2.1
+Poloxamer 188 (0.4%) DT NE	178.3±2.4	0.09	−60.1±3.4
+Poloxamer 188 (0.6%) DT NE	182.2±3.1	0.10	−58.5±0.8
+Solutol HS15 (0.2%) DT NE	180.4±1.2	0.11	−62.3±3.8
+Solutol HS15 (0.4%) DT NE	183.9±1.9	0.13	−61.7±2.4
+Solutol HS15 (0.6%) DT NE	188.7±2.5	0.16	−59.9±2.5

The NEs were all prepared at four cycles and 1,000 bars homogenization pressures. Each value represents the means of three measurements± SD. The ratio of the additives is weight ratio (percent)

As the results shown in Fig. 2, the intracellular ATP and GTP reduction of cells treated by the blank NEs with co-emulsifiers were all slight. It was concluded that the co-emulsifiers (P188 and S15) were all almost nonirritant.

The effects of DT NEs with co-emulsifiers additives on intracellular ATP and GTP levels are shown in Fig. 3. Post-Dunnett's tests following ANOVA shows that the intracellular ATP and GTP reduction of the cells exposed to the S15 containing DT NEs were higher than that of the control DT NE significantly ($p<0.05$), and the intracellular ATP and GTP reduction aggravate with the increase of the S15 concentration in the NE. In contrast, the P188 containing DT NEs significantly restore the intracellular ATP and GTP compared to the control DT NE ($p<0.01$), and the DT NEs with lower concentrations of P188 cause the intracellular ATP and GTP less decrease ($p<0.01$). This suggests that the incorporation of lower concentration P188 could significantly increase the endothelial cells compliance of DT NEs.

It had been generally accepted that the venous irritation of drug-loaded NE should be attributed to the free drug in the aqueous phase. The results of free DT concentrations in the aqueous phase of DT NEs containing co-emulsifiers are presented in Fig. 4. The DT concentrations are dependent on the concentration of co-emulsifier in NEs, namely, the free DT concentrations increase with the increase of the S15 concentration, and they are all higher than that of the control DT NE. However, the addition of 0.2% and 0.4% of P188 result in the decrease of the free DT concentrations, with the exception that the 0.6% of P188 causes the increase of the DT concentrations. Furthermore, the DT concentrations in the aqueous phase of NEs containing S15 are all higher than that of NEs containing P188 when the concentrations of the two co-emulsifiers are the same.

It is obvious that the intracellular ATP and GTP reduction and the DT concentrations in the aqueous phase of DT NEs had strong positive relationship. In other words, the higher free DT concentrations resulted in more intracellular ATP and GTP reduction. Thus, these findings suggest that modulation of the DT concentration in the aqueous phase of DT NEs was an effective method to reduce the venous irritation of DT NE for intravenous delivery.

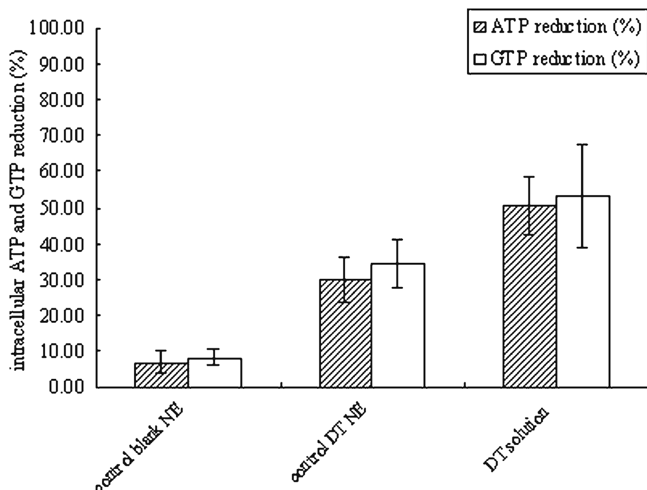

Fig. 1. Intracellular adenosine triphosphate and guanosine triphosphate reduction (percent) in HUVEC CRL-1730 cell line treated with control blank nanoemulsion (NE), control diallyl trisulfide (DT) NE, and DT solution. Each value represents the means of three measurements±SD

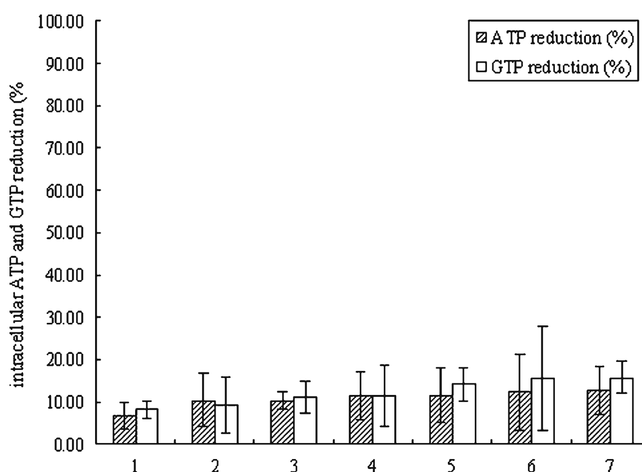

Fig. 2. Effect of co-emulsifiers on intracellular adenosine triphosphate and guanosine triphosphate reduction (percent) in HUVEC CRL-1730 cell line treated with nanoemulsions (NEs). (*1*) Control blank NE; (*2*) Poloxamer 188 (0.2%) blank NE; (*3*) Poloxamer 188 (0.4%) blank NE; (*4*) Poloxamer 188 (0.6%) blank NE; (*5*) Solutol HS15 (0.2%) blank NE; (*6*) Solutol HS15 (0.4%) blank NE; (*7*) Solutol HS15 (0.6%) blank NE. Each value represents the means of three measurements±SD

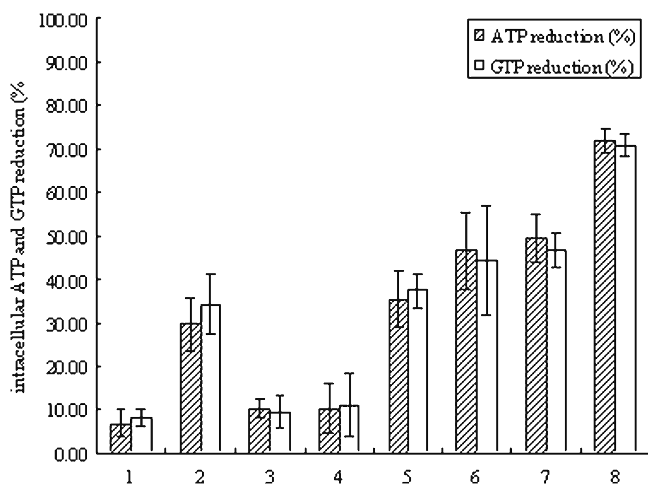

Fig. 3. Effect of the nanoemulsions with various co-emulsifiers on intracellular adenosine triphosphate and guanosine triphosphate reduction (percent) in HUVEC CRL-1730 cell line. (*1*) Control blank nanoemulsion (NE); (*2*) control diallyl trisulfide (DT) NE; (*3*) Poloxamer 188 (0.2%) DT NE; (*4*) Poloxamer 188 (0.4%) DT NE; (*5*) Poloxamer 188 (0.6%) DT NE; (*6*) Solutol HS15 (0.2%) DT NE; (*7*) Solutol HS15 (0.4%) DT NE; (*8*) Solutol HS15 (0.6%) DT NE. Each value represents the means of three measurements±SD

Modulation of the DT Concentration in the Aqueous Phase of Nanoemulsions

We investigated the effects of the co-emulsifiers on the DT concentrations in the aqueous phase of NEs. The theoretical mechanism of drug interfacial transport based on Fick's first law of diffusion is applied to predict the mass transport phenomena at an interfacial membrane of oil-in-water NE *in vitro*. Hosokawa *et al.* (37) deduced the percentage of the drug in the aqueous phase of oil-in-water emulsion, *Qa* (percent), which is given by Eq. 1:

$$\%Qa = 100 - 100 \times \exp\left(-\frac{PS}{Vo} \times t\right) \tag{1}$$

where *Vo* is the volume of oil, *S* is the surface area of interfacial membrane, *t* is the unit time, and *P* is the permeability of drug across the interfacial membrane. Assuming all the particles have a mean particle size, *d*, measured by dynamic light scattering, a term of *S/Vo* in Eq. 1 can be represented as 6/d, and the Qa can be expressed as ($Ca \times Va$), *Ca* is the drug concentration in the aqueous phase, and *Va* is the volume of aqueous phase, hence, rearrangement of Eq. 1 yields

$$\%(Ca \times Va) = 100 - 100 \times \exp\left(-\frac{6P}{d} \times t\right) \tag{2}$$

Furthermore, the permeability *P* is given by:

$$P = \frac{Ds}{Kds \cdot \Delta s} \tag{3}$$

where *Kds* is the solute (drug) partition coefficient between the dispersed phase (oil) and the continuous phases (surfactant solution), *Ds* is the effective diffusion coefficient through the surfactant layer (interfacial membrane), and *Δs* is the surfactant layer thickness (38).

Considering both Eqs. 2 and 3, when the particle sizes of DT NEs with co-emulsifiers, *d*, are almost the same (Table I), the DT concentrations in the aqueous phase of DT NEs, *Ca*, are determined by the three parameters: *Kds*, *Ds*, and *Δs*. As far as the DT NEs is concerned, the interfacial membranes are all made of the phospholipids, and the amounts of the phospholipids in the formulations are the same. Thus, it is speculated that the parameters of *Ds* and *Δs* are almost the same and *Kds* is the critical one in the determination of the DT concentration of the aqueous phase.

As the logarithm of *Kds* are shown in Fig. 5, lg*Kds*-P (the lg*Kds* of DT between oil and P188 buffer solution) is higher than lg*Kds*-S (the lg*Kds* of DT between oil and S15 buffer solution) when their concentrations are the same. This indicates that the free drug concentration in aqueous phase of NE containing S15 would be higher than that of NE containing P188. The predicted results are well in accordance with the experimental results that are presented in Fig. 4. From which, we can see that the *Kds*-P and the *Kds*-S relate to the surfactants concentration. The trend well illustrates the DT concentrations in the aqueous phase of NEs. Therefore, it is concluded that the free DT concentrations of NEs containing co-emulsifiers may be largely decided by the partition coefficient of DT between oil and surfactant buffer solution.

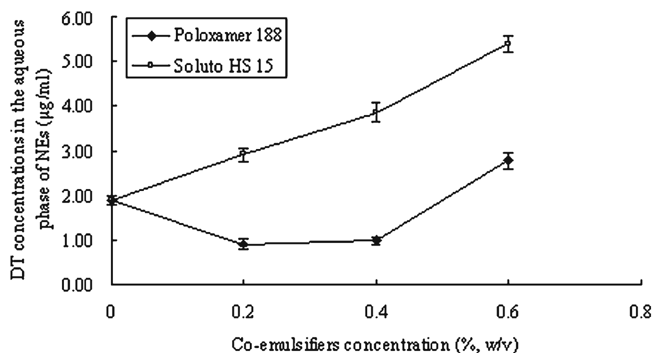

Fig. 4. Effect of Poleaxes 188 and Solutol HS 15 additives on the concentrations of diallyl trisulfide in aqueous phases of the nanoemulsions. The data are means of three measurements±SD

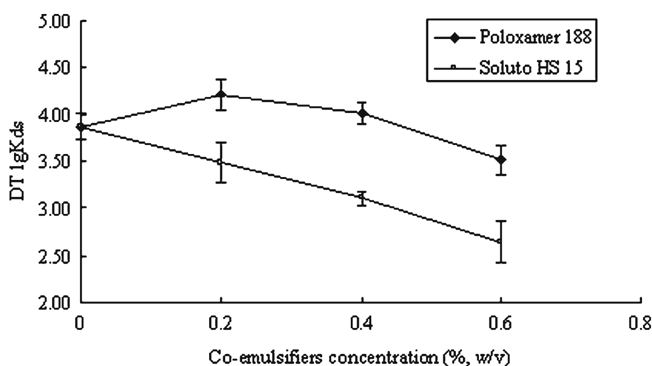

Fig. 5. Effect of Poloxamer 188 and Solutol HS 15 concentration on the logarithm of oil/surfactant buffer solution partition coefficients of diallyl trisulfide (0.05 M phosphate buffer, ionic strength 0.2, 25°C). The data are means of three measurements±SD

CONCLUSION

The DT NEs for intravenous delivery were prepared and evaluated using *in vitro* cell model. Various co-emulsifiers were selected to prepare stable NEs for investigating their effects on the venous irritation of DT NEs. The incorporation of P188 and S15 in the DT NEs led to a considerable decrease of particle size. The incorporation of the co-emulsifiers could regulate the venous irritation of the DT NEs. In addition, the DT NEs containing 0.2% P188 showed the best compatibility and the lowest venous irritation compared to the control DT NE without co-emulsifier. The incidence of HUVEC compatibility varied in a free DT concentration-dependent manner. It was deduced that the co-emulsifiers tend to regulate the free DT concentrations by the partition coefficients of DT between the oil and surfactant buffer solution. In conclusion, NE was an appropriate delivery system for lipophilic and venous irritant drug, DT, and optimization of the composition of emulsifiers was an effective method to alleviate the venous irritation of DT NEs.

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