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Preparation and preclinical evaluation of intravenous lipid emulsion containing a novel thiochromanone derivative (CF) with low toxicity

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ABSTRACT

In this study, (Z)-3-(chloromethylene)-6-fluorothiochroman-4-one (CF), a novel thiochromanone derivative was organized. Lipid emulsion containing CF (CF-LE) was developed by high pressure homogenization to improve its solubility. The physicochemical properties such as morphology, particle size, zeta potential and yielding efficiency of CF-LE were studied. Stability and safety assessment (including hemolytic test, vascular irritation test and paw lick test) of CF-LE was also investigated. The optimum CF-LE, consisting of 10% MCT, 1.2% egg yolk lecithin, 0.6% F68, 0.4% oleic acid and 2.25% glycerol, was prepared at 800 bar for 10 cycles. The average particle size of CF-LE was 234.7nm, z-potential was -30.76 mV, and entrapment efficiency was 95%. CF-LE showed a sufficient physicochemical stability in the long-term centrifugation and dilution with 0.9% sodium chloride injection and 5% glucose injection for 6 h. It did not cause any hemolysis and blood coagulation in vitro and did not cause obvious intravenous irritation. The results suggested that CF-LE was a promising drug delivery system for CF intravenous administration.

INTRODUCTION

Z-3-(chloromethylene)-6-fluorothiochroman-4-one (CF) is a novel synthetic thiochromanone derivative [1] and its structural formula is shown in Fig. 1. CF is highly lipid soluble and low water-soluble sulfur atom heterocyclic compound [1,2]. It has a wide range of physiological activity and can easily permeate fungal cell membranes, alter fungal cell ultrastructure, damage their cell wall structure and function of cell membranes, and then cause fungal death [3-5]. Studies have shown that fluorothiochroman has a strong inhibitory activity to several important pathogenic bacteria such as cryptococcus neoformans, saccharomycetes, mycetin and trichophyton [6-9]. Although CF showed a strong antifungal activity, there is a big challenge to develop a formulation of CF for its practically insoluble in water. It could not be injected directly as an aqueous solution. Therefore, developing a new CF preparation to overcome its insolubility is of great significance for its clinical application.

Lipid emulsion (LE) as drug delivery systems for poorly water-soluble drugs and oil soluble drugs has been successfully developed over the last few decades [10-12]. Compared with liposome, it has the advantages of relatively simple manufacturing process. It also had the unique properties of low toxicity, improving patient compliance and decreasing the adverse reaction for drugs incorporated into the interior oil phase and the oil-water interfacial [13-18]. Preparation of lipid emulsion without using organic solvent can directly reduce irritation to the vascular [15]. The excellent characteristics of using non-toxic materials, high drug loading, increased stability [20-23] of the drug make LEs as an attractive carrier for CF.

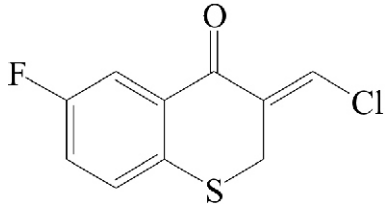

Figure 1 : The chemical structure of CF

High pressure homogenization is often employed for the method of large scale production of lipid emulsion. A narrower particle size range of emulsion can be got by processing liquid

passed through homogenization under high pressure [24]. In this study, high pressure homogenization was used to prepare the CF-LE and the physical and chemical properties of emulsion were also evaluated. The physicochemical properties such as morphology, particle size, zeta potential of CF intravenous lipid emulsion were studied. The stability of CF-LE after long-term centrifugation and dilution with 0.9% sodium chloride injection and 5% glucose injection for 6 h were investigated in detail [25]. Preliminary safety assessment of lipid emulsion included hemolytic test, vascular irritation test and paw lick test was also studied.

MATERIALS AND METHODS

Materials

(Z)-3-(chloromethylene)-6-fluorothiochroman-4-one (CF) (purity>99.0%) was synthesized by pharmaceutical chemistry of Hebei University (baoding, China). Lipoid E80 was purchased from Lipoid KG, (Ludwigshafen, Germany). Soybean oil and medium-chain triglyceride (MCT) was purchased from Tieling Beiya Pharmaceutical Co (Tieling, China). Poloxamer 188 (Pluronic F68) was a kind gift from BASF AG (Ludwigshafen, Germany). Glycerol was kindly provided by Zhejiang Suichang Glycerol Plant (Zhejiang, China). Oleic acid is purchased by Tianjin Hengxing Chemicals Manufacturing. All chemicals and reagents used in this experiment were analytical or chromatographic grade.

Preparation of CF-LE

Intravenous CF-LE was prepared by high pressure homogenization method. Briefly, egg yolk lecithin (1.2%,w/v) was dissolved in MCT (10%,w/v) with moderate ethanol under stirring and then CF (0.05%,w/v), oleic acid (0.40%, w/v) were also added in MCT at 60 °C to obtain a homogeneous oil phase. F68 (0.60%,w/v) and glycerol (2.25%, w/v) were dissolved in water as water phase. Then water phase was added to oil phase at the 60 °C with high-speed dispersion for 15 min to obtain the primary emulsion. The pH was adjusted to 6.0 using 0.1 mol/L NaOH solution, and the volume was made up to 100% with distilled water. The primary emulsion was passed through a high pressure homogenizer(ATS Engineering Inc AH-2010) at 800 bar for ten cycles to produce final emulsion. Finally, the lipid emulsion was sealed in vials and autoclaved at 100 °C for 45 min to obtain CF-LE.

Characterization of CF-LE

The size distribution and zeta potential of LE were determined using Nicomp particle sizing system. The emulsion was diluted to a certain concentration with distilled water before measurement.

The morphology of CF-LE was observed by appearance and TEM (JEM-100SX, JEOL, Japan). A drop of the diluted sample was spread on a copper grid, and then negatively stained with 1% phosphotungstic acid for 5min. Then the grid was dried at room temperature and was observed by TEM later.

The entrapment efficiency (EE) of CF-LE was determined. 5mL LE was put in centrifuge tube, and then the sample was put in the high speed refrigerated centrifuge at 17000 rpm for 3h at 4℃. Total amounts of CF in the formulation and water phase were measured by high-performance liquid chromatography (HPLC), and the EE were calculated according to the following equation:

$$EE\,(\%) = (C_T V_T - C_W V_W) / C_T V_T * 100\% \quad (1)$$

Where C_T and V_T were drug concentration and drug volume of CF-LE, and the C_W and V_W were those in water phase, respectively.

HPLC assay

CF content was measured using HPLC method with a C18 column (5μm, 250 × 4.6mm). The mobile phase was composed of acetonitrile/ammonium acetate (containing 0.05% glacial acetic acid) (70:30, V/V). The flow rate and wavelength were 1.0mL/min and 245nm. After filtration by 0.22μm membrane, 20μL of sample was injected for analysis.

Safety assessment

Hemolytic Test

For hemolytic test, blood samples were obtained from ear vein of rabbit, and fibrin was removed by stirring. Then the red erythrocyte cells were washed several times with normal saline, then the red blood cells was diluted to 2% erythrocyte dispersion with 0.9% physiologic saline. Various volume of CF-LE (0.1, 0.2, 0.3, 0.4 and 0.5 mL) ($1^{\#}$-$5^{\#}$) were added to the tubes along with 2.5 mL volumes of the erythrocyte dispersion. Distilled water was served as positive control ($6^{\#}$) and normal saline was served as negative control ($7^{\#}$). Then normal saline was added to the tubes to obtain a final volume of 5mL, respectively. The tubes were incubated at room temperature and observed for 4 hours.

Intravenous irritation assessment

Rabbits weighing 2.5 ± 0.22 kg were randomly divided into two groups with three in each group. CF-LE was injected into the marginal ear vein of at a daily dose of 0.2 mg/mL for 3 days as the experimental group. The other group was given an equivalent volume of 0.9% saline as the control. The rabbits were sacrificed at 24 h after the last administration and then vascular tissues around the injection site were excised and fixed in 10% formaldehyde for histological examination. Microscope was then used for observing and capturing images of histological changes, vascular endothelium subcutaneous tissue inflammation and thrombosis etc.

Licking experiment

10 rats were randomly divided into two groups with 5 rats in each group. Experimental group was given a single injection of 0.2 mL of CF-LE into the foot pad of the right hind paw of the rat. Meantime, control group received an injection of 0.2 mL saline solution. The onset time of paw licking, the average time of paw licking within 30 min and the total time of licking were recorded.

Stability study

Dilution stability

The physical stabilities of CF-LE in 0.9% sodium chloride (NaCl) and 5% glucose injections were investigated. Samples were diluted 5 fold and 50 fold with 0.9% NaCl and 5% glucose, respectively. The morphology of samples was measured at intervals of 0.5, 1, 2, 3, 4 and 6 h at room temperature, respectively.

Sterilization stability

CF-LE was autoclaved at 100°C for 45min, 115°C for 30min and 121°C for 15min, respectively. Then the physical appearance, content and particle size were evaluated.

RESULTS

Physical characteristics

The morphology of CF-LE studied by appearance and TEM analysis were shown in Fig.2A and Fig.2B. CF-LE showed homogeneous white emulsion.TEM showed a uniform droplet size of CF-LE. It also was shown that droplet was almost uniform spherical shape and there was no aggregation or adhesion among droplet of emulsion. The average diameter and zeta potential of CF-LE was 234.7 nm and -30.76mV, respectively. The entrapment efficiency was 95%.

Figure 2A : Micrograph of CF-LE

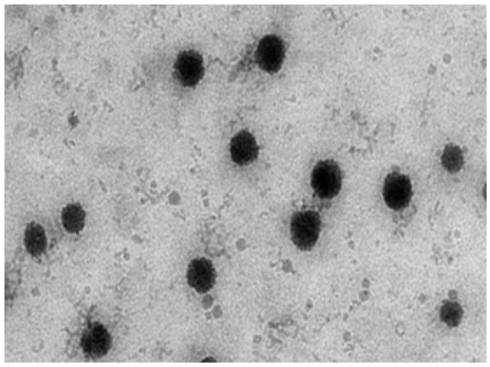

Figure 2B : TEM of CF-LE

Safety test

Hemolysis test

Hemolysis test results of CF-LE were shown in Fig. 3A. It was easy to see that complete hemolysis was observed in 7# positive control tube. The upper solution of 1#-5# tubes (experimental group) and negative control group showed the original color of CF-LE, and erythrocytes of 1#-5# tubes were precipitated at the bottom, indicating that the lipid emulsion did not cause any hemolysis in vitro.

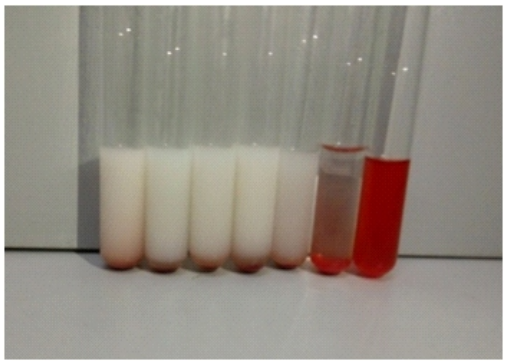

Figure 3A : TResult of hemolysis test for 4h (1# - 7# tubes from left to right)

The bottom solution of 1# tube, 6# negative control and 7# positive control tube were drawn on the slide and observed under optical microscope, the results were shown in Fig. 3B. There was no hemolysis in 1# tube (drug group) and 6# negative control group, and complete hemolysis could be seen in 7# positive control tube. The results indicated that CF-LE did not cause any hemolysis and blood coagulation in vitro and could be used for intravenous injection.

Intravenous irritation assessment

Observing the rabbit ear-border vein by naked-eye, there was no hyperemia swelling or erythema phenomenon at injection site before and after drug administration. There was no significant difference between experimental group and saline control group. Fig.4 showed the appearance and histopathology examination of rabbit ear vein after intravenous injection. It indicated that CF-LE had not cause obvious irritation. There was no obvious edema and vascular congestion and fester or other irritation changes in both of experimental group and saline control group. No inflammatory phenomena were found in both groups. The results showed CF-LE did not cause obvious intravenous irritation and it could be used for intravenous injection.

Paw licking test

Within 30 minutes, the average and total licking time of experimental group were higher than the control group (Table 1). The first licking time of drug group was earlier than that of control group indicating that lipid emulsion had certain irritation to the subcutaneous tissue, it need to prevent liquid emulsion from leaking to the subcutaneous tissue when CF-LE was given in intravenous injection way.

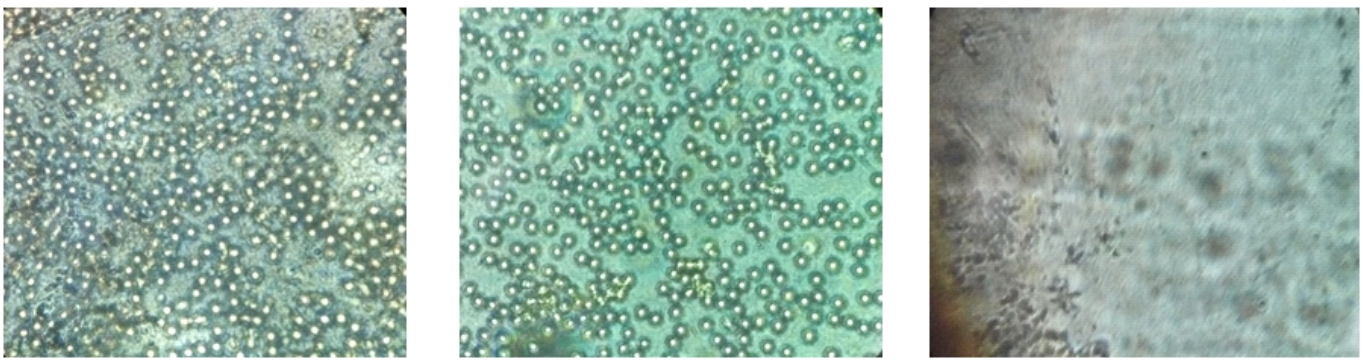

Figure 4 : ROptical microscope images of test tube of experimental group, negative control group, and positive control group

Table 1. : Results of rat paw lick test

Group of mice	First time licking (min)				Total time licking (s)		The average time licking (s)	
	Control groups	Experimental group	Control groups	Experimental group	Control groups	Experimental group	Control groups	Experimental group
1	12	6	16.6	4.6	100	104	25.2	77
2	20	3			15	95		
3	18	5			3	85		
4	15	4			5	66		
5	18	5			3	35		

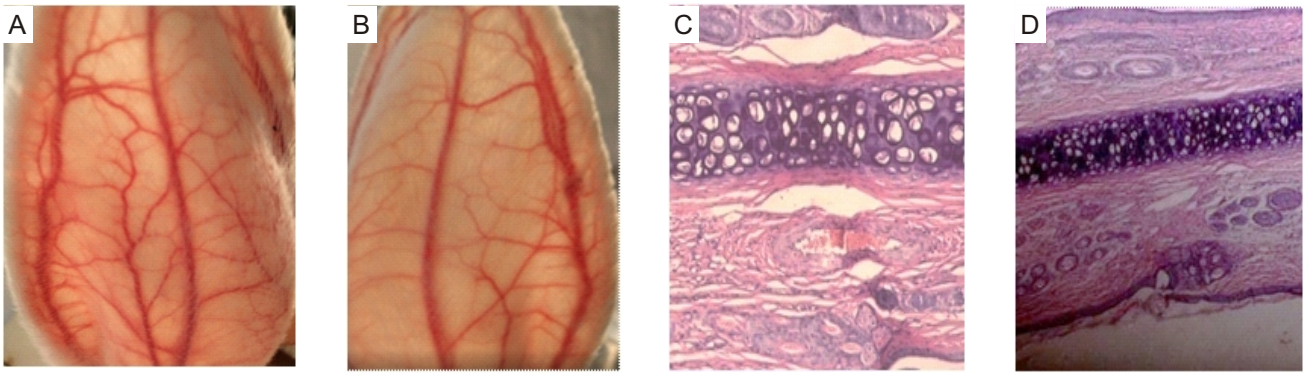

Fig. 4. : The appearance examination of rabbit ear vein, experimental group(A), normal saline (B) and histopathology examination of rabbit ear vein, experimental group (C), normal saline (D).

Stability study

Dilution stability of CF-LE

The result of physical stabilities of CF-LE in 0.9% sodium chloride and 5% glucose injections were investigated and the results showed CF-LE samples diluted 5 fold and 50 fold with 0.9% NaCl and 5% glucose did not produce flocculation or precipitation within 6h at room temperature, respectively. CF-LE showed good compatibility with glucose and NaCl and can be diluted for clinical application.

Sterilization stability

Thermal sterilization is very necessary for intravenous injection used for clinical application. The appearance stability of the formulation set as the index to evaluate the CF-LE. The autoclaving conditions were 100 ℃ for 45min, which showed the physical and chemical stability of CF-LE.

DISCUSSION

LE as a parenteral drug carrier could offer organ targeting and sustained release. Emulsifier was an important component of LE, the sorts and amount of emulsifiers and the process could significantly affect the quality of LE. As one of the emulsifiers, egg yolk lecithin is regarded as well tolerated and non-toxic compounds with good biocompatibility, so it is suitable for preparing parenteral lipid emulsion. F68 as a water-soluble non-ionic emulsifier also has a strong emulsifying effect and can stabilize the created interface, and it is essential to form the emulsion with good physicochemical property. A single emulsifier of egg yolk lecithin was insufficient to maintain the stability of the emulsion. The combination of F68 and egg yolk lecithin could help further emulsify the oil phase and form a tight complex interfacial film between the water phase and oil phase. Water-soluble co-emulsifier of oleic acid could produce a negative charge by adsorbing on the oil-water interfacial film and enhance the electrostatic repulsion between emulsion droplets. Thus the incorporation of oleic acid could strongly remain the stability of the emulsion, and keep pH value nearly unchanged and increase the absolute zeta potential.

In this study, the stability and particle size of emulsion was set as the evaluation index for the emulsion preparation. The dosage of egg yolk lecithin and F68 were set at the concentration 1.2% (w/v) and 0.6% (w/v). The oleic acid was at 0.4% (w/v). Glycerin is important for the intravenous lipid emulsion as an isotonic adjustment agent and the amount at 2.25% (w/v) is often selected to adjust the osmotic pressure. High-speed shear mixing for 15 min was selected to prepare the coarse emulsion, then high pressure homogenization was used to produce consistent fine droplets with a narrow size distribution. The mean particle size reduced as the homogenization cycle increased from 4 to 10, above 10 cycles, the particle size did not change obviously. Likewise, the homogenization pressure was up to 800 bar, excess

did not obviously decrease the mean particle size. Consequently, 800 bar for 10 cycles was chosen as the optimum homogenization parameters.

CONCLUSION

CF-LE was successfully prepared by high pressure homogenization and the physicochemical properties, safety and stability were investigated in detail. The CF-LE containing 10% MCT, 1.2% egg yolk lecithin, 0.6% F68, 0.4% oleic acid and 2.25% glycerol, was prepared at 800 bar for 10 cycles. The average particle size was 234.7nm, z-potential was -30.76 mV, and entrapment efficiency was 95%. Safety assessment demonstrated that CF-LE did not cause any hemolysis and blood coagulation in vitro and no obvious intravenous irritation were found. Paw licking test showed that CF-LE had little tissue irritation. It was also showed good dilution stability with 0.9% sodium chloride and 5% glucose solution. Above all, LE provided a relative useful potential carrier for parenteral administration of CF.

Declaration of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Acknowledgments

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REFERENCES

1. C.N. Li, C.L. Yang, Y.X. Liu, *T.F.* Liu, Q. Liu, Antitumor activities of (Z) -3- (chloromethy-lene) -6-methylthiochroman -4- one in vitro (in Chinese), Chinese. Sci. Bull. 55 (2010) 3027-3031.
2. Z.Y. Ma, G.Y. Yan, G.L. Yang, S.G. Zhu, L. GUAN, Synthesis and Antifungal Activities in Vitro of 2, 3, 3a,4-Tetrahydrothiochromeno[4, 3-c]pyrazole Derivatives, Chin. Pharm. J. 44 (2009) 630-633.
3. L.Fang, Z.G. Dai, G.L. Zhang, Synthesis of Thiochromones, Thiochromanones and Study of Their Antifungal Activity, J. Shenyang. Pharm. Univ. 15 (1998) 116-119.
4. H. Zhang, D.L. Bai, Research development of antifungal medicines, Chin. J. Med. Chem. 7 (1997) 303-310.
5. Z.G. Zhu, G.L. Yang, Z.Y. Ma,C.L. Wei, L.D.Guo, Synthesis and antifungal activity of chlorobenzothiopyrano [4,3-c]pyrazolines, Chin. J. New. Drugs. 17 (2008) 1034-1036.
6. P. Qi, Y.H. Jin, C. Guo, *L. Fang,* Synthesis of 3-benzylthioehromanones and their In vitro antifungal activity, Chin. J. New. Drugs. 13 (2004) 141-143.
7. C. Olano, C .Méndez, J.A. Salas, Antitumor compounds from marine actinomycetes. Mar Drugs. 7 (2009) 210-248.
8. Q.G. Zhu, L. Fang, G.L. Zhang, Synthesis and Antifungal Activities of the Mannich Baes of Thiochromanones Derivatives, Chin. J. Med. Chem. 10 (2000) 5-8.
9. G.Y. Yu, T. Xiao, Recent advances in the research of chromone and thiochromone derivatives as antifungal agents, Chin. J. Antibiot. 36 (2010) 481-484.
10. D.E. Turner-Lawrence, L.W. Kerns, Intravenous fat emulsion: a potential novel antidote, J. Med. Toxicol. 4(2008):109-114.
11. J. Nordenström, Y.A. Carpentier, J.Askanazi, A.P. Robin, D.H. Elwyn, T.W. Hensle, J.M. Kinney, Metabolic utilization of intravenous fat emulsion during total parenteral nutrition, Ann .Surg. 196 (1982) 221-231.
12. A.A. Date, M.S. Nagarsenker, Parenteral microemulsions: an overview, Int. J. Pharm. 355 (2008) 19-30.
13. D.Cao, K. Heard, M. Foran, A. Koyfman, Intravenous lipid emulsion in the emergency department:a systematic review of recent literature, J. Emerg. Med. 48(2015) 387-397.
14. M.A. Rosenblatt, M. Abel, G.W. Fischer, C.J. Itzkovich, J.B. Eisenkraft, Successful use of a 20% lipid emulsion to resuscitate a patient after a presumed bupivacaine-related cardiac arrest, Anesthesiology, 105(2006) 217-218.
15. G. Cave, M. Harvey, Intravenous lipid emulsion as antidote beyond local anesthetic toxicity: a systematic review, Acad. Emerg. Med. 16 (2009) 815-824.
16. M.M. Berger, The 2013 Arvid Wretlind lecture: evolving concepts in parenteral nutrition, Clin.Nutr. 33 (2014) 563-570.
17. G. Cave, M. Harvey, A. Graudins, Intravenous lipid emulsion as antidote: a summary of published human experience, Emerg. Med. Australas. 23 (2011) 123-141.
18. L. Rothschild , S. Bern, S. Oswald, G. Weinberg, Intravenous lipid emulsion in clinical toxicology, Scand. J. Trauma. Resusc. Emerg. Med. 18 (2010) 2-8.
19. R.H. Müller, S.Schmidt, I. Buttle, A . Akkar, J.Schmitt, S. Brömer, SolEmuls-novel technology for the formulation of i.v. emulsions with poorly soluble drugs, Int. J. Pharm. 269(2004) 293-302.
20. S. Collins, J. Neubrander, Z. Vorst, B. Sheffield, Lipid Emulsion in Treatment of Local Anesthetic Toxicity, J. Perianesth. Nurs. 30 (2015) 308-320.
21. R.H. Müller, S. Schmidt, I. Buttle, A. Akkar, J. Schmitt, S. Brömer, Solemuls-novel technology for the formulation of i.v. emulsions with poorly soluble drugs, Int. J. Pharm. 269 (2004) 293-302.
22. R.G. Strickley, Solubilizing excipients in oral and injectable formulations, Pharm. Res. 21 (2004) 201-230.
23. F. Ishii, T.Nii, Properties of various phospholipid mixtures as emulsifiers or dispersing agents in nanoparticle drug carrier preparations, Colloids. Surf. B. Biointerfaces. 41(2005) 257-262.
24. M.R. Zahi,P.Wan, H. Liang, Q. Yuan, Formation and stability of D-limonene organogel-based nanoemulsion prepared by a high pressure homogenizer. J. Agric. Food. Chem. 62 (2014) 12563-12569.
25. Y.J. Shao, S. Li, B. Tian, L.L. Su, C.G. Zhang, Y.Q. Wang, X. Tang, Y.J. Wang, Evaluation of the stability and pharmacokinetics of cabazitaxel-loaded intravenous lipid microspheres: Beneficial effect of cholesterol. Eur. J. Lipid Sci. Technol. 117 (2015) 460-470.