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Formulation and thermal sterile stability of a less painful intravenous clarithromycin emulsion containing vitamin E

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Abstract

The purpose of this study is to develop a less painful intravenous clarithromycin emulsion (ClaE) and investigate its thermal sterile stability. The formulation of ClaE is composed of clarithromycin 0.25% (w/v), vitamin E 5% (w/v), medium chain triglyceride (MCT) 10% (w/v), egg lecithin 1.0% (w/v), Cremophor EL-40 (EL-40) 2% (w/v), Pluronic F-68 (F-68) 0.2% (w/v), Tween80 0.2% (w/v), glycerol 2.5% (w/v) and L-cysteine 0.05% (w/v) in water. High-pressure homogenization, photon correlation spectroscopy (PCS) and electrophoretic light scattering (ELS) technology, light microscopy and high-performance liquid chromatography (HPLC) methods were used in the preparation and evaluation of ClaE. Investigation of thermal sterile stability included the effects of different thermal sterile methods, thermal sterile time, drug concentrations and pH values. Sterilization in a 100 °C rotating water bath for 30 min was finally adopted as the sterilization method. The drug remaining was 98.6% and 96.5%, respectively, before and after thermal sterilization. Moreover, the pH value, particle size distribution (PSD), ζ-potential and entrapment efficiency (EE) of ClaE after sterilization were 7.95, 213.6 nm, −22.29 mV and 96.35%, respectively. This showed that ClaE had sufficient physicochemical stability to resist the sterilization process. Tests using animal models demonstrated that there was a marked pain reduction following the injection of ClaE compared with clarithromycin solution. Overall, ClaE described in this paper may be very suitable for industrial-scale production and clinical application.

Keywords: Clarithromycin; Formulation; Thermal sterile stability; Intravenous emulsion; Less painful; Vitamin E

1. Introduction

Clarithromycin (Fig. 1) is a synthetic macrolide antibiotic with a methoxy group (–OCH_3) attached to the C_6 position of erythromycin, which makes it more acid stable than erythromycin. Clarithromycin has been incorporated into many dosage forms because of its high sustained concentration in blood and more effective antibacterial activity than erythromycin. The main marketable products are tablets, capsules, dry suspensions and intravenous injectable clarithromycin lactobionate. However, the main problem associated with its use is that the intravenous injectable dosage form causes venous irritation which results in serious pain in some patients. For this reason, it is necessary to develop a new dosage form for intravenous administration of clarithromycin.

A range of lipid emulsions has been developed in the last 30 years. The first prototype formulation was described as Intralipid™. It was well known as a source of calories and essential fatty acids for patients. Drug-loaded emulsions have also been rapidly developed for their unique properties, low toxicity and reduced irritation. Simultaneously, lipid emulsions can also enhance the solubilization or stabilization of the incorporated drugs to obtain sustained release and targeting (Floyd, 1999; Venkateswarlu and Patlolla, 2001; Dan, 2005).

Lovell et al. (1994) reported a formulation for a less painful clarithromycin emulsion contained lipophilic counterions of hexanoic acid and oleic acid. However, the final emulsion was unable to undergo thermal sterilization, which was replaced by sterile filtration using a 0.22 μm Nalgene® nylon filter. The main reason for the failure of thermal sterilization was thought to be the presence of lipophilic counterions. When heated by thermal stress, the counterions would be unstable and might separate from the interfacial film of the emulsifiers. This would break up

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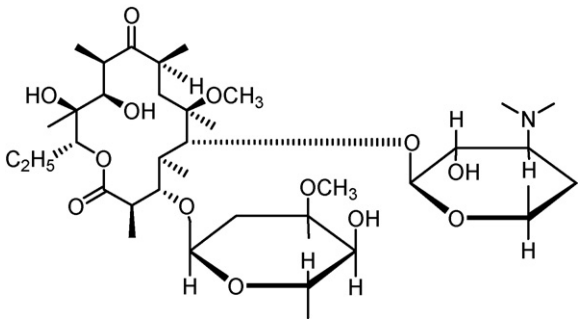

Fig. 1. Molecular structure of clarithromycin.

the emulsion. In addition, the separated lipophilic counterions of hexanoic acid and oleic acid could induce the degradation of clarithromycin during thermal sterilization since clarithromycin is quite unstable in acid (Nakagawa et al., 1992). Furthermore, the sterile filtration also limited the potential for industrial-scale production.

Based on the mentioned background above, a new clarithromycin emulsion formulation containing vitamin E for intravenous administration was investigated in this study, which could survive thermal sterilization. Vitamin E acted as a novel oil solvent for clarithromycin. The experimental results demonstrated that ClaE containing vitamin E was still stable after sterilization in a 100 °C rotating water bath for 30 min, both in terms of its physical and chemical properties. Two animal models, the rat paw lick test and the rabbit ear vein test, were used to evaluate the pain reduction and reduced irritation of ClaE compared with clarithromycin solution. According to the results obtained, it appears that the industrial production and clinical application of this intravenous clarithromycin emulsion containing vitamin E is distinctly feasible.

2. Materials and methods

2.1. Materials

The following materials were obtained from the sources in brackets: Clarithromycin (Zhejiang Huayi Pharma Ltd. Co., Zhejiang, China), Vitamin E (Zhejiang Medicine Ltd. Co., Zhejiang, China), MCT (Lipoid KG, Ludwigshafen, Germany), Egg lecithin (EPIKURON 170, PC72%, Degussa Food Ingredients, German), long-chain triglyceride (LCT) (TieLing BeiYa Pharmaceutical Co., Tieling, China), F-68 (BASF AG, Ludwigshafen, Germany), Tween80 for parenteral use and EL-40 (Shenyu Medicine and Chemical Industry Ltd. Co., Shanghai, China), L-cysteine (Tianjin Chemical Agents Co., Tianjin, China), glycerol (Zhejiang Suichang glycerol Plant, Zhejiang, China), potassium dihydrogen phosphate (Guangdong ·Shantou Xilong Chemical Plant, Shantou, China), triethylamine, acetonitrile and methanol (Tianjin Concord Technology Ltd. Co., Tianjin, China). All chemicals and reagents used were of analytical or chromatographic grade.

2.2. Methods

2.2.1. Solubility of clarithromycin in different oils and in phosphate buffer solutions with different pH values

Excess clarithromycin powder was put into several 100 ml conical flasks, followed by the addition of 50 ml phosphate buffer solutions (PBS) of pH (5.0, 6.8 and 7.4), MCT, long-chain triglyceride (LCT) and vitamin E. The mixtures above were equilibrated in an HZQ-C air bath agitator (Harbin Dongming Medical Equipment Factory, Harbin, China) at 25 °C for 48 h and then kept at the same temperature without agitation for another 24 h. Finally, the supernatants of the different samples were analyzed by high-performance liquid chromatography.

2.2.2. Emulsion preparation

Clarithromycin emulsion containing vitamin E was prepared by high-pressure homogenization. 0.25% (w/v) clarithromycin was dispersed in 5% (w/v) vitamin E and 1.0% (w/v) egg lecithin was dispersed in 10% (w/v) MCT separately at 55 °C until dissolved, and then they were mixed together as the oil phase. The aqueous phase consisting of 2.5% (w/v) glycerol, 0.2% (w/v) F-68, 0.2% (w/v) Tween80, 2.0% (w/v) EL-40 and 0.05% (w/v) L-cysteine was also heated in the water bath until it was uniformly dispersed at 55 °C. Then the coarse emulsion was prepared by high shear mixing (ULTRA TURRAX® T18 basic, IKA® WORKS Guangzhou, Germany) by adding the water phase to the oil phase rapidly at 10,000 rpm. The high shear mixing process was carried out for 3 min and repeated three times. The final emulsion was obtained by high-pressure homogenization using Niro Soavi NS 10012K homogenization (Niro Soavi S.p.A., Via M.da Erba Edoari, 29/A-43100PARMA, Italy), at 600 bar for eight cycles. The temperature of the entire homogenization process was controlled at 40 °C using ice-water bath. Then the volume was adjusted to 100 ml with double-distilled water and the pH value of the final emulsion was adjusted to 8.0 with 0.1 mol/L HCl or 0.1 mol/L NaOH. Finally, the emulsion was transferred to vials after adding nitrogen gas and sterilized in a 100 °C rotating water bath for 30 min.

2.2.3. Characterization of clarithromycin emulsion containing vitamin E

The particle size distribution of ClaE was measured by the PCS technique using a Nicomp™ 380 Particle Sizing system (Zeta Potential/Particle Sizer NICOMP™ 380ZLS, Santa Barbara, California, USA). The system covered the range from 5 nm to approximately 3 μm. The emulsion sample was diluted 1:5000 with double-distilled water immediately before the measurement at 25 °C. The Nicomp™ 380 system was also used to determine the ζ-potential by the ELS technique. Before measurement, double-distilled water used to dilute the emulsion samples was adjusted to the same pH value as the emulsion using 0.01 mol/L HCl or NaOH solutions. Then the emulsion sample was diluted 1:50 with the water as described above. The determination was carried out at 25 °C. The pH of the bulk emulsion was measured using a pH-meter (Leici®, Shanghai Precision Science Instrument Ltd., Shanghai, China) with a microelectrode at room temperature (25 ± 2 °C). The EE of

ClaE was obtained by measuring the free clarithromycin concentration in the dispersed medium. Ultrafiltration was carried out three times using a Vivaspin 4 apparatus (provided by Beijing Genosys Tech-Trading Co. Ltd., Beijing, China) operated at 3000 rpm, and for 15 min. The Vivaspin 4 mainly consists of a filter membrane with a molecular weight cut-off of approximately 10,000 Da. The centrifuged free drug in the aqueous phase was determined by HPLC. The microscopic assessment was carried out using a Motic DMBA 450 microscope (MoticChina Group Co. Ltd., Beijing, China). The emulsion samples were investigated without being diluted using an oil immersion with 1000-fold magnification; typically 20 microscopic fields were analyzed for the detection of residual drug crystals in the emulsions.

2.2.4. Thermal sterile stability studies for ClaE

2.2.4.1. Effect of pH. A series of 2.5 mg/ml ClaE according to Section 2.2.2 were prepared by adjusting pH value with 0.1 mol/l HCl or NaOH to pH 3–9. The thermal sterile stability influenced by pH was investigated by sterilizing the emulsion samples in a 100 °C rotating water bath for 30 min. Then, all the samples before and after sterilization were analyzed by HPLC.

2.2.4.2. Effect of thermal sterile methods. ClaE emulsion samples containing 2.5 mg drug/ml were prepared according to Section 2.2.2. Then, the samples were sterilized under different sterilization conditions in a 100 °C rotating water bath for 30 min; in a 100 °C rotating water vapor bath for 15 min; in a 100 °C rotating water vapor bath for 30 min; in a 115 °C rotating autoclave by water vapor for 30 min and in a 121 °C rotating autoclave by water vapor for 20 min, respectively. Then, all the samples before and after sterilization were analyzed by HPLC.

2.2.4.3. Effect of thermal sterilization time. ClaE (2.5 mg/ml) was prepared according to Section 2.2.2. The emulsions were packed in vials and then sterilized in a 100 °C rotating water bath. Samples were taken at different time points of 0, 15, 30, 45, 60, 90 and 120 min. In addition, all the samples were analyzed by HPLC and the particle size, pH values, ζ-potential and entrapment efficiency of ClaE were also determined.

2.2.4.4. Effect of drug concentration in ClaE (other components of ClaE and the pH value were the same as in Section 2.2.2). The emulsions with clarithromycin concentrations of 0.2, 0.5, 1.5 and 2.5 mg/ml were prepared for the thermal sterile stability study. Other conditions were the same as in Section 2.2.2. The samples were sterilized in a 100 °C rotating water bath for 30 min and then the samples were analyzed by HPLC.

2.2.5. Drug analysis

A reverse phase HPLC analytic method was used for drug analysis. An HiQ sil C_{18} column (5 μm, 4.6 mm × 250 mm, KYA TECH Corporation, Japan) was employed. The mobile phase consisted of PBS solution (9.11 g potassium dihydrogen phosphate was diluted in 1000 ml double-distilled water and 2 ml triethylamine by adjusting to pH 5.5 with phosphoric acid) and acetonitrile at a ratio of 3:2; the flow rate was 1.0 ml/min; the detection wavelength was 210 nm; the column temperature was 45 °C and the injection volume was 20 μl. The drug powder or drug-containing emulsion was diluted with methanol to the appropriate concentration before determination.

2.2.6. Rat paw lick test

In this test, groups of 10 Wistar rats received a subplantar injection of 0.1 ml ClaE and clarithromycin solution (CS) into the footpad of their right hind paws. Then, the number of times the animals licked their paws and the total time of the paws being licked were monitored over a period of 15 min (Lovell et al., 1994). The clarithromycin solution was prepared by dissolving clarithromycin in phosphoric acid solution of pH 1, and adjusting the pH to 6.0 with 10% sodium hydroxide. Then, the solution was passed through a 0.45 μm micropore filter, and sealed in vials after adding nitrogen gas; they were finally sterilized in a 100 °C rotating water bath for 30 min. The concentration of the clarithromycin solution was the same as for ClaE.

2.2.7. Rabbit ear vein test

Groups of three rabbits were given an infusion of 2.5 mg/ml ClaE and clarithromycin solution (positive control) into their marginal ear veins (Lovell et al., 1994). The rate of infusion was maintained 20 drops/min at a dose of 12 mg/(kg day) for 4 days. Following infusion, visual observations of the vascular reaction were made every day. Forty-eight 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 for histopathological examination.

3. Results and discussion

3.1. Formulation investigations of ClaE

3.1.1. Vitamin E as a novel oil solvent for clarithromycin

Vitamin E can be an excellent solvent for water insoluble drugs and is compatible with other co-solvents, oils and surfactants. Vitamin E emulsions can also offer an appealing alternative for the parenteral administration of poorly soluble drugs (Constantinides et al., 2004). Fig. 2 shows that clarithromycin was nearly insoluble in MCT, LCT and water of

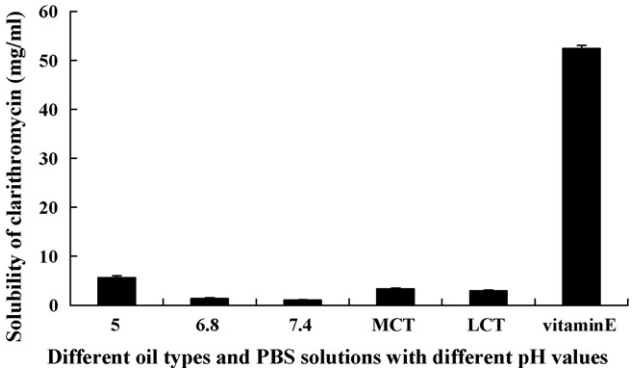

Fig. 2. The solubility of clarithromycin in different oils and in PBS solutions with different pH values at 25 °C (Mean ± SD, $n = 3$).

Table 1
The effect of three oil phase ratios on the characterization of ClaE (other components, pH value and thermal sterilization method for the ClaE formulation were the same as in Section 2.2.2)

Each 100 ml ClaE	MCT:vitamin E	Physical appearance after sterilization	Particle size distribution (nm)	Entrapment efficiency (%)
Formulation A	1:1	Emulsion breaking	ND	ND
Formulation B	1:2	Good	213.6 ± 58.4	96.35
Formulation C	1:3	Good	232.5 ± 83.7	96.17

ND: not determined.

different pH values. In contrast, the solubility of clarithromycin in vitamin E was 52.25 mg/ml, which was much more than the values of 3.39 and 2.91 mg/ml in MCT and LCT, respectively. The good solubility of clarithromycin in vitamin E can be explained by the well-established solution theory and principles pioneered by Hildebrand, Illum et al. (1997) (Constantinides et al., 2004) and this is mainly because clarithromycin is highly soluble in chloroform and poorly soluble in methanol. Hildebrand, Illum et al. (1997) proposed a "solubility in vitamin E" parameter (SVE) to predict the solubility of a compound in vitamin E. The SVE is defined as the solubility in chloroform divided by the solubility in methanol, expressed in mg/ml for both solvents. Moreover, an SVE of at least >10, preferably >100, indicates the solubility in vitamin E (Constantinides et al., 2004). Therefore, in the ClaE formulation, vitamin E was selected as a novel oil solvent. In addition, the high solubility of clarithromycin in vitamin E could also maintain the solution stability during the thermal sterilization and storage of ClaE. Due to the fact that the dosage of ClaE was 2.5 mg/ml in this paper, 5 g vitamin E was selected per 100 ml ClaE.

3.1.2. Oil phase composition and oil phase ratios in ClaE

3.1.2.1. Oil phase composition in ClaE. The oil phase composition in emulsions plays an important role in emulsion formulations. The oil phase composition influences the physicochemical properties and the stability of parenteral lipid emulsions (Jumaa and Muller, 1997). As mentioned in Section 3.1.1, clarithromycin is very soluble in vitamin E. Additionally, the lipid emulsion containing MCT might provide more stable all-in-one admixtures (Driscoll et al., 2000), MCT can reduce the toxicity compared with emulsions prepared with pure LCT (Smyrniotis et al., 2001) and it can also reduce the high amount of linoleic acid and balance the fatty acid pattern (Carpentier and Hacquebard, 2006). Moreover, it has been reported that oil phases with relatively higher viscosities need a higher homogenization pressure to obtain the fine emulsion droplets which are needed for safe intravenous administration. However, excess high homogenization pressure is a disadvantage for industrial production and uses a lot of energy (Nakagawa et al., 1992). So MCT was selected to adjust the viscosity of the oil phase in ClaE. Hence, in this study, vitamin E and MCT were selected as the oil phase.

3.1.2.2. Oil phase ratios in ClaE. Moreover, three oil phase ratios of 10%, 15%, 20% in ClaE with MCT–vitamin E 1:1, 1:2, 1:3 were employed to investigate the effects of different oil phase ratios in ClaE and the other components in all of the three formulations were the same as in Section 2.2.2. The thermal sterilization was carried out in a 100 °C rotating water bath for 30 min and the results are shown in Table 1. In addition, the emulsion images of a 15% and 20% oil phase in ClaE are shown in Fig. 3.

From Table 1, it appears that the oil phase of vitamin E–MCT 1:1 underwent emulsion breakage. The reason for this might be that when insufficient oil was used in the emulsions, it could not encapsulate the drug effectively, in addition, the oil film was not strong enough to remain stable during thermal sterilization. The ratios of Formulations B and C employed resulted in good physical stability. The EE was 96.29% and 96.17%, respectively, which was nearly unchanged. This suggested that 10 g MCT per 100 ml ClaE was enough for encapsulating clarithromycin and using 15 g MCT produced no further improvement in the EE. At the same time, the particle size of Formulation C was approximately 20 nm larger than Formulation B. This might

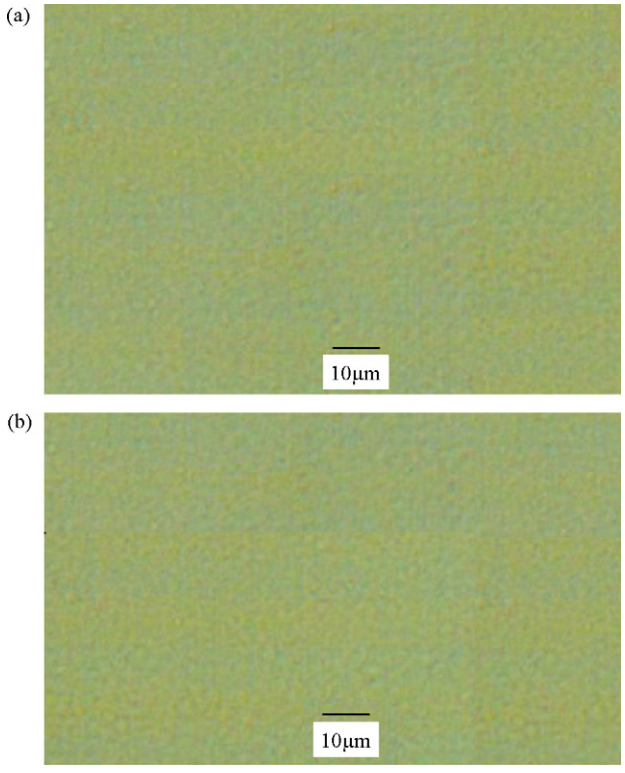

Fig. 3. The images of ClaE obtained by a Motic DMBA 450 microscope: (a) 15% oil phase of vitamin E:MCT 1:2 and (b) 20% oil phase of vitamin E:MCT 1:3.

Table 2
The effect of different amounts of egg lecithin on the characterization of ClaE with constant amounts of F-68 0.2% (w/v), Tween80 0.2% (w/v) and EL-40 2% (w/v) (pH value and thermal sterile method of ClaE were the same as in Section 2.2.2)

Each 100 ml ClaE	Egg lecithin (%, w/v)	Physical appearance after sterilization	Particle size distribution (nm)	ζ-Potential (mV)
Formulation I	0.5	Emulsion breaking	ND	ND
Formulation II	1.0	Stable	213.6 ± 58.7	−22.49
Formulation III	1.5	Stable	218.6 ± 80.4	−23.88

ND: not determined.

be because excess MCT resulted in small free oil drops with a relatively large particle size present in ClaE during sterilization, resulting in an increase in mean particle size and standard deviation. So, Formulation B was more suitable for obtaining good physicochemical characteristics compared with Formulation C. In addition, Fig. 3 proves that Formulations B and C were physically stable after thermal sterilization since the microscope photos showed no drug crystals and no oil drops larger than 5 μm in ClaE. Furthermore, the good physical appearance of Formulation B proved that the oil phase with vitamin E–MCT 1:2 also produced a proper viscosity and interfacial tension for the ClaE emulsion, which exhibited sufficient stability during the thermal sterilization. Therefore, the ratio of the oil phase in the ClaE Formulation was selected as 15% with vitamin E–MCT 1:2.

3.1.3. Emulsifier composition in ClaE

Emulsifiers are another essential component of lipid emulsions. It is well known that the emulsification of lipid emulsions is related to the emulsifiers used, and the emulsification process significantly influences the stability of lipid emulsions. Also, it is obvious that the emulsifiers play an important role in the emulsification process (Yamano and Seike, 1983; Weingarten et al., 1991; Yamaguchi et al., 1995a,b).

In ClaE formulations, egg lecithin was selected as the main emulsifier. The egg lecithin used in ClaE consisted of approximately 72% phosphatidylcholine and a small amount of anionic phospholipids. It has been reported that the stability of lipid emulsions is influenced by the purity of the lecithin. For example, Yamaguchi et al. (1995a,b) showed that Vt^{max} (maximum total interaction energy) varied for different lecithin. For the egg lecithin composed of 72% phosphatidylcholine in ClaE, the Vt^{max} value was very large. Hence, it did not readily undergo flocculation and coalescence. A small amount of anionic phospholipids can also increase the ζ-potential of the emulsion surface, owing to the enhancement of the electrostatic repulsion between emulsion droplets (Joseph, 1990; Chansiri et al., 1999). The electrostatic repulsion between emulsion droplets markedly affects the stability of emulsions during the thermal sterilization and storage process. Moreover, a single emulsifier of egg lecithin was not sufficient to maintain the stability of the emulsion, since it is well known from the literatures that phospholipids are only stable at an alkaline pH. This is because of the high sensitivity of phospholipids to changes in pH values, especially in the presence of electrolytes (Dawes and Groves, 1978; Chaturvedi et al., 1992; Jumaa and Muller, 2002). Therefore, a combination of different emulsifiers is effective and essential for thermal sterile stability. In this paper, F-68, Tween80 and EL-40 were investigated as co-emulsifiers.

In our study, the amounts of each emulsifier used, including egg lecithin, F-68, Tween80 and EL-40, were investigated alongside other emulsifiers at a constant level. Tables 2–5 show the typical formulations designed in our study. The stability of a parenteral emulsion mainly refers to maintaining its main physical property, namely the particle size distribution. Moreover, the safety of application is to a high degree dependent on the particle size distribution, since particles larger than 5 μm given intravenously can lead to emboli in vivo (Davis, 1974). Hence, PSD is an essential parameter for investigating the stability of emul-

Table 3
The effect of different amounts of Tween80 on the characterization of ClaE with constant amounts of egg lecithin 1.0% (w/v) and EL-40 2% (w/v) and without F-68 (pH value and thermal sterilization method of ClaE were the same as in Section 2.2.2)

Each 100 ml ClaE	Tween80 (%, w/v)	Physical appearance after sterilization	Particle size distribution (nm)	ζ-Potential (mV)
Formulation IV	0	Emulsion breaking	ND	ND
Formulation V	0.2	Visible oils drops	ND	ND
Formulation VI	0.4	Visible oils drops	ND	ND

ND: not determined.

Table 4
The effect of different amounts of F-68 on the characterization of ClaE with constant amounts of egg lecithin1.0% (w/v), Tween80 0.2% (w/v) and EL-40 2% (w/v) (pH value and thermal sterilization method of ClaE were the same as in Section 2.2.2)

Each 100 ml ClaE	F-68 (%, w/v)	Physical appearance after sterilization	Particle size distribution (nm)	ζ-Potential (mV)
Formulation II	0.2	Stable	213.6 ± 58.7	−22.49
Formulation VII	0.4	Stable	195.3 ± 65.4	−23.15

Table 5
The effect of different amounts of EL-40 on the characterization of ClaE with constant amounts of egg lecithin1.0% (w/v), Tween80 0.2% (w/v) and F-68 0.2% (w/v) (pH value and the thermal sterilization method of ClaE were the same as in Section 2.2.2)

Each 100 ml ClaE	El-40 (%, w/v)	Physical appearance after sterilization	Particle size distribution (nm)	ζ-Potential (mV)
Formulation VIII	0.5	Emulsion breaking	ND	ND
Formulation IX	1.5	Visible oil drops	ND	ND
Formulation II	2	Stable	213.6 ± 58.7	−22.49
Formulation X	3	Stable	192.6 ± 75.4	−24.05

ND: not determined.

sions. Furthermore, the ζ-potential also plays an important role in stabilizing drug-containing emulsions through electrostatic repulsion (Washington, 1996) and the ζ-potential for relatively stable emulsions is usually below −20 mV. Therefore, in this part, the physical appearance after sterilization was taken as a qualitative description while the particle size distribution and ζ-potential were used for quantitative descriptions of the physical stability of ClaE.

As shown in Table 2, the emulsion breaking of Formulation I took place because 0.5% (w/v) egg lecithin was not enough to emulsify the 15% oil phase in ClaE to obtain a good emulsification effect. Also, 0.5% (w/v) egg lecithin could also not adequately strengthen and uniformly disperse in the emulsifier interfacial film. Formulations II and III displayed good physical stability after thermal sterilization with a suitable ζ-potential of −22.49 and −23.88 mV, respectively. However, Formulation III suggested that with the increase of egg lecithin, the standard deviation of PSD became larger compared with Formulation II. This might be because the excess egg lecithin could form micelles with a smaller particle size and liposomes with a relatively larger particle size. Moreover, although Formulation III was physically safe for intravenous administration, the excess egg lecithin was also presumed to be the reason for the deviation from the chylomicron-like behavior, and, thus, a different metabolic fate of the lecithin-coated oil droplets; it was also suspected to consist of liposomes which caused enhanced formation of abnormal 'Lipoprotein-X', increased cholesterol release and other dislipidemic effects (Bach et al., 1996). Hence, 1.0% (w/v) in Formulation II was chosen as the amount of egg lecithin.

Table 3 shows that Tween80 as a co-emulsifier plays an important role in ClaE since the physical thermal sterile stability was obviously improved, which was demonstrated by the fact that Formulation V used Tween80 with only a few oil drops compared with Formulation IV which was emulsion breaking without Tween80 after thermal sterilization. However, the visible oil drops that appeared in Formulation V could not be dissolved by adding more Tween80 since Formulation VI with 0.4% (w/v) Tween80 also displayed visible oil drops after thermal sterilization. Therefore, other emulsifiers with a better emulsifying effect need to be added to ClaE. Also, it proved that the incorporation of F-68 as a co-emulsifier might be necessary and important, as seen in Table 4. Table 4 shows the effect of the total amount of F-68. F-68 (dissolved in water) is used as the water-soluble non-ionic emulsifier, which has a strong emulsifying effect. It could stabilize the newly created interface immediately and within only one homogenization cycle maximum dispersion was already achieved and could not be further reduced by repeated homogenization (Eccleston, 1992). In addition, the combination of egg lecithin and F-68 leads to the formation of a close-packed mixed film, which confers improved stability, which is attributed to the steric stabilization of the non-ionic surfactant (Weingarten et al., 1991; Jumaa and Muller, 2002). By adding 0.2% (w/v) F-68, ClaE was physically stable enough to undergo thermal sterilization shown as Formulation II with a suitable ζ-potential of −22.49 mV. Also, the result of Formulation VII suggested that with 0.4% (w/v) F-68, the mean particle size was slightly reduced since more F-68 further emulsified the oil phase giving a ζ-potential of −23.15 mV. However, since F-68 and Tween80 are not endogenous, the less used, the better. Hence, the amount of F-68 and Tween80 were both selected to be 0.2% (w/v) for ClaE.

Table 5 shows the effect of EL-40 used in ClaE. Formulations VIII and IX displayed emulsion breaking because less than 2% (w/v) EL-40 in ClaE might not enough to emulsify the 15% oil phase, especially to emulsify the vitamin E contained in the oil phase. Based on the results of Formulations II and X, it appears that when using at least 2% (w/v) EL-40, ClaE could produce a satisfactory emulsifying effect. However, the amount of EL-40 was not the more, the better because EL-40 has been associated with some adverse toxic effects, such as bronchospasm and hypotension (Lorenz et al., 1977; Dye and Watkins, 1980). Therefore, the amount of EL-40 was chosen as 2% (w/v) finally as shown in Formulation II, which also provided a suitable ζ-potential of −22.49 mV.

Ultimately, besides 1.0% (w/v) egg lecithin, 0.2% (w/v) F-68, 0.2% (w/v) Tween80 and 2% (w/v) EL-40 were employed as co-emulsifiers. Also, the enhanced emulsion stability produced by using co-emulsifiers was probably due to the strengthening of the complex interfacial film composed of emulsifier mixtures. In addition, 2.5% (w/v) glycerin was used to adjust osmotic pressure; L-cysteine was also used in the ClaE for its antioxidant effect. In summary, the final ClaE formulation consisted of clarithromycin 0.25% (w/v), vitamin E 5% (w/v), MCT 10% (w/v), egg lecithin 1.0% (w/v), EL-40 2% (w/v), F-68 0.2% (w/v), Tween80 0.2% (w/v), glycerin 2.5% (w/v) and L-cysteine 0.05% (w/v) in water. Drug analyses before and after thermal sterilization were carried out by HPLC. The amount of drug remaining was 98.6% and 96.5%, respectively, which proved the chemical stability of ClaE was sufficient to undergo thermal sterilization in a 100 °C rotating water bath for 30 min.

Table 6
Characterization of ClaE underwent thermal sterilization in a 100 °C rotating water bath for 30 min and stored at 10 °C for 3 months (the formulation and pH value of ClaE were the same as in Section 2.2.2)

Characterization parameters	Before thermal sterilization	After thermal sterilization	3 months (stored at 10 °C)
pH value	8.0	7.95	7.81
Particle size distribution (nm)	212.8 ± 32.6	213.6 ± 58.4	225.7 ± 65.3
ζ-Potential (mV)	−20.46	−22.29	−24.58
Entrapment efficiency (%)	96.60	96.35	95.49

3.1.4. Characterization of ClaE underwent thermal sterilization and stored at 10 °C for 3 months

Thermal sterilization is necessary for lipid emulsions for clinical application. Also, the emulsions should display sufficient stability during the sterilization procedure (Hansrani et al., 1983; Jumaa and Muller, 1999a,b). The pH value, particle size, ζ-potential and entrapment efficiency are essential and common parameters for evaluating lipid emulsions and these were determined before and after the thermal sterilization in a 100 °C rotating water bath for 30 min, in order to confirm the physical stability of ClaE as far as thermal sterilization was concerned. The results are shown in Table 6.

Based on Table 6, the mean particle size of ClaE was nearly unchanged during the sterilization process together with the reduction in pH and increase in ζ-potential. The standard deviation of the particle size became more than 20 nm larger after sterilization because the thermal sterilization was a relatively severe thermal stress process, which caused more rapid Brownian motion leading to aggregation of some emulsion particles. The changes in pH value and ζ-potential were mainly because of the degradation of phospholipids and/or oils, which involved oxidation and hydrolysis (Grit and Crommelin, 1993). Oxidation can be avoided by adding an oxidation inhibitor, L-cysteine, and filling the emulsion containers with nitrogen. However, hydrolysis cannot be avoided and it produced free fatty acids (FFA). It is known that the pH value and ζ-potential are slightly changed by the presence of FFA (Herman and Groves, 1992; Buszello et al., 2000). The external FFA contributed to the emulsion stability as it increased the electrostatic repulsion between emulsion droplets, as shown by the ζ-potential. In addition, the entrapment efficiency of ClaE was 96.6% and 96.35%, before and after sterilization. This suggests that almost all the clarithromycin was located in the oil phase, which was not influenced by thermal sterilization. Furthermore, a light microscopy study was also carried out to discover whether there were any residual drug crystals in ClaE since microscopic analysis to detect drug crystals in undiluted emulsions is an established method to characterize drug incorporation of intravenous administration emulsions (Müller and Heinemann, 1992; MÜller et al., 2004). The observed results of the ClaE, before and after thermal sterilization, proved that no drug crystals were present in ClaE. These results suggest that the ClaE is safe for intravenous administration, ruling out the possibility of embolism formation during infusion (Jeppsson et al., 1976; Laval-Jeantet et al., 1982). In addition, the same parameters above were also determined after 3 months of storage at 10 °C. The data in Table 6 prove that ClaE is still stable and safe for intravenous administration after 3 months of storage at 10 °C.

3.2. Thermal sterile stability studies of ClaE

3.2.1. Effect of pH

A suitable pH value is necessary for intravenous administration of lipid emulsions. The range of pH values for emulsions is from 4 to 9 since the pH value of human blood is 7.4. The thermal sterile stability of ClaE with different pH values was investigated by sterilization in a 100 °C rotating water bath for 30 min. The results of drug remaining before and after thermal sterilization are shown in Fig. 4.

The results indicate that, at a pH above 4, ClaE displayed physical stability without emulsion breaking. However, the drug sharply degraded at pH 4 and 5 because clarithromycin is unstable under acid conditions (Nakagawa et al., 1992). From pH 6 to 9, the reduction in drug remaining after sterilization was less than 10%, and moreover less than 5% from pH 8 to 9. This suggests that different pH values significantly affect the chemical stability of the ClaE emulsion during thermal sterilization. Considering both physicochemical thermal sterile stability and the patient compliance with regard to ClaE, the pH of ClaE was finally adjusted to pH 8–9.

3.2.2. Effects of thermal sterilization methods and thermal sterilization time

Different thermal sterile methods consisting of sterilizing in a 100 °C rotating water bath for 30 min (I); a 100 °C rotating water vapor bath for 15 min (II); a 100 °C rotating water vapor bath for 30 min (III); a 115 °C rotating autoclave water vapor bath for

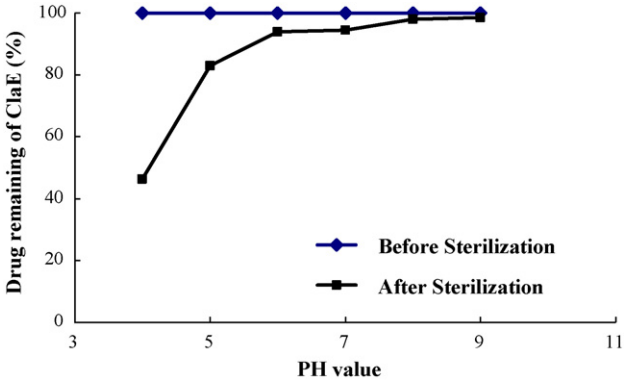

Fig. 4. Drug remaining in ClaE with different pH values before and after being sterilized in a 100 °C rotating water bath for 30 min (the formulation in ClaE was the same as in Section 2.2.2).

Table 7
Physical appearance and drug remaining in ClaE after different thermal sterilization methods (the formulation and pH value of ClaE were the same as in Section 2.2.2)

	BS	I	II	III	IV	V
Physical appearance	Good	Good	Good	EB	EB	EB
Drug remaining (%)	98.6	96.5	94.96	ND	ND	ND

BS, before sterilization; EB, emulsion breaking; ND, not determined.

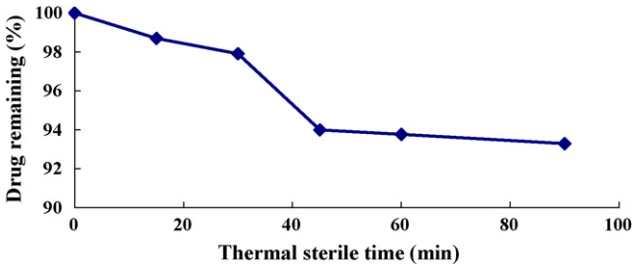

Fig. 5. Drug remaining in ClaE at different time points during sterilization in a 100 °C rotating water bath (the formulation and pH value of ClaE were the same as in Section 2.2.2).

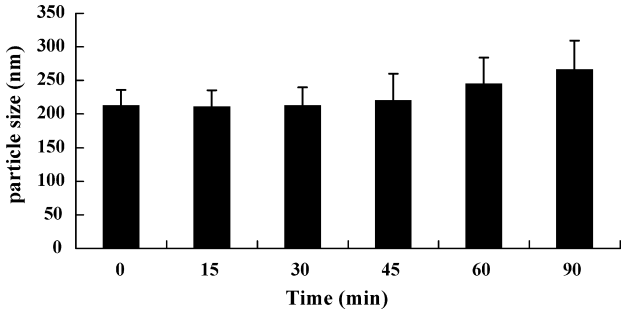

Fig. 6. The particle size of ClaE at different time points during sterilization in a 100 °C rotating water bath (the formulation and pH value of ClaE were the same as in Section 2.2.2).

30 min (IV) and a 121 °C rotating autoclave water vapor bath for 20 min (V) were employed in this study to optimize the thermal sterile method. Table 7 shows the study results.

In summary, ClaE could not resist autoclaving (IV and V) for the ClaE sterilized in IV and V in this test exhibited emulsion breaking. The emulsion sterilized in a 100 °C rotating water vapor bath for less 30 min, i.e. for 15 min, in Table 7 displayed a good physical appearance. However, there was 1.54% more drug remaining in drop form compared with thermal sterilization in a 100 °C water bath for 30 min. In addition, water vapor thermal sterilization involves a lack of heat uniformity, which might affect the sterile stability of ClaE. Hence, following various investigations of sterile conditions, sterilizing in a 100 °C rotating water bath for 30 min was selected as the ClaE thermal sterilization method.

Based on the results obtained under the different sterile conditions, ClaE samples were further evaluated by treating samples in a 100 °C rotating water bath for different time. At different time points, samples were selected and analyzed by HPLC. The drug remaining at different time points is shown in Fig. 5. Furthermore, as the essential factor to evaluate intravenous emulsions, the particle size distribution is shown in Fig. 6. Other characterization results of ClaE are shown in Table 8.

According to Figs. 5 and 6, within 30 min ClaE was slightly degraded from 100% to 97.9% with only 2.1% as drops. After 30 min sterilization, the degradation produced more than 5% as drops. In addition, from 45 to 90 min, the degradation was reduced. This shows that the time point of 30 min was critical for controlling the drug remaining during the thermal sterilization. Hence, 30 min was adopted as the most suitable thermal sterilization time. The particle size distribution of ClaE was not rapidly changed by increasing sterilization time over 30 min. However, after 45 min, the particle size increased rapidly, possibly because the longer thermal sterilization caused coalescence of the emulsion drops. Furthermore, based on the results shown in Table 8, the ζ-potential and pH value were slightly increased and reduced, respectively, after sterilization. The reason for the changes may be because the egg lecithin underwent hydrolysis and produced FFA and the larger ζ-potential strengthened the repulsion between emulsion particles. Moreover, the entrapment efficiency in this test during the 45 min sterile process remained above 90% and, therefore, almost all the clarithromycin was present in the oil phase. However, when sterilized for 60 min or longer, the EE sharply decreased. It is suggested that the interfacial film consisting of emulsifier mixtures was destroyed by excess thermal stress which induced the drug leakage. Hence, it appears that the formulation selected in this study is quite stable during thermal sterilization in a 100 °C rotating water bath for 30 min and is safe enough for intravenous administration.

3.2.3. *Effect of different drug concentrations in ClaE*

Since the incorporation of drug into lipid emulsions may influence the characterization of the emulsions, ClaE of different

Table 8
Characterization of ClaE at different time points during sterilization in a 100 °C rotating water bath (the formulation and pH value of ClaE were the same as in Section 2.2.2)

Time points	0 min	15 min	30 min	45 min	60 min	90 min	120 min
ζ-Potential (mV)	−20.52	−21.20	−22.35	−23.67	−24.09	−25.85	Not determined
EE (%)	96.12	96.18	95.86	95.3	78.5	48.3	Not determined
pH value	8.08	8.05	8.04	7.99	7.88	7.76	Not determined

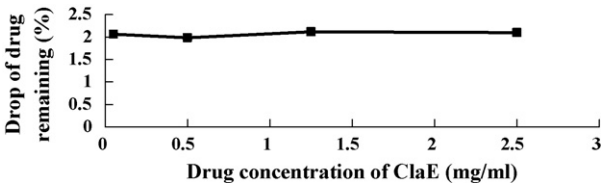

Fig. 7. Reduction in drug remaining in ClaE with different drug concentrations after sterilization in a 100 °C rotating water bath for 30 min (other components and pH value of the ClaE formulation are same as in Section 2.2.2).

concentrations were prepared to study this. The experimental data are shown in Fig. 7. It appears that different clarithromycin concentrations had no effect on the drug remaining during thermal sterilization. This suggests that the dose of ClaE (2.5 mg/ml) chosen for the formulation was suitable. In addition, the drug degradation during the thermal sterilization was independent of the drug concentration. Moreover, it appears that the degradation process of ClaE is based on the inherent physicochemical properties of the drug.

Finally, according to the experimental results of the thermal sterile investigations above, sterilizing in a 100 °C rotating water bath for 30 min was selected as the most suitable thermal sterilization method for ClaE.

3.3. Rat paw lick test

In order to evaluate the level of pain reduction for selecting a lipid emulsion as drug delivery, an effective animal model called the rat paw lick test was used. The basis of the test is that the more painful the formulations is, the greater the number of animals that perform paw licking; in addition, the number of times and the total time an animal licks its paw also increases while the first licking time decreases (Lovell et al., 1994; Celozzi et al., 1980). Table 9 summarizes the data from the rat paw lick study. When clarithromycin solution was injected, all the animals licked their paws. The mean number of times of each rat licked its paw was about 20, which corresponds to an average total licking time of 96.2 s. However, when ClaE was administrated, just 80% of the animals licked their paws and the number of times the rats licked their paws was reduced to 7.9 times, accompanied by a total licking time of 31.2 s. These results show that a significant pain reduction was obtained with ClaE compared with clarithromycin solution (CS).

3.4. Rabbit ear vein irritation test

The rabbit vein irritation test was carried out to measure the irritation caused by ClaE following i.v. injection and the results were compared with CS. In the group of CS, all three rabbits exhibited a slight discoloration following infusion. Also, 24 h after administration, all three animals showed some damage around the administration site. However, there was no obvious visible damage in the ClaE group during infusion and 24 h after it. The histopathological slides of the rabbit ear-rim auricular vein are shown in Fig. 8. From the macroscopic observations, vascular engorgement and dropsy were seen at the injection site, simultaneously angiectasia and erythrocyte aggregation were investigated at or away from the site of administration in the CS group. However, these phenomena were not apparent in the emulsion group. Based on these investigations, the ClaE formulation produced less irritation than CS.

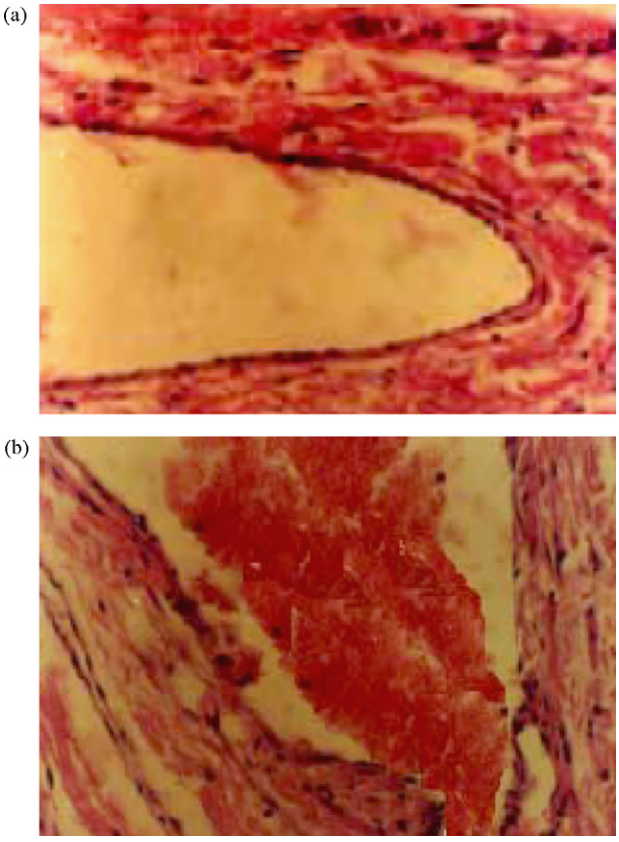

Fig. 8. The histopathological slides of rabbit ear-rim auricular vein: (a) administration by ClaE and (b) administration by CS.

4. Conclusions

In conclusion, a new formulation for intravenous clarithromycin emulsion was developed in this study, which consisted of clarithromycin 0.25% (w/v), vitamin E 5% (w/v), MCT 10% (w/v), egg lecithin 1.0% (w/v), Cremophor EL-40 2% (w/v), Pluronic F-68 0.2% (w/v), Tween80 0.2% (w/v), glycerol 2.5% (w/v) and L-cysteine 0.05% (w/v) in water. Sterilization

Table 9
Results of the rat paw lick test (the formulation, pH value and thermal sterile method of ClaE were the same as in Section 2.2.2)

Formulation test	Number of animals licking	First licking time (s)	Average number of times each rat licked	Average total licking time (s)
ClaE	80% (8/10)	245.2	7.9	31.2
CS	100% (10/10)	37.5	20.1	96.2

in a 100 °C rotating water bath for 30 min was finally adopted as a suitable thermal sterilization method for ClaE. Also, determination of the drug assay, pH value, particle size, ζ-potential, entrapment efficiency and light microscopy studies were carried out for the evaluation of ClaE with regard to its physicochemical stability. Advanced technologies, such as high-pressure homogenization, PCS and ELS, were applied in our study. The results of animal models tests showed that ClaE reduced pain and/or irritation significantly compared with CS. Furthermore, the potential of ClaE for industrial-scale production and clinical administration needs further studies.

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