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Formulation of an intravenous emulsion loaded with a clarithromycin–phospholipid complex and its pharmacokinetics in rats

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ABSTRACT

The purpose of this paper is to prepare a new formulation of clarithromycin emulsion (ClaE) with the clarithromycin–phospholipid complex which was analyzed by DSC. High-pressure homogenization, Nicomp™ 380 Particle Sizing system, and HPLC were used to prepare and investigate ClaE, while UPLC/MS/MS for pharmacokinetic study. Clarithromycin and soybean lecithin were reacted in dehydrated alcohol at a ratio of 1:10 for 3 h at 65 °C to prepare the complex. The ClaE formulation consisted of, according to quality percentage, the complex (clarithromycin 0.25% in ClaE), LCT 4%, MCT 16%, soybean lecithin 1.0%, F68 0.2%, Tween80 0.2%, glycerol 2.5%, sodium oleate 0.1% and L-cysteine 0.02%. ClaE was sterilized in a 100 °C revolving water bath for 30 min. The drug content, particle size distribution and entrapment efficiency of ClaE before and after sterilization and over 6 months storage at 10 °C were almost unchanged, while ζ-potential increased from −20.32 mV to −23.71 mV. These results show that ClaE has enough physicochemical stability to undergo sterilization and storage. The pharmacokinetic study showed that both ClaE and ClaS fitted a three-compartment model, their pharmacokinetic curves were similar and the main parameters showed no significant difference except Vss. ClaE has a great potential for clinical applications and industrial-scale production.

1. Introduction

Clarithromycin (Fig. 1) is a synthetic 6-methoxy derivative of the 14-member macrolide antibiotic erythromycin, and the methoxy group (—OCH_3) attached to the C_6 position makes it more acid stable than erythromycin (Nakagawa et al., 1992; Lovell et al., 1994). Many dosage forms of clarithromycin, such as tablets and capsules, have been developed based on its wide antibacterial spectrum. Clarithromycin has been extensively used for treatment of infections caused by Chlamydia, Mycoplasma, Legionella, Helicobacter pylori, and Mycobacterium avium complex (Vorbach et al., 1998). Clarithromycin has also been proved to be active against HIV-related opportunistic infections (Zimmermann et al., 2001; Alvarez-Elcoro and Enzler, 1999; Kissinger et al., 1995; Roger et al., 1999). Therefore, it is of great importance to develop new dosage forms for clarithromycin with improved therapeutic effects.

Because of the serious irritation produced by intravenous clarithromycin lactobionate, its clinical application using intravenous administration has been restricted. Accordingly, it is very important to develop a new i.v. dosage form of clarithromycin to reduce its venous irritation and improve patient compliance. With this in mind, a clarithromycin o/w emulsion has just been developed (Lovell et al., 1994, 1995; Yan et al., 2008). Over 10 years ago, Lovell et al. (1994) developed a formulation for clarithromycin emulsion contained lipophilic counterions, hexanoic acid and oleic acid, which was sterilized by sterile filtration using a 0.22-μm Nalgene® nylon filter. And this work solved the problem of the solubility of clarithromycin in oil phase and did great contribution to the development of i.v. clarithromycin emulsion. However, the formulation and sterile method limited its industrial production. This is because the lipophilic counterions in the formulation would be unstable and might separate from the interfacial film of the emulsifiers by thermal sterilization. This would break up the emulsion (Yan et al., 2008). And the separated 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. The use of vitamin E in emulsions is well known (Illum et al., 1997) and it is widely employed as an antioxidant at amounts less than 1% (w/v) (Chansiri et al., 1999). In addition, taking vitamin E as a solvent to dissolve some poorly soluble drugs has been reported (Han, 2000). Hence, further research on clarithromycin was carried out using vitamin E as the oil phase because of its good solubility, and such clarithromycin emulsion

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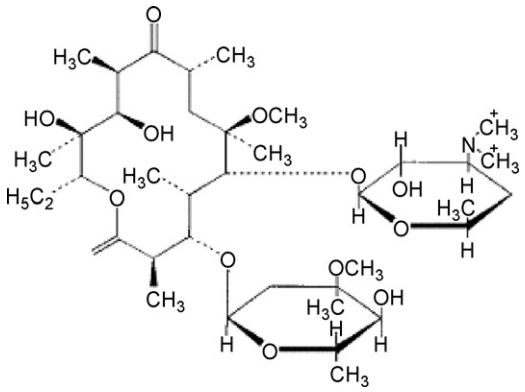

Fig. 1. Molecular structure of clarithromycin.

can undergo complete thermal sterilization, which significantly reduces the venous irritation compared with clarithromycin solution (ClaS) (Yan et al., 2008). However, the tolerability of vitamin E must be considered for routine applications. As reported, the daily intravenous dose of vitamin E for patients is 2300 mg/m^2 for 9 consecutive days (Helson, 1984) and little is known about higher doses or more prolonged use (Panayiotis et al., 2004). Besides this, it is suggested that low concentrations of vitamin E have a better antioxidation capacity by reducing the autooxidation of linoleic acid and methyl linoleate while excess vitamin E could accelerate such oxidation (Junmin, 2005) and so might cause degradation of the drug loaded in the emulsion during storage. Hence, there could be certain limitations with regard to the application of vitamin E to prepare emulsions. Furthermore, Cremophor EL used in the literature (Yan et al., 2008) can cause serious allergic responses after intravenous administration, such as bronchospasm, respiratory distress and hypotension, which might prove fatal to patients. Moreover, Cremophor EL can leach toxic substances from a variety of containers, which might result in adverse reactions, such as venous irritation (Panayiotis et al., 2004; Dye and Watkins, 1980). Thus, in this paper, a new kind of clarithromycin o/w emulsion (ClaE) loaded with a clarithromycin–phospholipid complex was examined. The preparation procedure of the clarithromycin–phospholipid complex was systemically investigated. Furthermore, the formulation and pharmacokinetics of ClaE together with its stability on short-term storage are discussed. All the experimental results proved that ClaE in this study could survive thermal sterilization and exhibit increased stability and safety with regard to clinical administration. Moreover, the ClaE in this paper should be suitable for industrial-scale production and clinical applications.

2. Materials and methods

2.1. Materials

The following materials were purchased or obtained from the sources in parentheses: clarithromycin (Zhejiang Huayi Pharma Ltd., Co., Zhejiang, China), Egg lecithin (Lipoid E80®) and medium-chain triglyceride (MCT) (Lipoid KG, Ludwigshafen, Germany), Soybean lecithin (EPIKURON 170, Degussa Food Ingredients, German), long-chain triglyceride (LCT) (TieLing BeiYa Pharmaceutical Co., Tieling, China), Poloxamer 188 (Pluronic F68®) (BASF AG, Ludwigshafen, Germany), Tween80 for parenteral use and L-cysteine (Tianjin Chemical Agents Co., Tianjin, China), vitamin E (Zhejiang Medicine Ltd., Co., Zhejiang, China), glycerol (Zhejiang Suichang Glycerol Plant, Zhejiang, China), potassium dihydrogen phosphate (Guangdong Shantou Xilong Chemical Plant, Shantou, China), triethylamine, acetonitrile, methanol, dehydrated alcohol, acetone, acetic ether, isopropanol, *N*-hexane and dichloromethane (Tianjin Concord Technology Ltd., Co., Tianjin, China). Sodium sulfite anhydrous (Tianjin Boya Chemical Industry Ltd., Co., Tianjin, China), roxithromycin (Dalian Meiluo Large Drug Factory, Dalian, China), ammonium acetate (Dima Technology Inc., Richmond Hill, USA), sodium oleate (Nation Drug Group Chemical Agents Ltd., Co., Shanghai, China). All chemicals and reagents used were of analytical or chromatographic grade.

All the animals used in this study were purchased from the Experimental Animal Center (Shenyang Pharmaceutical University, Shenyang, China).

2.2. Methods

2.2.1. Preparation of the clarithromycin–phospholipid complex

The complex was prepared with clarithromycin and phospholipids at a suitable ratio. The required amount of clarithromycin and phospholipids for a 100-mL emulsion were put in a 50-mL round bottom flask and 20-mL reaction solvent was added. The mixture was refluxed at a suitable temperature for 3 h. Then, the settled solution was evaporated to obtain the dried clarithromycin–phospholipid complex. In addition, the drug and soybean lecithin, and the dried clarithromycin–phospholipid complex were weighed by electronic balance, respectively, before and after reaction to make sure dehydrated alcohol have been totally evaporated as possible.

2.2.2. Investigations of the clarithromycin–phospholipid complex

2.2.2.1. Reaction solvents. Acetone, acetic ether and dehydrated alcohol were selected as the reaction solvents to prepare the clarithromycin–phospholipid complex. The complex was prepared according to Section 2.2.1 with clarithromycin and soybean lecithin at a ratio of 1:2 (g/g) for 3 h at 65 °C. Then, the reaction solution was evaporated to obtain the dried residue.

2.2.2.2. Ratio of clarithromycin and phospholipids. Taking dehydrated alcohol as the reaction solvent, the complex was prepared according to Section 2.2.1 at 65 °C for 3 h. The ratios of clarithromycin to soybean lecithin were 1:2, 1:8, and 1:10. In addition, 20% (w/v) oil phase in 100 mL emulsion with MCT:LCT 8:2 was used to evaluate the solubility of the complex with different ratios of clarithromycin and phospholipids.

2.2.2.3. Phospholipids types. Lipoid E80® (E80®) and soybean lecithin were employed to investigate the influence of different types of phospholipids on the complex. The clarithromycin–phospholipid complex was prepared according to Section 2.2.1 with a ratio of clarithromycin to phospholipids of 1:10 at 65 °C for 3 h. The emulsions loaded with E80® complex and soybean lecithin complex were prepared as described in Section 2.2.3 to investigate the stability of the emulsions with different complexes. The two formulations above were also combined with 0.02% (w/v) L-cysteine as antioxidant, another 1% (w/v) soybean lecithin, 0.2% (w/v) F68 and 0.2% (w/v) Tween80 as emulsifiers, 0.1% sodium oleate for adjustment of the ζ-potential and 2.5% (w/v) glycerol for adjustment of the osmotic pressure.

2.2.2.4. Differential scanning calorimetry (DSC). Samples were sealed in aluminum crimp cells and heated at a rate of 10 °C min^{-1} from 30 °C to 300 °C in a nitrogen atmosphere (DSC-60, SHIMADZU, JAPAN). The peak transition maximum temperatures of clarithromycin, soybean lecithin, the complex of clarithromycin and soybean lecithin and the physical mixture of clarithromycin

and soybean lecithin were determined and compared using a Thermal Analyzer (TA-60 WS, SHIMADZU, JAPAN).

2.2.3. Emulsion preparation

The clarithromycin emulsion (ClaE) used in this paper was prepared by high-pressure homogenization. The clarithromycin–phospholipid complex for 100 mL ClaE was prepared according to Section 2.2.1. Then, the complex was dissolved in a 20% (w/v) oil phase (MCT and LCT with a proper ratio) at 55 °C, in which some of the soybean lecithin had already been uniformly dissolved. The aqueous phase consisting of glycerol, F68, Tween80, L-cysteine and sodium oleate was uniformly dispersed at 80 °C in a water bath. Then, the coarse emulsion was prepared by high shear mixing (ULTRA TURRAX® T18 basic, IKA® WORKS Guangzhou, China) by rapidly adding the aqueous phase to the oil phase 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 equipment (Niro Soavi S.p.A., Via M.da Erba Edoari, 29/A-43100 Parma, Italy) at 700 bar for 7 cycles. The temperature of the whole homogenization process was maintained below 40 °C in an ice-water bath. Then, the volume was adjusted with water for injection and the pH value was adjusted to 8.0 with 0.1 mol L^{-1} HCl or 0.1 mol L^{-1} NaOH. Finally, the emulsion was transferred to vials after adding nitrogen gas and sterilized in a 100 °C rotating water bath for 30 min.

2.2.4. Characteristics of ClaE

The mean PSD of ClaE was determined by PCS technique using a Nicomp™ 380 Particle Sizing system (Zeta Potential/Particle Sizer NICOMP™ 380ZLS, Santa Barbara, CA, USA). 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 measure the ζ-potential by the ELS technique. The emulsion sample was diluted 1:50 with double distilled water which had been adjusted to the same pH value as the emulsion using 0.1 mol L^{-1} HCl or NaOH solutions. The measurements were 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 25 °C. Ultrafiltration was selected to determine the entrapment efficiency (EE) of ClaE 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) at 3000 rpm for 15 min. The Vivaspin 4 contains 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.

2.2.5. Drug analysis in vitro

A reverse phase HPLC analytical method was employed for drug analysis. An HiQ sil C_{18} column (5 μm, 4.6 mm × 250 mm, KYA TECH Corporation, Japan) was used. The mobile phase consisted of PBS solution (9.11 g potassium dihydrogen phosphate was dissolved in 1000 mL double distilled water and then 2 mL triethylamine was added. Then, phosphoric acid was used to adjust the pH to 5.5) along with acetonitrile at a ratio of 3:2; the flow rate was 1.0 mL min^{-1}; the detection wavelength was 210 nm; the column temperature was maintained at 45 °C and the injection volume was 20 μL. The drug powder or drug-loaded emulsion was diluted with methanol to the appropriate concentration before determination. Furthermore, the linear range of clarithromycin in vitro was 5.0–250.0 μg mL^{-1} and the recovery, intra-day R.S.D. and inter-day R.S.D. of this HPLC method was 100.3%, 0.94% and 1.9%, respectively.

2.2.6. Pharmacokinetics studies of ClaE

2.2.6.1. Sample collection and disposal. A comparative pharmacokinetic study was carried out with ClaE and clarithromycin solution (ClaS). Male Wistar rats (weighting 200 ± 20 g) were divided into two equal groups of 6 rats per group. Based on the drug-loading capability of ClaE and ClaS, as well as the tolerance of the injection volume by patients, the dose of ClaE and ClaS for the rats in the animal experiments was selected as 22.5 mg kg^{-1} calculated according to the skin surface area conversion table. The administration was carried out via the femoral vein. At designed times (5 min, 10 min, 15 min, 20 min, 30 min, 1 h, 1.5 h, 2 h, 4 h, 6 h, 8 h, 12 h and 24 h), the rats were anesthetized with ether and 0.5 mL blood samples were obtained by retro-orbital puncture, transferred to heparinized tubes, and then immediately centrifuged at 5000 rpm for 10 min to obtain plasma samples. The plasma samples were stored at −20 °C for subsequent analysis.

For the analysis, 20 μL methanol solution (methanol:water 1:1), 30 μL roxithromycin methanol solution (4 μg mL^{-1}) as the internal standard, and 100 μL Na_2CO_3 solution (0.5 mol L^{-1}) were sequentially added to 100 μL plasma. The mixture was vortexed for 30 s in a Liquid Fast Mixer (YKH-3, Liaoxi Medical Apparatus and Instruments Factory, China) and then extracted with 3 mL extraction solvent (*N*-hexane:dichlormethane:isopropanol 300:150:15, v/v/v) by vortexing for 10 min. After centrifugation (FULGOR Refrigerated Centrifuge, GL-20B, Shanghai FULGOR Analytical Apparatus Ltd., Co., Shanghai, China) at 10,000 rpm for 5 min, the supernatant was transferred to a clean tube and evaporated to dryness in a centrifugal concentrator at 40 °C (Labconco Corp., MO, USA). The residue was reconstituted in 1 mL methanol and then 5 μL was injected into the UPLC/MS/MS system.

The pharmacokinetic study in this paper conformed to the National Institutes of Health Guide for Care and Use of Laboratory animals.

ClaS was prepared by dissolving clarithromycin in phosphoric acid solution at pH 1, and then adjusting the pH to 6.0 with 10% sodium hydroxide solution. This solution was passed through a 0.22-μm micropore film, and then sealed in vials after adding nitrogen gas. The concentration of ClaS was equal to that of ClaE.

2.2.6.2. Sample determination. A UPLC/MS/MS system was employed for analysis.

Chromatography was performed on an ACQUITY™ UPLC system (Waters Corp., Milford, MA, USA). The separation was carried out on an ACQUITY UPLC™ BEH C_{18} column (50 mm × 2.1 mm i.d., 1.7 μm; Waters Corp., Milford, MA, USA). The column temperature was kept at 35 °C. The determination was performed by gradient elution using (A) acetonitrile and (B) water (containing 0.05 mmol mL^{-1} ammonium acetate) as the mobile phase. The gradient conditions are shown in Table 1. The injection volume was 5 μL and the partial loop mode was selected for sample injection.

The Waters ACQUITY™ TQD triple-quadrupole tandem mass spectrometer (Waters Corp., Manchester, UK) was connected to the

Table 1
Gradient conditions for UPLC

Time (min)	Flow rate (mL/min)	A (%)[a]	B (%)[b]	Curve
Initial	0.250	50.0	50.0	Initial
0.50.	0.250	80.0	20.0	6[c]
1.50	0.250	80.0	20.0	6
2.00	0.250	50.0	50.0	1[d]

[a] Acetonitrile.
[b] 0.05 mmol mL^{-1} ammonium acetate water.
[c] Linear.
[d] Pre-step.

Table 2
Transition reactions of the analyte and internal standard

Molecule	Transition	Dwell (s)	Cone voltage (V)	Collision energy (eV)
Clarithromycin	748.20 → 157.80	0.200	40.0	30.0
Roxithromycin	837.30 → 157.80	0.200	45.0	35.0

UPLC system via an electrospray ionization (ESI) interface. The ESI source was operated in positive ionization mode with the capillary voltage setting at 3.3 kV. The extractor and RF voltage was 3.0 V and 0.2 V, respectively. The temperature of the source and desolvation was set at 100 °C and 400 °C, respectively. Nitrogen was used as the desolvation gas (550 L h^{-1}) and cone gas (50 L h^{-1}). For collision-induced dissociation (CID), argon was used as the collision gas at a flow rate of 0.15 mL min^{-1}. The multiple reaction monitoring (MRM) mode was used for quantification. Transition reactions of the analyte and internal standard are given in Table 2. All data collected in centroid mode were acquired using Masslynx™ NT4.1 software (Waters Corp., Milford, MA, USA). Post-acquisition quantitative analyses were performed using a QuanLynx™ program (Waters Corp., Milford, MA, USA).

Furthermore, this UPLC/MS/MS method had an acceptable recovery of above 70% and was easy to perform. Also, the linear range of clarithromycin in plasma was 0.01–20.00 μg mL^{-1} while the intra-day variation was nearly 6% and the inter-day variation was less than 15%.

3. Results and discussion

3.1. Investigations of the clarithromycin–phospholipid complex

3.1.1. Reaction solvents

The research on the complex of drug and phospholipids was based on a patent published by an Italian scientist in 1987 (Gabetta et al., 1987). As reported, the formation of a complex with drug and phospholipids could improve the physicochemical properties of the drug, especially its liposolubility (Jianmei et al., 2001; Pei-xue et al., 2005). In this paper, the clarithromycin–phospholipid complex was used to increase the liposolubility of clarithromycin. The mechanism of complex formation appears to involve an interaction between the drug and phospholipids, such as the combination of hydrogen bonds or van der Waals forces (Maiti et al., 2007; Yanyu et al., 2006), and the complex may be a unit of several molecules combined (Kidd and Head, 2005).

There are no systematic studies describing the preparation conditions for phospholipid complexes and Bombardelli E had only reported some brief reaction conditions for phospholipid complexes (Gabetta et al., 1987; Bombardelli and Mustich, 1991). This was mainly because each drug has its own particular physicochemical properties and it is hard to use a single set of conditions such as the reaction solvent, phospholipid ratios and reaction time for different drug–phospholipid complexes. In this paper, acetone, acetic ether and dehydrated alcohol, which are commonly used to prepare phospholipid complexes, were selected as the reaction solvents. Their dielectric constants were 21.4, 6.1 and 26.8, respectively. As shown in Table 3, in acetone, clarithromycin and phospholipids form almost no complex and this might be because acetone is a dysolvent for phospholipids (Jianmei et al., 2001). It has been reported that a higher dielectric constant may induce a greater reverse electric potential and suppress the interaction between the reacting materials (Jianmei et al., 2001). However, in this study, dehydrated alcohol with a higher dielectric constant of 26.8 has a better effect on the formation of uniform clarithromycin–phospholipid complex than acetic ether of 6.1, which was in accordance with the published literature (Ying et al., 2006). Moreover, dehydrated alcohol has already been used as a solvent for intravenous dosage forms and, thus, is safer than acetic ether (Panayiotis et al., 2004). Therefore, dehydrated alcohol was used as the reaction solvent for the preparation of the clarithromycin–phospholipid complex.

Table 3
Appearance of the complexes prepared according to Section 2.2.1 with clarithromycin and soybean lecithin at a ratio of 1:2 for 3 h at 65 °C

Reaction solvents	Dielectric constant	Appearance of the dried residue
Acetone	21.4	Obviously separated white drug powder and yellow phospholipids
Acetic ether	6.1	Almost yellow color of phospholipids with a few white drug spots in it
Dehydrated alcohol	26.8	Uniformly yellow color of phospholipids

3.1.2. Ratio of clarithromycin to phospholipid

It is well known that the ratio of drug to phospholipid for preparing complexes is usually 1:1 or 1:2 and almost never more than 1:3 (Jianmei et al., 2001; Ying et al., 2006). However, it can be seen from Table 4 that in this study the ratio for the complex to make sure that clarithromycin dissolve sufficiently in the 20% (w/v) oil phase is 1:10. As known, the formation of drug–phospholipid complex is based on the interaction such as hydrogen bonds and van der Waals forces between drug and phospholipids (Maiti et al., 2007; Yanyu et al., 2006). Therefore, the result above might be explained by the hypothesis that the polar parts of clarithromythin and phospholipids exhibit a relatively weak interaction while small amounts of phospholipids cannot occupy each possible position of clarithromycin, which might be involved in the interactions with phospholipids by the formation of hydrogen bonds and intermolecular forces, and until the ratio raised to 1:10, it might allow clarithromycin to be surrounded by enough phospholipids and the apolar parts of the phospholipids are located around the spatial structure of clarithromycin. Such a clarithromycin–phospholipid complex structure effectively improves the liposolubility of clarithromycin. Hence, a clarithromycin–phospholipid complex with a ratio of 1:10 could produce sufficient solubility and stability in the oil phase to allow emulsion preparation.

3.1.3. Different phospholipids types

E80® has a higher content of phosphatidylcholine (PC) compared with that of soybean lecithin which is nearly 70%. It is suggested that a higher PC content is propitious to form a more stable phospholipid complex (Jing et al., 2006; Guihua et al., 2005). However, the results of this part of the study showed that the emulsion prepared with the E80® complex exhibited drug separation during storage. This phenomenon might be explained by the fact that the phospholipids used to prepare the clarithromycin–phospholipid complex also play an important role

Table 4
Dissolution of clarithromycin–phospholipid complexes prepared according to Section 2.2.1 with different phospholipids ratios in a 20% (w/v) oil phase consisting of MCT:LCT 8:2

Clarithromycin and soybean lecithin ratios	Dissolution status of clarithromycin–phospholipid complex
1:2	Precipitation separation
1:8	Precipitation separation
1:10	Settled solution

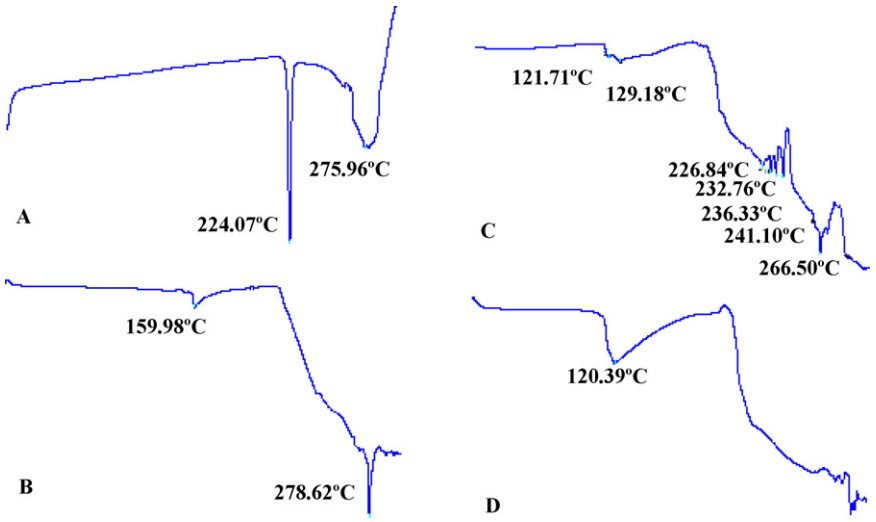

Fig. 2. DSC curves of (A) clarithromycin, (B) soybean lecithin, (C) the physical mixture of clarithromycin and soybean lecithin, and (D) the clarithromycin–phospholipid complex.

in emulsification. Soybean lecithin in this study had some anionic components besides PC, which influence the negative ζ-potential of the emulsions (Chansiri et al., 1999) while E80® with a higher PC content did not. It has also been reported that purer phospholipids, such as E80®, have a smaller value of Vt^{max} appearing to undergo flocculation readily while soybean lecithin, with a much larger Vt^{max} value, did not readily undergo flocculation and coalescence (Yamaguchi et al., 1995). In addition, emulsions with a greater negative ζ-potential are more stable to resist flocculation and coalescence because of the large electrostatic repulsion between the emulsion droplets, which might help to maintain the stability of emulsion (Yamaguchi et al., 1995). Accordingly, soybean lecithin was selected to prepare the clarithromycin–phospholipid complex in this study.

3.1.4. Differential scanning calorimetry of the clarithromycin–phospholipid complex

Differential scanning calorimetry is a rapid and reliable way to screen drug and excipient compatibility and it provides maximum information about possible interactions. Fig. 2 shows the DSC curves of clarithromycin (A), soybean lecithin (B), a physical mixture of clarithromycin and soybean lecithin (C) and the clarithromycin–phospholipid complex (D). Phospholipids exhibited two clearly different kinds of endothermal peaks. The first endothermal peak at 159.98 °C was mild, which suggested that the formation of this peak was due to hot movements of the polar part of the phospholipid molecule. Moreover, the second sharp endothermal peak could be formed owing to the phase transition from a gel-like state to a liquid crystal state, and the carbon-chain in the phospholipid may have perhaps undergone melting, isomeric or crystal changes (Maiti et al., 2007; Yanyu et al., 2006). The thermogram of clarithromycin exhibited two peaks. The first melting peak was sharp with a maximum at 224.07 °C while the second broad peak at 275.96 °C might be caused by the partial schizolysis of clarithromycin. In the DSC curve of the physical mixture of clarithromycin and phospholipids, there are some small sharp endothermal peaks between 225 °C and 242 °C, which were almost at the same temperature as the one in the clarithromycin thermogram of 224.07 °C. In addition, the broad peaks from 120 °C to 130 °C in the physical mixture curve might be due to the melting of the clarithromycin–phospholipid complex. This is because of the fact that when the temperature rises, clarithromycin and phospholipids partly interact to form a complex which has lower melting point than the individual components. Moreover, the sharp peak at 266.50 °C in the physical mixture curve also appeared at a lower temperature than the sharp peak at 278.62 °C appearing in the phospholipid curve, which is further proof of partial formation of the complex, which reduces the phase transition temperature of the phospholipids. The DSC curve of the clarithromycin–phospholipid complex shows that the original peaks of clarithromycin and phospholipids have disappeared. Also, the broad peak at 120.39 °C was in agreement with those between 120 °C and 130 °C in the physical mixture curve. In addition, the phase transition peak disappeared in the complex curve, indicating that the complex had a lower phase transition temperature than that of the phospholipids. It is evident by comparison of the four DSC curves that clarithromycin and phospholipids exhibit some interactions, such as the formation of hydrogen bonds and van der Waals forces. Due to the combination of the polar parts between clarithromyin and phospholipids, the carbon–hydrogen chain in the phospholipids can rotate freely and enwrap the polar parts of the phospholipid molecule, producing a sequential reduction between the phospholipid aliphatic hydrocarbon chains while the second endothermal peak of the phospholipids disappears and the phase transition temperature is reduced (Maiti et al., 2007; Yanyu et al., 2006).

3.2. Formulation investigations of ClaE

3.2.1. Oil phase ratio and oil phase composition of ClaE

Normally, long-chain triglycerides, medium-chain triglycerides, safflower oil and cotton seed oil are employed as the oil phase in fat emulsions intended for total parenteral nutrition (Muhannud and Bernd, 1998; Bivins et al., 1980; Hansrani et al., 1983; Sayeed et al., 1987). However, although LCT has been in clinical use for more than 30 years, its toxic effects, such as the triggering of immune dysfunction, accumulation in reticuloendothelial cells and deposition of adipochrome in the liver or lung after long-term use (Hongyao et al., 2008) must be taken into account when further applications are concerned. It has been reported that MCT in lipid emulsions reduces the large amount of linoleic acid, providing a better fatty acid balance (Carpentier and Hacquebard, 2006). It is also reported that the mixture of MCT and LCT can reduce the viscosity of LCT (Jumaa and Muller, 2001) and the particle size distribution of emulsion, as well promoting the solubility of drugs or increasing the entrapment

Table 5
Different oil phase ratios and oil phase composition in ClaE with MCT and LCT

The ratios of MCT and LCT	Total oil phase ratio in emulsion (w/v)	Oil phase appearance contained clarithromycin–phospholipid complex	Appearance of emulsions	Particle size distribution	Entrapment efficiency
8:12	20%	Drug separation	–	–	–
10:10	20%	Drug separation	–	–	–
12:8	20%	Drug separation	–	–	–
16:4	20%	Clear	Uniform	139.3 ± 45.7	91.52%
15:0	15%	Drug separation	–	–	–
18:0	18%	Clear	Uniform	134.2 ± 51.7	92.26%
20:0	20%	Clear	Uniform	136.0 ± 52.2	91.85%

efficiency. In addition, using an MCT and LCT mixture could reduce the interfacial tension which would increase the physical stability of emulsions (Muhannud and Bernd, 1998). Therefore, a mixture of LCT and MCT is currently used for commercial parenteral emulsions (Hongyao et al., 2008). This could simultaneously reduce the toxicity caused by total LCT and might also provide a more stable all-in-one mixture (Driscoll et al., 2000). Hence in this study, LCT and MCT were selected as the mixed oil phase. It has been reported that the oil phase volume has a marked effect on the mean particle size of the emulsions (Ishii et al., 1989), and this has, in turn, an effect on the physical stability and safe administration because larger particles may cause flocculation and embolism (Jeppsson et al., 1976; Laval-Jeantet et al., 1982). Moreover, oil phases with different ratios of LCT and MCT have different capacities for drug uptake and physicochemical properties, such as the viscosity of the oil phase, which directly affect the homogenization pressure and particle size distribution. As mentioned above, different oil phase ratios and oil phase compositions in ClaE with MCT and LCT were investigated. The results are shown in Table 5.

From Table 5 it can be seen that, by increasing the proportion of MCT, the oil phase with 20% MCT and LCT (w/v) in ClaE showed the increased solubility of the clarithromycin–phospholipid complex. This might be due to the higher polarity of MCT compared with LCT, resulting in an increased drug uptake capability for MCT. At an MCT: LCT ratio of 16:4, 18:0 and 20:0, the oil phase appeared clear after dissolving the complex. From Table 5 it can be seen that the values of EE and particle size among the three formulations remained almost unchanged while the standard deviation of PSD became slightly larger following the increase in the proportion of MCT. This is mainly because an excess of MCT may reduce the physical stability of the emulsion to induce aggregation of the emulsion droplets or the formation of oil droplets (Jia et al., 2006). In addition, oil droplets were observed in the formulation with 18% (w/v) and 20% (w/v) MCT, after thermal sterilization or during storage. Therefore, finally, a ratio of MCT:LCT of 16:4 of the 20% (w/v) oil phase in the ClaE formulation was employed.

3.2.2. Amount of soybean lecithin as emulsifier

Lecithin is regarded as a well-tolerated and non-toxic compound, making it suitable for long-term use and high-dose infusion. It is totally biodegraded and metabolized and is an integral part of biological membranes. Emulsions cannot be stabilized using pure PC; Hansrani (1980) and Yamaguchi et al. (1995) reported that certain 'impurities' were necessary to form stable emulsions. This is because some charged phospholipid components with negative ions contribute to more negative ζ-potentials and, thus, increase the stability of the emulsions to heat-stress or storage. Soybean lecithin contains more PA and PI than egg lecithin, and it has also been reported to exhibit excellent emulsifying properties (Chansiri et al., 1999; Rydhag, 1979). Hence, in this paper, soybean lecithin with about a 72% PC content and some other ionic components was selected as the main emulsifier. Different amounts of this soybean lecithin were investigated. The results are shown in Table 6.

As shown in Table 6, besides 2.5% (w/v) soybean lecithin used for preparing the clarithromycin–phospholipid complex, if no more soybean lecithin was added, the physical appearance of ClaE showed oil droplets after thermal sterilization. It is suggested that this is caused by a relatively large oil phase volume of 20% (w/v) and the small particle size in the nanometer range led to an increase in the interfacial surface in ClaE (Muhannud and Bernd, 1998), which requires more emulsifier for emulsification. Hence, to make a sufficiently emulsified large oil phase, more soybean lecithin was added as an emulsifier of 0.5% (w/v) and 1.0% (w/v) for further investigation. It can be seen from Table 6 that another 0.5% (w/v) soybean lecithin could still not totally stabilize the emulsion and adding 1.0% (w/v) soybean lecithin produced an emulsion with good physical stability following thermal stress and storage. The values of PSD and entrapment efficiency also confirmed its safety for intravenous administration.

3.2.3. The co-emulsifiers of Tween80, F68 and sodium oleate

PC alone is obviously insufficient to stabilize the freshly formed interface during high-pressure homogenization. The co-emulsifiers can help to further emulsify the oil phase and form a tight complex interfacial film with lecithin between the water phase and the oil phase to maintain the emulsion stability. Tween80, F68 and sodium oleate were used in this investigation. Tween80 is widely used as the co-emulsifier in emulsion preparations, while the toxicity of Tween80 should be considered for clinical applications (YanJiao et al., 2007), and the amount of 0.2% (w/v) was considered within safe limits. Pluronic® F68 is a non-ionic emulsifier and produces sterically stabilized emulsions (Eccleston, 1992). It stabilized the newly created interface immediately. Within only a single homogenization cycle, maximum dispersity had already been attained and could not be further reduced by repeated homogenization. Tween80 and F68 are not endogenous materials and, so, the less used the better. Therefore, 0.2% (w/v) Tween80 and 0.2% (w/v) F68, were finally employed in this study. It can be seen from Table 6 that such amounts confer good physical stability on ClaE.

Table 6
Effect of different amounts of soybean lecithin in ClaE

The amount of soybean lecithin (%w/v)	Physical appearance	PSD (nm)	Entrapment efficiency
0	Visible oil droplets were observed after thermal sterilization	–	–
0.5	Visible oil droplets were observed during storage	–	–
1.0	Good appearance without oil droplets	139.3 ± 45.7	91.52%

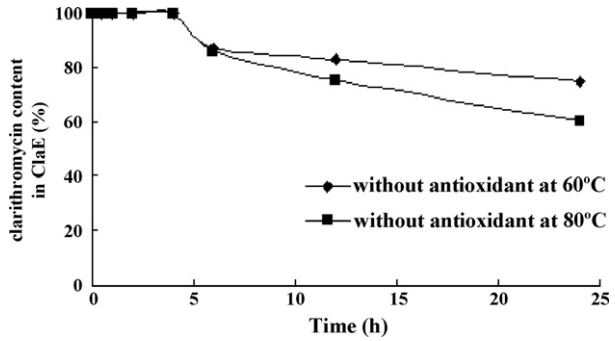

Fig. 3. The clarithromycin content in the ClaE formulation without any antioxidants at 60 °C and 80 °C for 24 h.

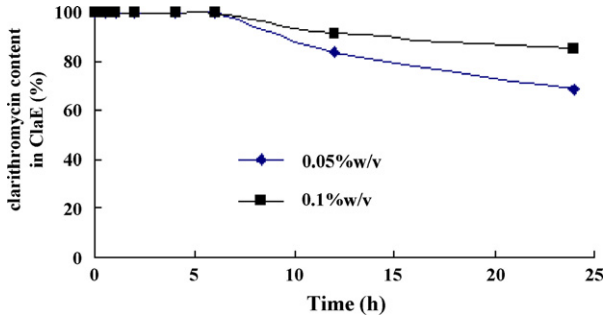

Fig. 4. The clarithromycin content in the ClaE formulation with 0.05% (w/v) and 0.1% (w/v) vitamin E at 80 °C for 24 h.

Sodium oleate, as a water-soluble co-emulsifier, can reach the interface rapidly and be incorporated at the oil–water interface. It can increase the electric repulsion of emulsion particles by forming an integral part of the emulsifier film giving them a negative charge, resulting in a higher resistance to the calescence of oil droplets (Hongyao et al., 2008; YanJiao et al., 2007), or allowing at least short-time stabilization of the new interface until PC gradually becomes available. This process is of great value in stabilizing the physical stability of emulsions. The effect of sodium oleate is reflected in the value of the ζ-potential, which is an important parameter for evaluating the stability of emulsions and it represents the repulsion among emulsion particles to some extent. In this paper, 0.1% (w/v) of sodium oleate was used in the ClaE formulation, and the ζ-potential of ClaE after sterilization was −21.26 mV, which is satisfactory.

3.2.4. Selections of effective antioxidants

Safe, effective and controllable are of great importance for clinical drug applications. Hence, L-cysteine, Na_2SO_3 and vitamin E were investigated as antioxidants to maintain the effective content of ClaE for safe administration in this study. Different amounts of the antioxidants were investigated compared with the formulation without antioxidants. Six different formulations with 0.02% (w/v) and 0.05% (w/v) L-cysteine, 0.05% (w/v) and 0.1% (w/v) vitamin E, and 0.1% (w/v) and 0.2% (w/v) Na_2SO_3 were examined separately by measuring the drug content at different time points for 24 h and the emulsions were put in 60 °C and 80 °C conditions, respectively. In addition, the pH values of all samples during the whole procedure were also determined.

As shown in Fig. 3, ClaE without any antioxidants exhibited a marked reduction of 25.44% and 39.88% in clarithromycin content at 60 °C and 80 °C after 24 h. As the chemical degradation of drug in emulsions can be manifested in terms of a change in the bulk pH, the pH values of all samples during the entire procedure in this part were determined. They were all above pH 7, which indicates that the pH has no influence on the drug content in this study because clarithromycin and ClaE are both stable above pH 7 (Yan et al., 2008). Also, there are no research reports that emphatically prove that clarithormycin is easily oxidized. So, the degradation of clarithromycin in ClaE might be due to the degradation of phospholipids and oils and the degradation pathways are mainly oxidation and hydrolysis while the main degradation products are free fatty acids (FFA). Moreover, in the ClaE formulation without any oxidants, phospholipids and oils easily undergo oxidation and hydrolysis and this process reduced the stability of clarithromycin–phospholipid complexes and resulted in partial complex dissociation. Then the dissociated clarithromycin was transferred from the oil phase to the oil–water interface or the aqueous phase which contained a relatively large amount of FFA. Also, the FFA might cause severe degradation of clarithromycin since it is acid unstable, which is exhibited as a marked reduction in drug content as shown in Fig. 3. Hence, it is of great importance to add some antioxidants to ClaE to minimize the degradation of phospholipids and oils and further to control the drug content within effective limits.

In the formulation with 0.1% (w/v) and 0.2% (w/v) Na_2SO_3, separation of clarithromyin and visible oil droplets appeared in ClaE after thermal sterilization, which proved that the antioxidant Na_2SO_3 might lead to breakdown of the oil–water interfacial film because of its inherent physicochemical properties and, hence, it cannot be used in ClaE as an antioxidant.

In the formulations with 0.05% (w/v) and 0.1% (w/v) vitamin E at 60 °C, ClaE showed no reduction in clarithromycin content. It is suggested that vitamin E produces effective antioxidation in ClaE. However, at 80 °C both formulations with 0.05% (w/v) and 0.1% (w/v) vitamin E showed a clear reduction in clarithromycin content and the reduction in clarithromycin content at 80 °C was 31.81% and 14.71%, respectively, as shown in Fig. 4. In addition, it appears that a greater amount of 0.1% (w/v) vitamin E in ClaE had a stronger antioxidant effect than that of 0.05% (w/v). It also appears that vitamin E produces an insufficient antioxidant effect in ClaE by increasing the temperature, which might reflect the fact that ClaE with vitamin E as an antioxidant cannot undergo oxidation during long-term storage.

Furthermore, the formulations with 0.02% (w/v) and 0.05% (w/v) L-cysteine at 60 °C and 80 °C both exhibited no reduction in drug content, which suggests that L-cysteine has a marked antioxidant effect in ClaE. A typical result for the clarithromycin content in ClaE with 0.02% (w/v) and 0.05% (w/v) L-cysteine at 80 °C for 24 h is shown in Fig. 5. Fig. 5 also shows that 0.02% (w/v) L-cysteine produces a sufficient antioxidant effect in ClaE. This antioxidant effect was also proved by the results of the stability studies of ClaE in part3.3, which showed no obvious change in clarithromyin

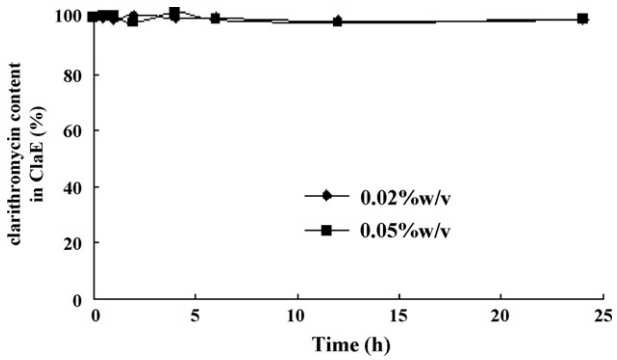

Fig. 5. The clarithromycin content in the ClaE formulation with 0.02% (w/v) and 0.05% (w/v) L-cysteine at 80 °C for 24 h.

Table 7
Characteristics of ClaE before and after thermal sterilization

	Physical appearance	Content	PSD (nm)	ζ-Potential	EE (%)
Before thermal sterilization	Good	99.53%	134.3 ± 48.2	−20.32 mV	92.05%
After thermal sterilization	Good	99.16%	139.3 ± 45.7	−21.26 mV	91.52%

content during storage at 10 °C for 6 months. Therefore, 0.02% (w/v) L-cysteine was finally selected as the single antioxidant in ClaE.

3.3. Stability studies of ClaE

The physical appearance, content, PSD, entrapment efficiency and ζ-potential are the main parameters used to evaluate the physicochemical stability of emulsions and these were employed in this paper. Studies of both the thermal sterilization stability and storage stability were carried out and the results are shown in Tables 7 and 8, respectively.

As reported, the stability of ClaE during thermal sterilization is key and essential for further applications. It is reported that the thermal sterilization process might be a re-emulsifying procedure or an irreversible redistribution of emulsifier compounds within the oil and aqueous compartments (Herman, 1992; Herman and Groves, 1993). Based on the data shown in Table 7, ClaE prepared according to the final formulation had a uniform milky appearance. As far as the content was concerned, the PSD and entrapment efficiency were nearly unchanged after thermal sterilization, which proves that ClaE has sufficient physicochemical stability to thermal stress. In addition, the absolute values of the ζ-potential were slightly greater due to the free fatty acids which were produced by the hydrolysis of lecithin, co-emulsifiers and oil phase during thermal sterilization. Higher absolute value of ζ-potential could increase the electrostatic repulsion of emulsion droplets to obtain more stable emulsions. Hence, ClaE in this study could undergo thermal sterilization in a 100 °C rotating water bath for 30 min.

The short-term stability of ClaE was examined over 6 months while the emulsion was kept at 10 °C. The data determined after 6 months storage are shown in Table 8 and they indicate that the variations in the main parameters evaluated in this study changed very little compared with the data obtained after sterilization at zero time. In addition, all the parameters of ClaE after 6 months were still within the range required for safe intravenous administration. Hence, it can be concluded that the ClaE in this study has sufficient physicochemical stability for storage at 10 °C for 6 months. It is suggested that the ClaE in this paper can be used for further clinical studies and can be produced on an industrial scale.

3.4. Pharmacokinetic studies of ClaE

The pharmacokinetic experiment was carried out to compare ClaE and ClaS by determining the drug concentration in rat plasma up to 24 h after i.v. administration. The plasma was subjected to liquid–liquid extraction, which had an acceptable relative recovery of clarithronycin from plasma and was simple to perform. The mean plasma concentration–time profiles of ClaE and ClaS are presented in Fig. 6. The data were analyzed by drug and statistics (DAS) version 2.0 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The concentration–time curves of ClaE and ClaS in rat plasma fitted a three-compartment model with a weighting factor of $1/c^{-2}$.

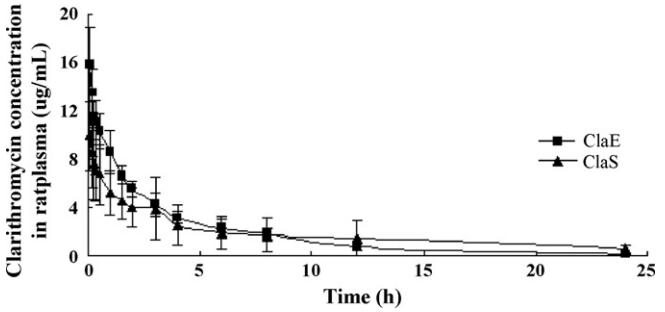

Fig. 6. Mean plasma concentration–time profiles after i.v. clarithromycin emulsion (ClaE) and clarithromycin solution (ClaS) given to rats at a dose of 22.5 mg kg^{-1} (n = 6).

As known a drug-loaded emulsion usually exhibits a slow release because the loaded drug needs time to transfer from the oil phase to the water phase. However, it can be seen from Fig. 6 that the elimination shown by the ClaE and ClaS curves was similar and there was no significant delayed release for ClaE compared with ClaS. It has been reported that the main factor for drug loading in an o/w emulsion for a poorly water and oil soluble drug is the oil–water interfacial film, which is mainly formed by different kinds of emulsifiers, such as lecithin, F68, Tween80 and sodium oleate (YanJiao et al., 2007; Akkar and Müller, 2003). Also, the drug in the interfacial film can be released more rapidly into the blood than that in the oil phase. Hence, the results shown in Fig. 6 indicate that part of the clarithromycin in ClaE might be in the oil–water interfacial film as well as in the intra-oil phase. Moreover, it appears from Fig. 6 that, at first few time points in the period up to 8 h, the drug concentration in ClaE was a little higher than that in ClaS. This is because after i.v. administration, ClaS is rapidly distributed and penetrates into tissues while the drug in ClaE needs more time to be released from the oil phase into blood. However, Fig. 6 shows that at the last two time points after 8 h, namely 12 h and 24 h, the plasma drug concentration of ClaS was a little higher than ClaE. This might show that the clarithromycin in ClaE undergoes faster elimination than ClaS. This might be due to the fact that lipid emulsions with a particle size between 10 nm and 1000 nm can undergo phagocytosis and be eliminated by the endothelial system after i.v. administration. This process helps the drug reach higher levels in the liver, spleen and brain which causes a greater reduction in the drug-plasma concentrations produced by the emulsion compared with the injected solution. In addition, such distribution characteristics of emulsions results in better targeting and a better therapeutic effect.

The main pharmacokinetic parameters were also calculated by the statistical moment method using the DAS 2.0 software. The

Table 8
Characteristics of ClaE after thermal sterilization and over 6 months storage at 10 °C

	Physical appearance	Content	PSD (nm)	ζ-Potential	Entrapment efficiency
After thermal sterilization	Good	99.16%	139.3 ± 45.7	−21.26 mV	91.52%
After 6 months storage	Good	98.96%	149.6 ± 56.2	−23.71 mV	89.31%

Table 9
Pharmacokinetic parameters in rats after i.v. administration of ClaE and ClaS

Parameters	ClaE	ClaS
k_e ($1\,h^{-1}$)	0.200 ± 0.045	0.165 ± 0.089
AUC_{0-t} ($mg/(L\,h^{-1})$)	44.963 ± 7.222	38.52 ± 28.455
$AUMC_{0-t}$	188.627 ± 49.979	239.902 ± 263.605
MRT (h)	4.162 ± 0.653	4.896 ± 2.809
$T_{1/2}$ (h)	3.605 ± 0.761	5.272 ± 2.472
CL ($L/(h\,kg^{-1})$)	0.5 ± 0.098	0.697 ± 0.428
Vss ($L\,kg^{-1}$)	2.54 ± 0.423	4.339 ± 1.702

The data are mean ± S.D. ($n = 6$). k_e, elimination rate constant; $T_{1/2}$, half life; AUC_{0-t}, area under the concentration–time curve; $AUMC_{0-t}$, area under the cross-product of the time and plasma concentration–time curve; MRT, mean residence time; CL, clearance; Vss, steady-state apparent volume of distribution.

results are shown in Table 9 and most of the parameters showed no statistically significant differences ($n = 6$, $P > 0.05$) except Vss ($n = 6$, $p < 0.05$) between ClaE and ClaS analyzed by SPSS. Also, the Vss of ClaS was 1.7 times higher than that of ClaE. This result was in agreement with the results of propofol (Sandeep and Ebling, 1998), tirilazad (Wang et al., 1999) and vinorelbine lipid microsphere (Hongyao et al., 2008). This phenomenon could be explained by the fact that, after i.v. ClaS, clarithromycin underwent rapidly distribution and penetration into tissues, while in the case of ClaE, since the drug is located in the oil phase or in the oil–water interfacial film, this structure can delay penetration and distribution into tissues for clarithromycin and, moreover, free clarithroycin needs time to dissociate from the complex and be transferred into water phase. Therefore, ClaE produces higher plasma concentrations together with a lower Vss and CL than ClaS, while the AUC_{0-t} of ClaE was slightly higher than that of ClaS, which might suggest that the availability of ClaE was increased somewhat. Also, ClaE in this study was sufficiently stable to thermal sterilization and might markedly reduce vein irritation while increasing patient compliance. Therefore, ClaE has a great potential for clinical applications and can be produced on an industrial scale.

4. Conclusion

In conclusion, based on systematic investigations, a clarithromycin–phospholipid complex was finally prepared in which clarithromycin and soybean lecithin were reacted in dehydrated alcohol at a ratio of 1:10 for 3 h at 65 °C. Furthermore, a new ClaE formulation loaded with the complex was developed, which was composed of clarithromycin 0.25% (w/v) and 2.5% (w/v) soybean lecithin to prepare a complex, long-chain triglyceride (LCT) 4% (w/v), medium-chain triglyceride (MCT) 16% (w/v), another soybean lecithin 1.0% (w/v), F68 0.2% (w/v), Tween80 0.2% (w/v), glycerol 2.5% (w/v), sodium oleate 0.1% (w/v) and L-cysteine 0.02% (w/v). In addition, ClaE was sterilized in a 100 °C revolving water bath for 30 min. High-pressure homogenization, DSC, PCS, ELS and HPLC were used for the preparation and evaluation of ClaE in vitro by determining essential parameters, such as the drug content, PSD, ζ-potential and EE of ClaE. The results obtained proved that ClaE had sufficient physicochemical stability to sterilization and short-term storage at 10 °C. The pharmacokinetic study in vivo using the UPLC/MS/MS system indicated that there were no significant differences in the main parameters of ClaE and ClaS, except Vss. Hence, ClaE has a great potential for clinical applications and can be produced on an industrial scale.

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