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Optimization of tocol emulsions for the intravenous delivery of clarithromycin

Jie Li, Shufang Nie, Xingang Yang, Changguang Wang, Shuxia Cui, Weisan Pan *

Shenyang Pharmaceutical University, Shenyang, China

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Abstract

In the present study, novel less-painful tocol emulsions for the intravenous delivery of clarithromycin were prepared and optimized. The therapeutically effective concentration of clarithromycin, 5 mg/ml, was achieved using tocopherol succinate (TS) combined with oleic acid as lipophilic counterions. The possibility of employing the microdialysis technique to investigate the distribution of the drug in emulsions was explored. A three-level three-factorial Box–Behnken experimental design was utilized to conduct the experiments. The effects of selected variables, tocopherol succinate/oleic acid relation, poloxamer 188 content and 0.1 M NaOH amount, on three considered responses were investigated. The particle size, ζ potential and the oil phase distribution of clarithromycin for the optimized formulation were observed to be 138.5 nm, −32.16 mV and 97.28%, respectively. The emulsions prepared with the optimized formula demonstrated good physical stability during storage at 4 °C and room temperature. The histopathological examination for rabbit ear vein irritation test indicated that the irritation of clarithromycin could be eliminated by formulating the drug in a tocol emulsion.

Keywords: Clarithromycin; Tocol emulsion; Tocopherol succinate; Microdialysis; Box–Behnken design; Vein irritation

1. Introduction

Clarithromycin (CLA) is a new semisynthetic macrolide antibacterial with a 14-membered ring. Its chemical structure differs from erythromycin by the methylation of the hydroxyl group at position 6 on the lactone ring. These chemical modifications are responsible for CLA being acid stable, having an increased spectrum of activity, better pharmacokinetic properties and fewer gastrointestinal adverse effects than erythromycin (Rodvold, 1999). CLA is currently marketed in tablet form as Biaxin®. However, CLA has the potential for vein irritation during intravenous administration, which may result from the intrinsic property of the drug molecule (Cannon et al., 1995).

Parenteral emulsions that are formulated using a biocompatible emulsifying agent to disperse oil in an aqueous phase are used extensively for drug delivery (Tamilvanan, 2004). The benefits of emulsions are numerous. In general, they are biocompatible, biodegradable, physically stable (particularly nanoemulsions and microemulsions) and relatively easy to produce on a large scale using proven technology. Lipophilic drugs are incorporated into the interior oil phase, which are sequestered from direct contact with the nerve ending in the venous wall. Thus, emulsions can minimize the pain associated with intravenously administrated drugs by exposing the tissue to lower concentrations of the compounds. Unfortunately, emulsions based largely on vegetable oils are unsuitable, since the poor solubility of CLA in long-chain triglycerides (LCTs) and medium-chain triglycerides (MCTs) (Lovell et al., 1994). A recent advance was the use of tocopherol or derivatives thereof as the oil phase of emulsions (tocol emulsions) for drug solubilization and parenteral delivery (Constantinides et al., 2004). More recently, a tocol emulsion for the intravenous delivery of CLA was prepared and demonstrated a marked pain reduction (Lu et al., 2008). However, the concentration of drug in the formulation was 2.5 mg/ml which was not high enough to be therapeutically useful. It was disclosed that the dimethylamino group of CLA could be exploited through lipophilic ion pairing

Abbreviations: CLA, clarithromycin; LCT, long-chain triglyceride; MCT, medium-chain triglyceride; TS, tocopherol succinate.

* Corresponding author at: Department of Pharmaceutics, Shenyang Pharmaceutical University, PO Box 122, Wenhua Road 103, 110016 Shenyang, China. Tel.: +86 24 23986313; fax: +86 24 23953241.
E-mail address: weisanpan@gmail.com (W. Pan).

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to capture the drug in the oil phase of emulsions (Lovell et al., 1994). A tocol emulsion using tocopherol succinate (TS) as lipophilic counterions to improve CLA solubility was reported in the literature (Constantinides et al., 2002). However, relatively high content of tocopherol and the need for bioincompatible surfactants for formulation led to regulatory issues (Chen, 2005).

The evaluation of distribution of CLA in emulsions is important for predicting their irritation effects in vivo. Microdialysis technique is a sampling technique based on the passive diffusion of substances along their concentration gradients across a semi-permeable membrane (Abrahamsson and Winsö, 2005). Up to now, this technique has not been proposed for investigating the distribution of compounds in emulsion systems. Microdialysis technique is volume-neutral, i.e. there is little removal of fluid volumes from periprobe fluid (Chaurasia et al., 2007). Thus, compared with other conventional techniques, the disturbing effect of microdialysis on the distributional equilibrium of compounds between aqueous and oil phase of an emulsion is minimal.

In the present study, a novel less-painful tocol emulsion for the intravenous administration of CLA was developed. The therapeutically effective concentration of CLA, 5 mg/ml, was achieved using TS combined with oleic acid as lipophilic counterions. The possibility of employing the microdialysis technique to investigate the distribution of CLA in emulsions was explored. Based on the principles of design of experiments, response surface methodology (Nutan et al., 2005) was utilized to evaluate the effects of selected variables and to optimize the formulation parameters.

2. Materials and methods

2.1. Materials

CLA (Purity 97.4%) was purchased from Huayi Corp. (Yiwu, China). TS was obtained from Xinchang Corp. (Zhejiang, China). Oleic acid was supplied by Yuwang Corp. (Shandong, China). Lipoid E80 was purchased from Lipoid Corp. (Ludwigshafen, Germany). Poloxamer 188 (Lutrol F68) was furnished by BASF. Purified soybean oil was purchased from Beiya Corp. (Tieling, China) and Miglyol 812 (MCT) was obtained from Huls (Witten/Ruhr, Germany). All other ingredients were of pharmaceutical grade.

2.2. Preparation of emulsions

The emulsions were prepared by the phase-inversion technique. Briefly, 1% lecithin was dissolved in a 6% oil phase containing a mixture of soybean oil with MCT (1:1, w/w). TS and oleic acid were added and the drug (0.5%) was completely dissolved in the oil phase. Poloxamer 188, lecithin (0.5%), and various amounts of NaOH solution (0.1 M) were dissolved in 2.5% aqueous solutions of glycerol (for adjustment of isotonicity). The oil phase and the aqueous solution were heated separately to about 70–75 °C. The aqueous solution was added dropwise to the oil phase and this mixture was pre-emulsified using an Ultra-Turrax T18 (Janke & Kunkel, Staufen, Germany) at 8000 rpm for 3 min. Final emulsification was carried out by passing the coarse emulsion through a high-pressure homogenizer (YSNM-1500 Nanomizer SYSTEM, Japan) six times at a pressure of 11,600 psi. The emulsions were filled into 10-ml vials, flushed with nitrogen and sterilized in a 100 °C rotating water bath for 30 min.

2.3. HPLC analysis

The HPLC system consisted of a 200 mm × 4.6 mm RP-18 5 μm BonChrom column (Agela Tech., China), Shimadzu LC-10AT pump and a Shimadzu SPD-10A ultraviolet detector (Shimadzu, Kyoto, Japan). The mobile phase consisted of a mixture of 0.067 M aqueous solution of KH_2PO_4 (adjusted to pH 5.5 with H_3PO_4) and acetonitrile (60:40, v/v). The flow rate and injection volume were set to 1 ml/min and 20 μl, respectively. The detection was at a wavelength of 210 nm.

2.4. Particle size and ζ potential

The particle size and ζ potential of the emulsions were determined by a laser scattering method (Nicomp PSS ZW 380, Santa Barbara, CA, USA). The formulations were diluted with double-distilled water for the measurements.

2.5. Calibration of the microdialysis system

2.5.1. Microdialysis equipment

The microdialysis system consisted a tubular dialysis fiber of 100 mm membrane length (Cuprophan ALF-120, Nikkiso, Japan) which was connected with an inlet and an outlet tube, a subject (CLA standard solution or emulsion), and a perfusion pump (Shenyang Pharmaceutical University, China). A perfusion fluid entered the probe through the inlet tubing at a constant flow rate (1.0 μl/min), passed the membrane and was then transported through the outlet tubing and collected in a microvial (dialysate) (Plock and Kloft, 2005). The experiments were conducted under moderate stirring.

2.5.2. Method of no-net-flux

The microdialysis experiment was not performed under equilibrium conditions because the perfusate was constantly being pumped through the probe (Höcht et al., 2007). Hence, the concentration found in the dialysate would always be only a fraction of the actual analyte concentration in the periprobe fluid. This fraction is called recovery (Michalowski et al., 2004), and should be determined by suitable methods for quantification of microdialysis data (Lange et al., 2000). For no-net-flux calibration, different concentrations of CLA saline solution were prepared. Before the sampling, the probe had an equilibrium period of 1 h. The time interval for sampling was 2 h. Three samples were collected (Abrahamsson and Winsö, 2005). A linear regression analysis was performed, for the relationship between $(C_{in} - C_{out})$ and $(C_{in} - C_m)$. The slope of the straight line was

the recovery as could be seen from the following equation:

$$R\ (\%) = \frac{C_{in} - C_{out}}{C_{in} - C_m} \times 100 \quad (1)$$

where R was the recovery for the microdialysis system, and C_{in}, C_{out}, C_m were the concentration of CLA in the perfusion medium, dialysate and surrounding medium, respectively (Stahle, 2000).

2.5.3. Method of retrodialysis

Retrodialysis was defined as the diffusive loss of CLA from the dialysis perfusate (24.7, 51.9, 71.9, 83.1, and 96.1 μg/ml CLA saline solution) into the surrounding medium (blank emulsion) (Elmquist and Sawchuk, 1997). As the diffusion process was assumed to be quantitatively equal in both directions, the substance loss through the membrane was the same as its recovery (Plock and Kloft, 2005). It could be calculated by the following equation:

$$R\ (\%) = 1 - \left(\frac{C_{out}}{C_{in}}\right) \times 100 \quad (2)$$

2.6. Distribution of CLA in emulsions

The microdialysis probe was placed in CLA tocol emulsions, and the drug free normal saline was chosen as the perfusion fluid. Three samples were collected. For the determination of the distribution of CLA in the oil phase of an emulsion, the following equation was used:

$$D\ (\%) = 1 - \left(\frac{C_{out}}{RC_{total}}\right) \times 100 \quad (3)$$

where D was the distribution of CLA in the oil phase of an emulsion, C_{total} was the strength of the emulsion determined by HPLC method, and R was the recovery for the microdialysis system determined by the retrodialysis method.

2.7. Experimental design

A three-level three-factorial Box–Behnken experimental design (Design Expert, Version 7.0.2, Stat-Ease Inc., Minneapolis, MN) was used for conducting the experiments. This design is suitable for exploring quadratic response surfaces and constructing second order polynomial models. The design consists of replicated center points and the set of points lying at the midpoint of each edge of the multidimensional cube that defines the region of interest. The non-linear quadratic model generated by the design is of the form:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$$

where Y represents the response associated with each factor level combination, b_0 is an intercept and b_1–b_{33} are the regression coefficients of the factors (Box and Behnken, 1960; Chopra et al., 2007). The factors chosen and settings of factor levels were presented in Table 1.

Table 1
Variables in Box–Behnken experimental design

Factor	Level		
	−1	0	+1
X_1: tocopherol succinate (%, w/w)	30	53	76
X_2: poloxamer 188 (%, w/v)	0.5	1.0	1.5
X_3: 0.1 M NaOH (%, v/v)	10	15	20

Response	Constraints
Y_1: particle size (nm)	Minimize
Y_2: ζ potential (mV)	Maximize
Y_3: distribution (%)	Maximize

2.8. Rabbit ear vein irritation test

Groups of three rabbits received an infusion of 5 mg/ml CLA emulsion, 5 mg/ml CLA lactobionate solution in saline (positive control), and an equivalent volume of saline solution into their marginal ear vain (Lovell et al., 1994). The rate of administration was maintained at 1 ml/min and the total drug dose was 40 mg/(kg day) for 2 days. Following infusion, visual observations of the site of infusion were made and three rabbits from each group were killed after the last administration for histopathological examination (Lu et al., 2008). The experimental protocol was approved by the institutional animal ethical committee and was in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

3. Results and discussion

3.1. Preparation of emulsions

The oil mixtures consisted of four components—soybean oil, MCT, TS and oleic acid. Both LCT and MCT either alone or MCT in combination with LCT have been known for their long-term commercial acceptability in parenteral emulsions. MCTs are reported to be 100 times more soluble in water than LCTs and to have an enhanced solubilizing capability (Tamilvanan, 2004). Also, MCTs may reduce the toxicity associated with pure LCT-based parenteral emulsions and may yield more stable formulations (Driscoll et al., 2002).

Because of their biocompatibility and stability, the naturally derived purified phospholipids continue to be the emulsifiers most frequently employed in injectable emulsion formulations (Floyd, 1999; Driscoll, 2006). In this study, Lipoid E80 was present in both the oil and aqueous phase before emulsification. Preliminary experiments indicated that this would kinetically facilitate the rapid formation of a stable emulsion since the phospholipids could approach the oil–water interface from both phases.

The ability of surfactant molecules to give the necessary curvature of the interfacial film required to form fine emulsions has been related to the packing geometry, which is the ratio between hydrocarbon volume, optimum head group area and tail length of the molecule at the interface (Trottaa et al., 2002). The incorporation of certain drugs in an emulsion formulation

with phospholipids as the sole emulsifier has been found to reduce stability and cause phase separation. A combination of phospholipids and a non-ionic copolymer (poloxamer 188) surfactant leads to the formation of close-packed mixed film of high surfactant coverage, as well as of sufficient viscosity to prevent creaming and promote stability, which is attributed to the steric stabilization of the non-ionic surfactant (Jumaa and Müller, 2002).

A combination of TS and oleic acid was added to the oil phase as ion pairing agents for a high loading of CLA. Alkali fatty acids are commonly added to emulsions for total parenteral nutrition to adjust the pH of the formulations to physiological values (Buszello et al., 2000). The inclusion of lipophilic counterions was also anticipated to facilitate drug partitioning from the oil–water interface into the core of emulsion droplets, and hence reduce pain upon intravenous administration. In addition, surplus lipophilic counterions required for the prevention of crystal growth during storage (Lovell et al., 1994) would meliorate the physicochemical properties of the oil phase, resulting in an enhanced solubilizing capacity for CLA. Moreover, besides CLA, other amphiphilic drugs, such as erythromycin, doxorubicin, amiodarone etc. can also benefit from formulation in the tocol emulsion system attributed to their cationic properties.

Emulsifiers can stabilize the emulsion droplets not just by formation of a mechanical barrier, but also by producing an electrical barrier (Trottaa et al., 2002). The presence of small amounts of phosphatidylserine and phosphatidylglycerol (2–5%) in Lipoid E80 resulted in a negative droplet surface charge (ζ potential). In addition, due to an accumulation of the negatively charged ionized carboxy groups on the interface, TS and oleic acid could also act as co-emulsifiers leading to more negative ζ potential, which resulted in a higher resistance to coalescence of the emulsion droplets (Jumaa et al., 1998).

3.2. Calibration of the microdialysis system

The microdialysis probe has a hollow fiber that is permeable to water and small molecules (Höcht et al., 2007), and when a perfusion fluid enter, exchange of CLA occurred in both directions across the semi-permeable membrane in favor of a gradient of concentration (Chaurasia et al., 2007). If C_{in} lies below C_m, CLA will diffuse through the membrane into the perfusate, resulting in a higher C_{out} than C_{in}. If concentrations are vice versa, then compared to the perfusate, C_{out} will be decreased (Plock and Kloft, 2005). The linear relationship between $(C_{in} - C_{out})$ and $(C_{in} - C_m)$ presented in Fig. 1 indicated that the membrane did not interact with the drug (Lange et al., 2000) and the extraction fraction was the same whether CLA exchange across the membrane occurred by either loss or gain.

The retrodialysis experiment was performed using the drug in the perfusate instead of in the surrounding medium. It was a one-point simplification of the no-net-flux method. If CLA was added to the unloaded emulsion, it could partially distributed into the oil phase, decreasing the free drug concentration in the aqueous phase of the system not allowing the calculation of the recovery properly (Lange et al., 2000). The microdialysis recoveries determined by no-net-flux and retrodialysis method were 85.5% and 86.2%, respectively. The high recovery of the microdialysis system for CLA could be attributed to the 100 mm length of the probe. According to Fick's law of diffusion the rate of perfusion across a membrane is proportional to its area. Therefore, increasing the length and thus the area of the microdialysis membrane will lead to an increase in recovery (Höcht et al., 2007). No significant difference between the results could be observed, which indicated that the presence of emulsion droplets did not interfere with the quantification of microdialysis data.

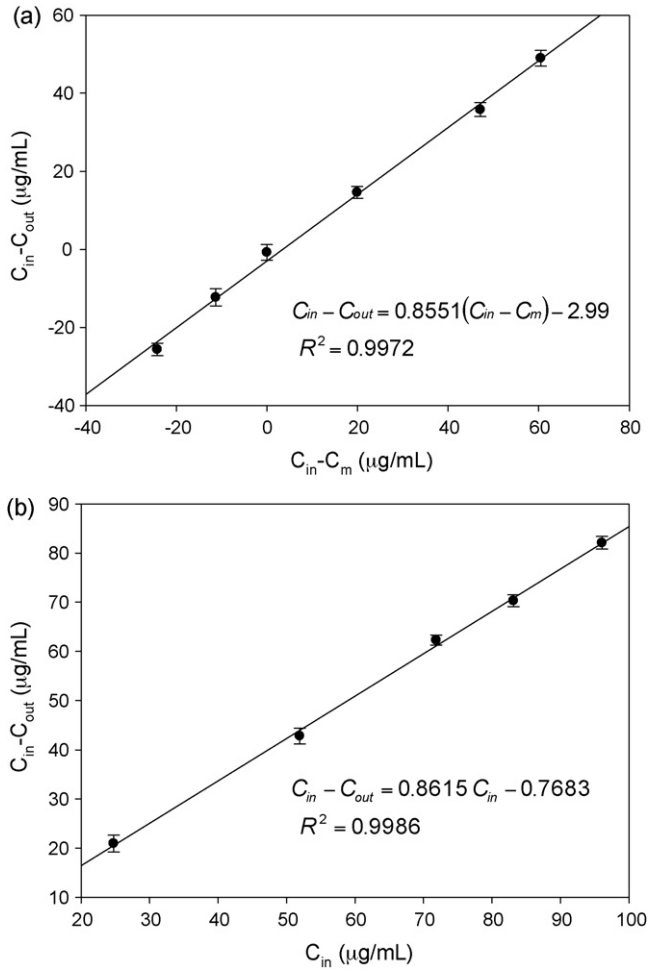

Fig. 1. The linear relationship between $(C_{in} - C_{out})$ and $(C_{in} - C_m)$, method of no-net-flux (a). The linear relationship between $(C_{in} - C_{out})$ and C_{in}, method of retrodialysis (b).

3.3. Box–Behnken experimental design

In the present study, 15 experiments were conducted in one block, composed of 12 factorial points, plus 3 center points. Three variables were considered: TS/oleic acid relation (TS%, X_1), poloxamer 188 content (X_2) and 0.1 M NaOH amount (X_3). In this trial, the total concentration of the lipophilic counterions was kept constant while the composition of the mixture was varied (Jumaa and Müller, 2002). CLA is a weak base and positively charged at formulation pH whereas, TS and oleic acid tend to be negatively charged due to the presence of ionizable carboxy

Table 2
Observed responses for the Box–Behnken design

Run	X_1	X_2	X_3	Y_1	Y_2	Y_3
2	76	0.5	15	138.4	30.72	97.53
5	30	1	10	196.5	10.72	97.97
12	53	1.5	20	144.6	28.86	87.63
3	30	1.5	15	124.7	28.68	91.72
15	53	1	15	161.7	23.29	94.86
1	30	0.5	15	167.7	19.84	91.74
8	76	1	20	182.7	22.42	96.37
14	53	1	15	149.8	21.11	95.37
7	30	1	20	163.9	23.72	91.33
13	53	1	15	153.9	22.48	95.43
6	76	1	10	167.4	24.3	94.57
9	53	0.5	10	149.7	17.73	92.28
4	76	1.5	15	190.6	23.09	91.50
10	53	1.5	10	169.5	24.3	94.12
11	53	0.5	20	166.5	28.92	95.44

groups. If the pH was too low, CLA would fully ionize while the ionization of lipophilic counterions totally suppressed. A significant fraction of the drug would partition into the aqueous phase of the emulsion, resulting in an enhanced vein irritation effect. Alternately, if the pH was too high, CLA would not be ionized, and failed to complex with the ion pairing agents leading to a poor drug-loading potency of the formulation. Thus, the proper selection of the amount of NaOH consisted in this system was critical (Washington, 1996).

The considered responses were the particle size (Y_1), absolute ζ potential value (Y_2) of the emulsion and the oil phase distribution of CLA (Y_3) determined by the microdialysis method. Response data for all experimental runs of Box–Behnken experimental design were presented in Table 2. The mean particle size of an intravenous emulsion is generally required to be smaller than 1 μm, and usually between 100 and 500 nm (Han et al., 2004). Therefore, the mean particle sizes of all the tocol emulsions prepared in this trial were suitable for intravenous applications. All the responses observed for 15 formulations prepared were fitted to first order, two-factor interaction and quadratic models. For the estimation of the significance of the model, the analysis of variance (ANOVA) was applied (Table 3). The resulted equations and the corresponding R^2-values were presented below:

- $Y_1 = 155.13 + 3.29X_1 + 0.89X_2 - 3.18X_3 + 23.8X_1X_2 + 11.98X_1X_3 - 10.43X_2X_3 + 10.13X_1^2 - 9.92X_2^2 + 12.36X_3^2, \quad R^2 = 0.9279$
- $Y_2 = 22.29 + 2.20X_1 + 0.97X_2 + 3.36X_3 - 4.12X_1X_2 - 3.72X_1X_3 - 1.66X_2X_3 - 0.69X_1^2 + 3.98X_2^2 - 1.32X_3^2, \quad R^2 = 0.9587$
- $Y_3 = 95.22 + 0.90X_1 - 1.50X_2 - 1.02X_3 - 1.50X_1X_2 + 2.11X_1X_3 - 2.41X_2X_3 + 0.30X_1^2 - 2.40X_2^2 - 0.46X_3^2, \quad R^2 = 0.9776$

An increasing fraction of TS (X_1) led to a positive effect on responses Y_2 and Y_3. This indicated that as a co-surfactant, TS had a higher capacity to stabilize the emulsions with regard to enhancing the surface charge of the droplets, and also, as a lipophilic counterion, it was more efficient to capture CLA in the oil phase of the emulsion than oleic acid. As might be expected, the presence of NaOH had a positive effect on response Y_2 as a result of the ionization of the lipophilic counterions. A significant interaction effect of X_1X_3 could be observed for all responses, which was favorable for response Y_3, whereas unfavorable for response Y_1 and Y_2. According to the analysis of variance, there were no significant main effects for factor X_2 (the concentration of poloxamer 188), while considerable quadratic effects and interaction effects with other factors on selected responses could be observed. This might be due to its proportional displacement of other ingredients in the interfacial layer of the emulsion system.

3.4. Contour plots and response surface analysis

Two-dimensional contour plots and three dimensional response surface plots, as presented in Figs. 2–5, are very useful to see interaction effects of the factors on the responses. These types of plots show effects of two factors on the response at a time. In all the presented figures, the third factor was kept at level zero. All the relationships among the three factors were non-linear, which indicated the presence of the strong interaction effects between variables. The effects of X_1 and X_2 and their interaction on Y_1 were given in Fig. 2. At low levels of X_1, Y_1 decreased with the increasing concentration of poloxamer 188. Conversely, at high levels of X_1, the particle size increased with

Table 3
The analysis of variance for responses Y_1, Y_2 and Y_3

Source	Y_1		Y_2		Y_3	
	F-Value	P-Value, Prob > F	F-Value	P-Value, Prob > F	F-Value	P-Value, Prob > F
Model	4820.34	0.0216	341.46	0.0058	105.44	0.0013
X_1	86.46	0.3318	38.59	0.0152	6.48	0.0146
X_2	6.30	0.7834	7.45	0.1726	18.09	0.0017
X_3	80.65	0.3470	90.25	0.0026	8.34	0.0089
X_1X_2	2265.76	0.0027	67.82	0.0049	9.04	0.0075
X_1X_3	573.60	0.0395	55.35	0.0075	17.81	0.0017
X_2X_3	434.72	0.0609	10.99	0.1113	23.29	0.0010
X_1^2	379.14	0.0743	1.74	0.4767	0.32	0.4495
X_2^2	363.10	0.0789	58.37	0.0067	21.19	0.0012
X_3^2	563.92	0.0406	6.40	0.2004	0.77	0.2630
Lack of fit	301.49	0.2779	12.30	0.2369	2.21	0.1213

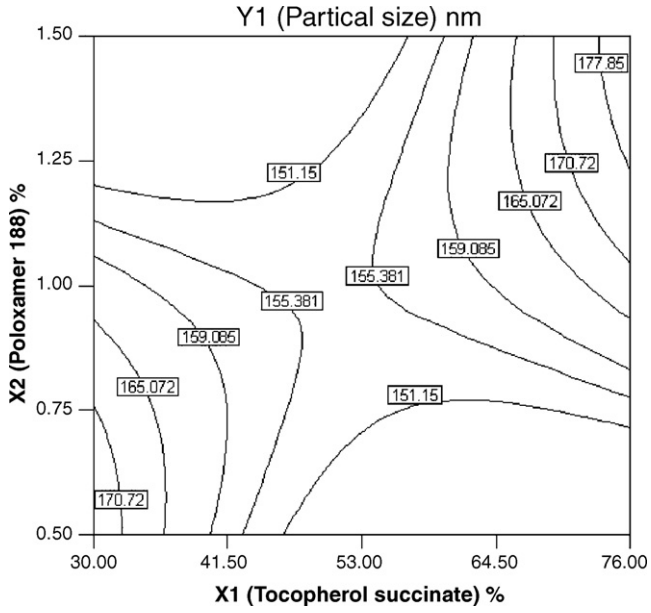

Fig. 2. The contour plot showing effects of X_1, X_2 on response Y_1.

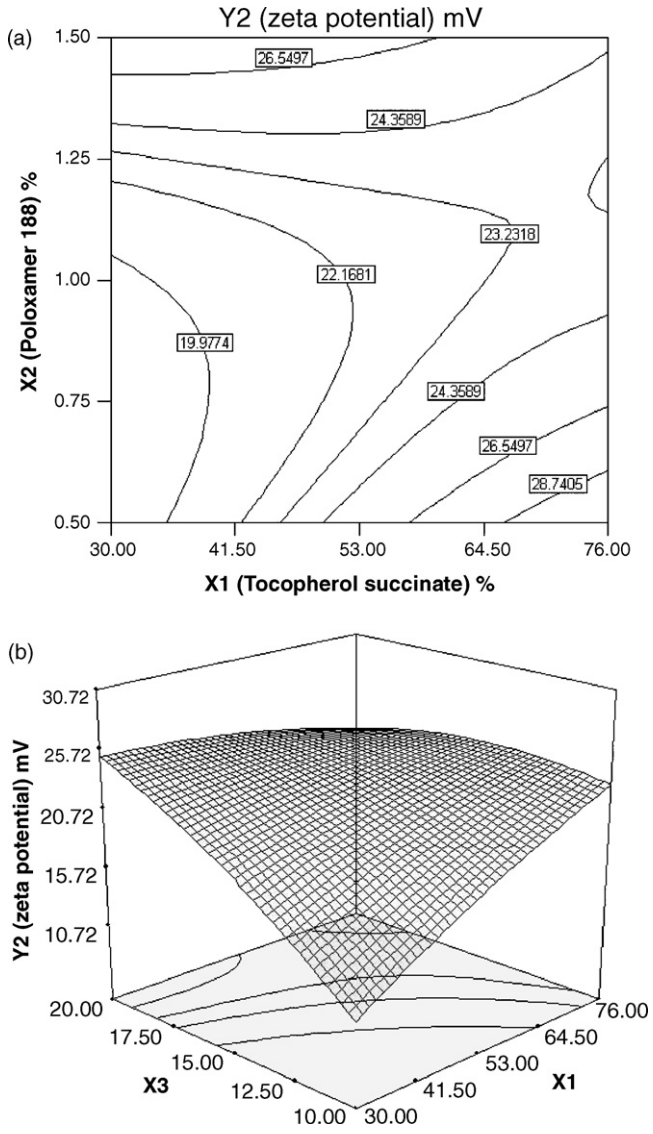

Fig. 4. The contour plot (a) and response surface plot (b) showing effects of X_1, X_2 and X_3 on response Y_2.

the content of poloxamer. The possible explanation for this is that CLA tocol emulsions were stabilized by two mechanisms: steric and electrostatic repulsion. At low fraction of TS, the steric stabilization effect offered by the incorporation of non-ionic surfactant played a major role. Alternately, with the increasing of the fraction of TS, the electrostatic repulsion between emulsion droplets attributed to the negative zeta potential increase (Fig. 4) instead of the steric effect became the overwhelming stabilizing mechanism for this system. The response surface plot developed by the model for X_1 and X_3 was shown in Fig. 3. The addition of NaOH led to an initial decrease in the particle size. However, a further increase of the amount of NaOH resulted again in an increase in the droplet size. A similar pattern could be observed for X_1. As a result, optimum fraction of TS and NaOH content were required to yield a formulation with minimized emulsion particle size. It was clear from Fig. 4b that absolute ζ potential

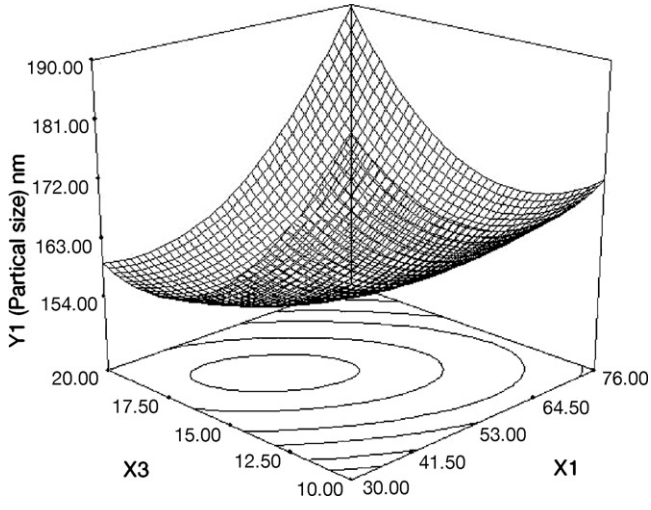

Fig. 3. Response surface plot showing effects of X_1 and X_3 on response Y_1.

value (Y_2) increase with the addition of NaOH irrespective of the TS/oleic acid ratio. On the contrary, higher level of X_3 would suppress the ionization of CLA as mentioned before, which was unfavorable for the distribution of the drug in the oil phase of the emulsion (Fig. 5b). It was evident from Fig. 5a that 1.5% poloxamer 188 led to a negative effect on Y_3. The reason for this behavior could be attributed to the micelle forming properties of the block copolymer. It was revealed that these micelles might increase the drug solubility in the aqueous phase and therefore facilitates the transfer of the drug from the oil phase into the aqueous of the emulsion (Han et al., 2004).

3.5. Formulation optimization

The optimum formulation was selected based on the criteria of attaining the minimum particle size (Y_1) and the maximum ζ

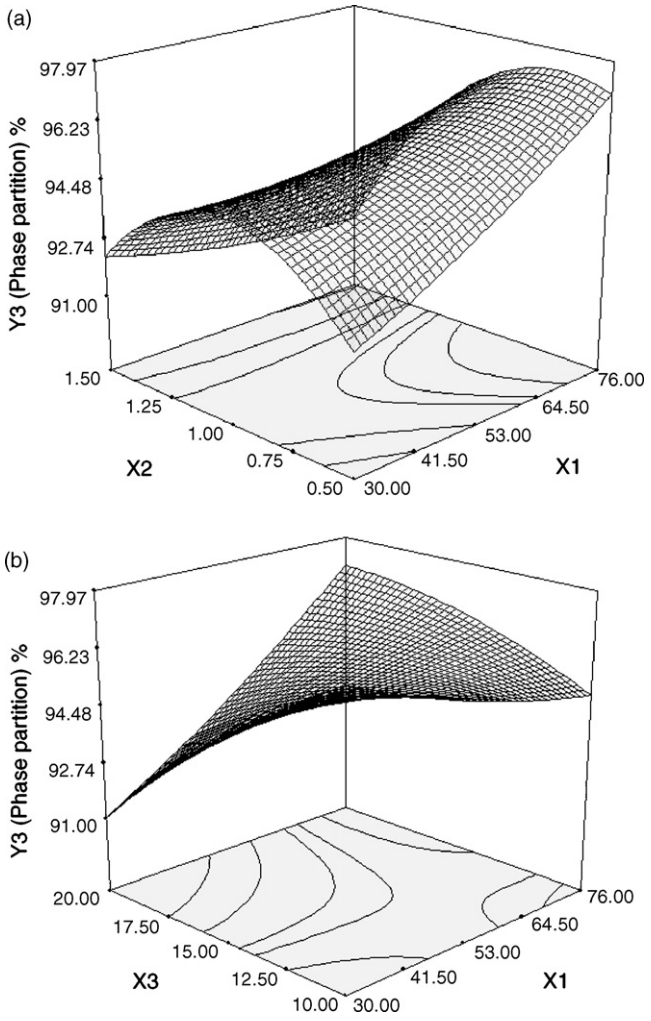

Fig. 5. Response surface plots showing effects of X_1, X_2 (a) and X_1, X_3 (b) on response Y_3.

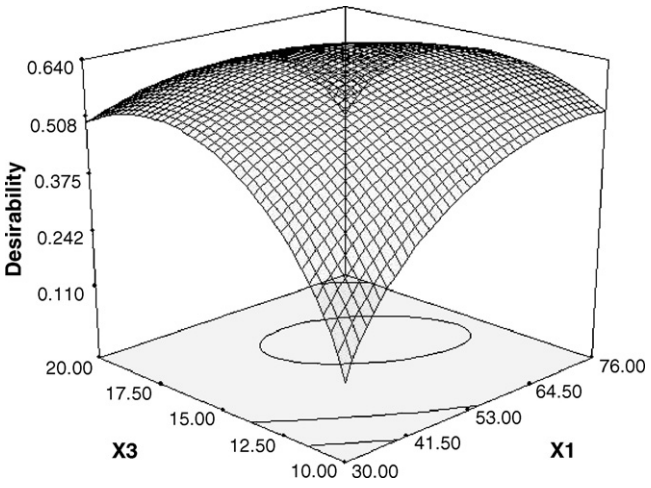

Fig. 6. The relation between overall desirability and variables X_1, X_3.

Table 4
The observed and predicted response values for the optimized formulation

Factor	Optimized level
X_1: tocopherol succinate (%)	76.0
X_2: poloxamer 188 (%)	0.5
X_3: 0.1 M NaOH (%)	15.4
Overall desirability	0.926

Response	Expected	Observed
Y_1: particle size (nm)	135.7	138.5
Y_2: ζ potential (mV)	31.03	32.16
Y_3: distribution (%)	97.33	97.28

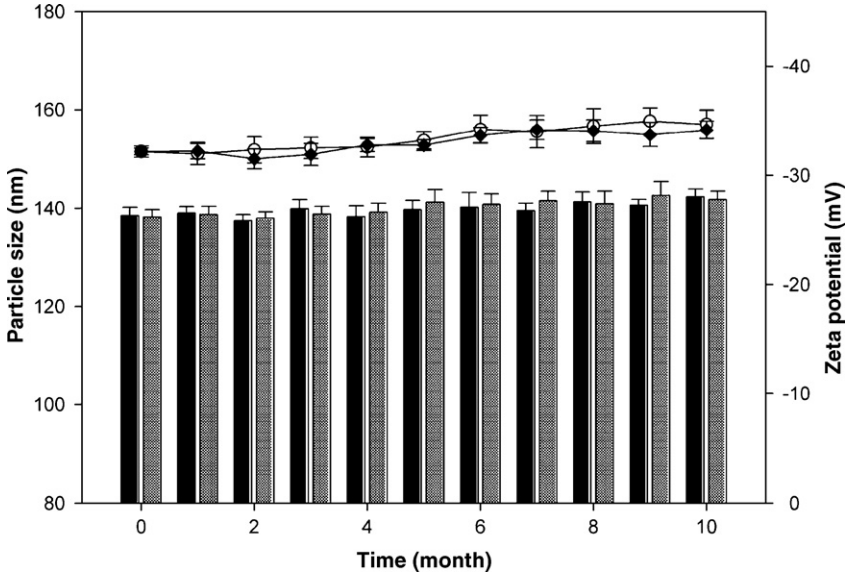

Fig. 7. Physical stability of the optimized CLA tocol emulsion upon storage at 4 °C and room temperature. Mean particle size ((■) 4 °C, 25 °C▩) and absolute ζ potential value ((⧫) 4 °C, (◯) 25 °C) over time of CLA tocol emulsions prepared with the optimized formula.

potential (Y_2), while maximizing the distribution of CLA in the oil phase of the emulsion (Y_3). An overall desirability function dependent on all the investigated formulation variables was used to predict the ranges of variables where the optimum formulation may occur (Paterakis et al., 2002). The desirable ranges are from 0 to 1 (least to most desirable, respectively). The relation between overall desirability and variables X_1, X_3 were illustrated by Fig. 6. To confirm the validity of the calculated optimal factors and predicted responses, a fresh batch with the optimized formula was prepared and evaluated in triplicate. The observed and predicted response values for the optimized formulation were presented in Table 4. The physical stability of emulsions was observed during storage at 4 °C and room temperature because the stability under stress conditions was not always the appropriate way to predict the long-term stability (Jumaa and Müller, 2002). The mean particle size and the absolute ζ potential value over time were shown in Fig. 7. No visible deterioration or crystals were observed under those storage conditions. The drug-loading potency, sterilization process and long-term physical stability of the optimized formulation compared to other conventional and tocol emulsions were presented in Table 5.

3.6. Rabbit ear vein irritation test

The CLA tocol emulsion was evaluated in the ear vein test relative to CLA lactobionate solution as the positive control and normal saline as the negative control ($n = 3$). There was a slight vascular injury in all groups including saline and emulsion-treated rabbits at the injection site related to trauma associated with venipuncture (Wang et al., 1999). However, there was no obvious discoloration along marginal ear vein in emulsion-treated or negative group, while severe reddish discoloration was observed in the positive control group. It was evident from the histopathological examination results presented in Fig. 8 that no apparent morphological changes could be observed in the emulsion-treated group compared with the normal saline control. In contrast, vascular angiectasia and erythrocyte aggregation were observed in the positive control group. These results demonstrated that vein irritation of CLA could be eliminated by formulating the drug in a tocol emulsion.

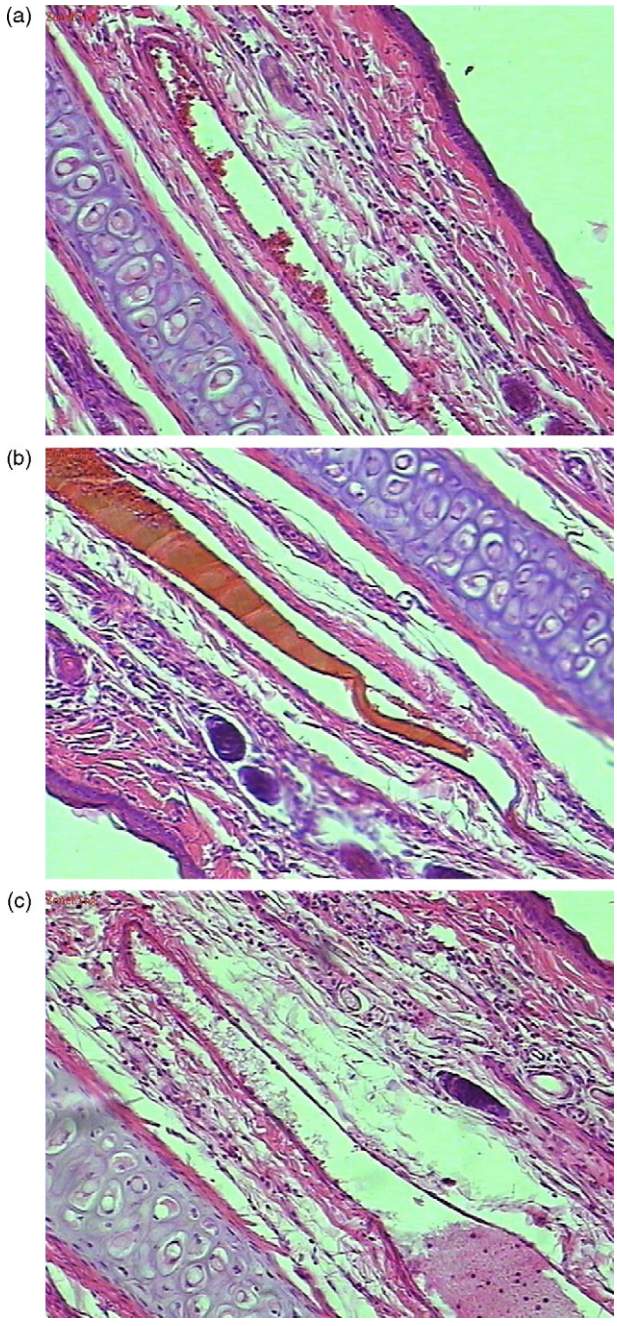

Fig. 8. Histopathology slides of rabbit ear-rim auricular vein following different infusions: normal saline (a), CLA-loaded tocol emulsion (b) and CLA aqueous solution (c).

Table 5
The product characteristics of the optimized formulation compared to other conventional and tocol emulsions

Technique	Drug loading (mg/ml)	Sterilization process	Vein irritation	Physical stability	References
Lipid emulsion with lipophilic counterions	5	Sterile filtration	Reduced	6 months under 5 °C and 30 °C	Lovell et al. (1994)
Tocol emulsion	2.5	100 °C rotating water bath	Reduced	3 months under 10 °C	Lu et al. (2008)
Tocol emulsion with lipophilic counterions	5	Sterile filtration	NA	>6 months under 4 °C	Constantinides et al. (2002)
Tocol emulsion with lipophilic counterions	5	100 °C rotating water bath	Reduced	>10 months under 4 °C and 25 °C	Present studies

NA: not available.

4. Conclusion

A less-painful injectable CLA tocol emulsion stabilized by relatively biocompatible surfactants was developed using TS combined with oleic acid as lipophilic counterions to achieve a therapeutically effective concentration of the drug. The microdialysis technique was proven to be effective to investigate the distribution of CLA in emulsion systems. The presence of lipophilic counterions acting also as co-emulsifiers yielded a formulation with good physical stability. The long-term stability evaluation of the optimized tocol formulation is ongoing.

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