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Design and *in vivo* pharmacodynamic evaluation of nanostructured lipid carriers for parenteral delivery of artemether: Nanoject

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ABSTRACT

The objective of the present investigation was to explore the potential of nanostructured lipid carriers (NLC) for the intravenous delivery of artemether (ARM), a poorly water-soluble antimalarial agent. The NLC of ARM (Nanoject) were formulated by employing a microemulsion template technique. The NLC were evaluated for particle size, encapsulation efficiency, *in vitro* drug release and *in vitro* hemolysis. The antimalarial activity of the Nanoject and conventional ARM injectable formulation was evaluated in *Plasmodium berghei* infected mice. The average particle size of Nanoject was 63 ± 28 nm and the encapsulation efficiency was found to be $30 \pm 2\%$. The Nanoject released ARM in a sustained manner. *In vitro* haemolytic studies showed that Nanoject had lower haemolytic potential (~13%) as compared to all the components when studied individually. Nanoject showed significantly higher ($P < 0.005$) antimalarial activity as compared to the marketed injectable formulation. The antimalarial activity of Nanoject lasted for a longer duration (more than 20 days) indicating that Nanoject may be long-circulating *in vivo*. Nanoject showed significantly higher survival rate (60%) even after 31 days as compared to marketed formulation which showed 0% survival (100% mortality). This clearly indicates that Nanoject offers several advantages over the currently marketed oily intramuscular formulation (Larither®).

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1. Introduction

Parasitic diseases are of immense global significance as around 30% of world's population experiences parasitic infections. Amongst various parasitic infections, malaria is the most life threatening disease and accounts for 1 million to 2 million deaths round the globe every year (Greenwood and Mutabingwa, 2002). The tropical countries such as India are more prone to the malaria and around 2 million cases are reported annually. In humans, malaria is caused by four distinct blood–borne Apicomplexan parasite species: *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae* and *Plasmodium ovale*. Amongst these, the most severe malaria is caused by *P. falciparum* which is responsible for almost all malaria related deaths. Existing treatments for malaria include a limited number of clinically effective antimalarial agents. Artemether (ARM) is a potent and rapidly acting antimalarial agent which is enlisted in WHO List of Essential medicines (WHO web siteWHO, 2007) for the treatment of severe multiresistant malaria. It is active against *P. vivax* as well as chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. It is also indicated in the treatment of cerebral malaria. However, poor aqueous solubility of ARM significantly hampers its therapeutic efficacy. Currently, ARM is available as tablets for oral therapy and as an intramuscular (IM) oily injection for the treatment of severe malarial infections. The oral bioavailability of ARM is low (~40%) due to its poor aqueous solubility and degradation in stomach acids (Karbwang et al., 1997) whereas the current oily intramuscular IM injection suffers from disadvantages such as pain on injection and slow and erratic absorption on intramuscular administration (Hien et al., 2004). The oily injection is not very suitable when quick eradication of the malarial infections is required. Furthermore, due to its oily nature, it cannot be administered by intravenous (IV) route. It has been demonstrated that IV delivery of ARM results in the highest availability to body as compared to all other routes and can lead to quick eradication of the malarial infection (Li et al., 1998). However, currently, no ARM product is available that enables IV delivery of ARM. Thus, the need of hour is to have an aqua based IV formulation of ARM that enables its quick availability to the body with concomitant reduction in the pain on injection. Lipid nanocarriers such as liposomes, nanoemulsions and nanoparticles have demonstrated a great potential in improved parenteral (IV) delivery of the hydrophobic agents since last two decades. The utility of the liposomes and nanoemulsions in the improved delivery of the antimalarial agents such as chloroquine

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(Owais et al., 1995) and primaquine (Dierling and Cui, 2005) has been established. Recently, we have reviewed (Date et al., 2007) the different polymeric, liposome based and lipid nanoparticle based approaches for the treatment of parasitic diseases. However, till date, the utility of the second-generation lipid nanocarriers such as nanostructured lipid carriers (NLC) has not been established for the parenteral delivery of antimalarial agents. NLC are lipid nanocarriers based on the mixture of biocompatible solid lipid and liquid lipid (oil) (Wissing et al., 2004). NLC have been regarded as an alternative to liposomes and nanoemulsions due to various advantages such as ease of manufacture, particulate nature, high drug loading and ability to sustain the release of the drug (Wissing et al., 2004). Furthermore, the aqueous nature of NLC, their nanostructure and the biocompatibility of the excipients would enable IV delivery of the therapeutic agents with concomitant reduction or abolishment in the pain on injection. Additionally, the ability of NLC to sustain the delivery of therapeutic agents could be useful in combating the recrudescence which is commonly observed with the ARM monotherapy (de Vries and Dien, 1996). In view of this, NLC appeared to be a novel approach for improving the delivery of ARM. In the present investigation, NLC of ARM (Nanoject) has been formulated by using biocompatible excipients and was evaluated for its potential in improving the antimalarial efficacy of ARM in comparison to the conventional IM oily formulation by means of a suitable *in vivo* model.

2. Materials and methods

2.1. Chemicals

Artemether was kindly provided by Ipca Laboratories Ltd., Mumbai, India. Glyceryl dilaurate from Anshul Agencies (Mumbai, India), Capmul MCM (glyceryl mono/dicaprylate) from Indchem International (Mumbai, India), Crodamol GTCC (caprylic capric triglyceride) from Croda India Ltd. (Mumbai, India), oleic acid, seasame oil, sunflower oil and cotton seed oil from Kamani Oil Industries Ltd. (Mumbai, India); Cremophor EL (PEG-35-hydrogenated castor oil) and Solutol HS 15 (PEG-660-12-hydroxystearate) from BASF India Ltd. (Mumbai, India) were received as gift samples. Acetonitrile (HPLC grade), tween 80 and dextrose were purchased from Merck India Ltd. PEG 400 (Polyethylene glycol 400), glycerine, propylene glycol (all AR grade) were purchased from s. d. fine Chemicals Ltd. (Mumbai, India). Dialysis bags (MW 100 kDa) were procured from Sigma Chemicals (NJ, USA). All the excipients and reagents were used as received. Double distilled water was prepared freshly whenever required.

2.2. Parasite

P. berghei ANKA strain was used for evaluation of antimalarial activity. This strain was examined and found to be free of contamination with *Eperythrozoon coccoides*. The strain is well characterized in our lab and it is known to provide high mortality in mice, providing a good model to estimate survival and antimalarial efficacy in reducing parasitemia. It is sensitive to all currently used antimalarial drugs.

2.3. Animals

Animal experiments were carried out according to the CPCSEA (Committee for the purpose of the control and supervision on experiments on animals) guidelines. In-house bred and eperythrozoon-free male swiss albino mice aged 2–4 weeks having body weight in the range of 30–45 g were used for the study. The animals, held at a temperature of 22 ± 3 °C and 65% relative humidity, were fed a standard mouse diet and provided with clean drinking water *ad libitum* throughout the experiments.

2.4. Screening of components for NLC formulation

The solubility of ARM in different liquid lipids (oils), surfactants and solubilizers was determined by using shake flask method. Briefly, an excess of ARM was added individually to the oils, surfactants and solubilizers (5 g each) in screw capped tubes. Mixtures were then shaken for 24 h in a water bath shaker (Remi, Mumbai, India) maintained at 25 ± 2 °C. After 24 h, each sample was centrifuged at 5000 rpm for 10 min, supernatant (0.5 ml) was diluted suitably and the amount of ARM solubilized in the vehicles was analyzed by HPLC.

2.5. HPLC analysis of ARM

The quantity of ARM solubilized in various vehicles was determined by using HPLC method by Chimanuka et al. (2002). The HPLC system consisted of Jasco PU 2080 Plus Intelligent HPLC Pump, Jasco, Japan, equipped with Lichrosphere 100 RP-18 (250 mm × 4 mm), 5-μm particle size column and a Jasco UV 2075 Intelligent UV-VIS Detector, Jasco, Japan, with a Rheodyne 7725 injector USA managed by Jasco Borwin Chromatography software version 1.05. The mobile phase (acetonitrile:water in the ratio of 75:25) was run at a flow rate of 1 ml/min and detection of ARM was carried out at 214 nm.

2.6. Pseudoternary phase diagram

NLC were fabricated by using microemulsion template technique first reported by us (Joshi and Patravale, 2008). The first step towards the formulation development was to determine the feasibility of the microemulsion formation. The boundaries of the microemulsion domains were determined with the aid of pseudoternary phase diagrams with the following components as the constituents of microemulsion. A mixture of glyceryl dilaurate (solid lipid) and Capmul® MCM (liquid lipid) at the ratio 1:1 was used as a lipid phase of the microemulsion. The surfactant phase consisted of a mixture of tween 80 and Solutol HS 15 (at the ratio of 1:1) while the aqueous phase was double distilled water. Briefly, mixtures of the lipid phase (glyceryl dilaurate + Capmul® MCM mixed in 1:1 ratio) and surfactant phase (tween 80 or Solutol HS 15 or tween 80 + solutol HS 15 mixed in 1:1 ratio) were prepared at ratios (w/w) of 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10 in pre-weighed test tubes. The lipid phase was heated to melt the solid lipid. The required quantities of surfactant phase and the lipid phase were heated to the same temperature and gently mixed to form a monophasic mixture that was slowly titrated with aliquots of distilled water and stirred at 60 °C for a sufficiently long time to attain equilibrium. After equilibrium was reached, the mixtures were checked visually for transparency and through crossed polarizers for optical isotropy. Only those systems which appeared black when visualized through the crossed polarizers were deemed to be within the microemulsion region. No attempts were made to completely identify the other regions of the phase diagrams.

2.7. Formulation of NLC preconcentrate of ARM (Nanoject)

The NLC preconcentrate was prepared by mixing the required amounts of glyceryl dilaurate, Capmul® MCM, tween 80 and solutol HS 15 by gentle heating at 60 °C without any water. The composition of this preconcentrate is highlighted by a dot in the phase diagram

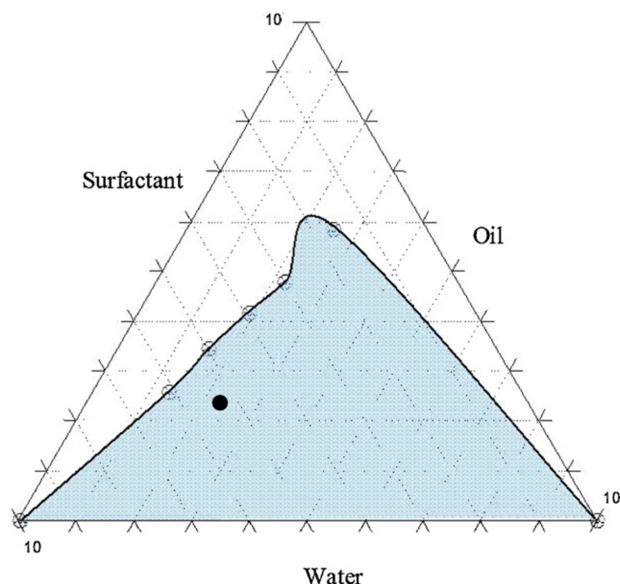

Fig. 1. Pseudoternary phase diagram for tween 80 + Solutol HS 15 – Capmul MCM + glyceryl dilaurate–water.

(Fig. 1) and it can hold 150 mg of ARM which is equivalent to the amount of ARM present in currently marketed IM formulation. The exact composition is as depicted in Table 1.

2.8. Determination of particle size

Particle size of NLC of ARM was determined by photon correlation spectroscopy. All measurements were performed in triplicate using a Beckman N4 plus submicron particle size analyzer (Wipro, India) at a temperature of 25 °C ± 2 °C and at 90° to the incident beam. The NLC preconcentrate of ARM was suitably diluted with double distilled water to ensure that the light scattering intensity (between 6E+004 to 1E+006) was within the instrument's sensitivity range. Double distilled water was filtered through 0.45 μm membrane filters (Pall Life sciences, Mumbai) prior to particle size determination. All data obtained were analyzed by *Contin* program.

2.9. Determination of drug encapsulation efficiency of ARM

NLC preconcentrate of ARM was diluted with double distilled water such that the concentration of the ARM in the nanodispersion is 2.08 mg/ml. Nanodispersion, 100 μl, was transferred to the upper chamber of Nanosep centrifuge tubes fitted with a ultrafilter (MWCO100KD, Pall Lifesciences, Mumbai, India). The Nanosep was centrifuged at 15,000 rpm for 40 min. The supernatant and the filtrate were diluted appropriately and the amount of drug in both the phases was determined by HPLC method as described earlier.

Table 1
Composition of artemether formulations

S. no.	Ingredient	NLC preconcentrate quantity required for one dose (gm)	NE preconcentrate quantity required for one dose (gm)
1	Artemether	0.15	0.15
2	Capmul MCM	0.20	0.40
3	Glyceryl dilaurate	0.20	–
4	Tween 80	0.50	0.50
5	Solutol HS 15	0.50	0.50

The entrapment efficiency was calculated by the following equation:

$$\text{Percentage entrapment efficiency} = \left[\frac{W_{\text{initial drug}} - W_{\text{free drug}}}{W_{\text{initial drug}}}\right] \times 100$$

where "$W_{\text{initial drug}}$" is the mass of initial drug added and the "$W_{\text{free drug}}$" is the mass of free drug detected in the filtrate of lower chamber of Nanosep after centrifugation of the aqueous dispersion.

2.10. Formulation of nanoemulsion of ARM

Nanoemulsion (NE) of the ARM was formulated by replacing the solid lipid present in Nanoject (glyceryl dilaurate) completely with the liquid lipid (Capmul MCM). The composition of the NE was similar to the Nanoject and is shown in Table 1. The NE of ARM was formulated to study the *in vitro* release of ARM in comparison to that of Nanoject of ARM.

2.11. In vitro drug release studies

It is well known fact that the release of the drug from nanocarriers is influenced by the structure and composition of the nanocarriers. Hence, the *in vitro* release of ARM from two different nanocarriers viz. NE and NLC was studied.

In vitro release was evaluated using a dialysis bag diffusion technique as described by Yang et al. (1999). The dialysis bags were hydrated in phosphate-buffered saline, pH 7.4 overnight before the experiment. NLC and NE containing ARM equivalent to 150 mg were placed in dialysis bags (molecular weight cut off: 100 kDa). The dialysis bags were tied at both ends and were placed in the basket of USP Type I dissolution apparatus (Electrolab, Mumbai, India). The baskets were immersed in 200 ml phosphate-buffered saline, pH 7.4, maintained at 37 °C. The baskets were rotated at the speed of 50 rpm. At regular intervals, 5 ml of dissolution medium was removed and was replaced with the fresh buffer. The amount of ARM in the aliquots was analyzed by HPLC method described earlier.

2.12. In vitro erythrocyte toxicity

The hemolytic activity has been suggested as a toxicity screen *in vitro* and it also serves as a simple and reliable measure for estimating the membrane damage caused by formulation *in vivo*. The *in vitro* hemolytic potential of the NLC preconcentrate and its individual components (at the concentration used in the NLC) was studied by using the method proposed by Jumaa et al. (1999). The samples tested for erythrocyte toxicity were as follows

1. Nanoject diluted with PBS to ARM concentration of 2.08 mg/ml;
2. 7% Capmul MCM diluted with PBS;
3. 7% glyceryl dilaurate diluted with PBS;
4. 18% tween 80 solution in PBS;
5. 18% Solutol HS 15 solution in PBS;
6. Triton X 100.

Blood was obtained from two human volunteers. Both volunteers signed written consent forms. Fresh blood was collected in a vial containing EDTA (anticoagulant). The blood was centrifuged for 5 min to remove WBC debris and suspended red blood cells (RBCs) were taken out. The RBCs were washed three times with isotonic saline solution (0.15 M NaCl and pH 7.4) before diluting with buffer to prepare erythrocyte stock dispersion. The washing step was repeated in order to remove debris and serum protein. The stock

Table 2
Details of different groups used for the *in vivo* antimalarial study ($n = 5$)

Group	Infection	Treatment
MV	+	Vehicle of marketed formulation, peanut oil (100 μl)
MF	+	0.208 mg/100 μl of marketed formulation
NV	+	NLC blank vehicle (100 μl)
ND I	+	0.208 mg/100 μl of NLC Nanoject formulation
ND II	+	0.0104 mg/100 μl of NLC Nanoject formulation
Control	+	No treatment

Blank NLC had composition similar to Nanoject of ARM (Table 1) except the ARM.

solution was refrigerated for a period of 24 h. Test sample (1 ml) was added to a 100 μl aliquot of the erythrocyte stock dispersion. Incubation was carried at 37 °C for a period of 1 h. After incubation under shaking, debris and intact erythrocytes were removed by centrifugation and 100 μl of resulting supernatant was dissolved in 2 ml of an ethanol/HCl mixture (ratio 39:1 99% ethanol, and HCl, w/v). This mixture dissolved all components and avoided the precipitation of haemoglobin. The absorbance of the mixture was determined at 398 nm by spectrophotometer monitoring against a blank sample. Control sample of 0% lysis (in buffer) and 100% lysis (in Triton X 100) were employed in the experiment.

The percent haemolysis caused by the test sample ($n = 3$) was calculated by following equation:

$$\%\text{haemolysis} = \frac{\text{Absorbance of test sample}}{\text{Absorbance at 100\% lysis}} \times 100$$

2.13. *Pharmacodynamic evaluation: in vivo antimalarial efficacy testing in P. berghei infected mice*

The protocol for animal studies was approved by the Institutional Animal Ethics Committee of the Tata Institute of Fundamental research (TIFR) and the work was carried out at TIFR premises. The protocol was slightly modified with that described by Chimanuka et al. (2002). The lethal ANKA strain of *P. berghei* was used for the experiments. In-house bred and mycoplasma free male swiss mice (weighing around 25 g each) were infected by intraperitoneal inoculation of donor mouse blood diluted in acid citrate dextrose (ACD) buffer containing approximately 10^6 infected RBCs on day '0'. The mice were randomly divided into various groups ($n = 5$ per group) as depicted in Table 2. On day '3' and day '5' post-infection, the different groups of mice were given intraperitoneal treatment as shown in Table 2. The Nanoject was sterilized by autoclaving and the dilutions were made in water for injection under aspectic conditions. There was no change in the particle size of NLC Nanoject due to autoclaving (data not shown). The final dilution was filtered through 0.22 μm filter (Millipore, USA) to avoid contamination. For making ND I and ND II, appropriate dilution of Nanoject preconcentrate was done so that the final dilution contains 0.208 and 0.0104 mg of artemether in 100 μl. Whereas NV was blank NLC preconcentrate having similar % lipid composition as ND I. From day 3 till the end of the study (day 20), the blood was withdrawn from tail vein on each day and the bloodsmears were prepared. Bloodsmears were fixed with methanol and stained with Giemsa's stain and the parasites were counted. Parasitemia was reported as percentage parasitemia after counting 1000 RBCs from each slide. Antimalarial activity was calculated by the following formula suggested in the standard protocol by Fidock et al. (2008).

$$\text{Activity} = 100 - \left[\frac{\text{mean parasitemia of treated group}}{\text{mean parasitemia of control group}}\right] \times 100$$

The number of surviving animals in all the groups were recorded on the 31st day.

Table 3
Solubility of artemether in different oils ($n = 3$)

Oil	Solubility (mg/g)
Capmul MCM	316 ± 7.5
Crodamol GTCC	24 ± 9.9
Oleic acid	14 ± 5.3
Sesame oil	60 ± 2.6
Sunflower oil	43 ± 6.4
Cottonseed oil	83 ± 5.3

2.14. *Statistical analysis*

Data was expressed as mean ± S.D. and parasitemia of the different groups were statistically assessed by unpaired *t*-test using Graphpad Instat Demo version. Differences were considered significant at $P < 0.05$.

3. Results

3.1. *Screening of components for NLC formulation*

Solubility studies were carried out to identify potential ingredients for the formulation of NLC from microemulsion template. Amongst the various oils that were screened, Capmul MCM exhibited highest solubilizing potential for ARM (Table 3). Capmul MCM was chosen as a liquid lipid for the formulation of NLC as it can give high ARM loading. Furthermore, recent reports suggest that Capmul MCM can be employed for IV delivery of the therapeutic agents like paclitaxel (Nornoo et al., 2008). The capmul MCM based parenteral microemulsions were well tolerated *in vivo* (Nornoo and Chow, 2008).

Amongst various surfactants, Cremophor EL demonstrated highest potential to solubilize ARM followed by tween 80 and Solutol HS 15 (Table 4). Cremophore EL was not selected for the formulation of NLC due to the adverse effects associated with its long-term parenteral use (Ten Tije et al., 2003). Tween 80 and Solutol HS 15 both exhibit fairly good parenteral acceptability and are being used in current parenteral formulations (Strickley, 2004). Hence, they were selected for the further studies. Commonly used cosolvents such as glycerine, PEG 400 and propylene glycol demonstrated poor solubilizing potential for ARM (Table 4). Hence, the cosolvents were not preferred for the formulation of NLC. For the formulation of NLC, glyceryl dilaurate was chosen as a solid lipid based on the initial screening (data not shown) by the method described by us. In our earlier study, we have also demonstrated that glyceryl dilaurate can form NLC with Capmul MCM (Joshi and Patravale, 2008). However, the surfactants and the solubilizers studied in the earlier investigation are different from those chosen for the current investigation. Appropriate studies have been done in our labs to establish parenteral acceptability of glyceryl dilaurate as per OECD guidelines (data not shown).

Table 4
Solubility of artemether in different surfactants and solubilizers ($n = 3$)

Surfactant/solubilizer	Solubility (mg/g)
Tween 80	12 ± 9.4
Cremophor EL	280 ± 1.4
Solutol HS 15	11 ± 6.9
PEG 400	2 ± 4.6
Glycerin	1.2 ± 8.7
Propylene glycol	3.1 ± 1.0

3.2. Pseudoternary phase diagram

It is well established fact that surfactants alone or in combination with another surfactant can yield microemulsions. Hence, the phase behaviour of three systems namely, tween 80 – Capmul MCM + glyeryl dilaurate–water, Solutol HS 15 – Capmul MCM + glyeryl dilaurate–water and tween 80 + Solutol HS 15 – Capmul MCM + glyeryl dilaurate–water was studied. It was observed that the system that contained tween 80 and Solutol HS 15 mixture demonstrated higher area of microemulsion formation as compared to the systems that contained individual surfactants (data not shown). This observation is in accordance with the reported literature (Li et al., 2005). The phase behaviour of the tween 80 + Solutol HS 15 – Capmul MCM + glyeryl dilaurate–water system is shown in Fig. 1. The black dot in Fig. 1 represents the composition of the microemulsion selected for the further studies. The microemulsion on further dilution with the water yields NLC. For the further studies, microemulsion without water (NLC preconcentrate) was used. This preconcentrate readily forms NLC on dilution with water and other IV fluids at room temperature.

3.3. Determination of particle size and encapsulation efficiency

The average particle size of NLC was found to be 63 ± 28 nm. The encapsulation efficiency of ARM in the nanoparticles was found to be 30 ± 2%. This indicates that 30% of the ARM is encapsulated in the NLC system while the remaining drug might be entrapped in the surfactant micelles. The solubilized drug would help in giving quick availability of ARM in the body whereas encapsulated drug would be released in a sustained manner which may help in the prevention of recrudescence.

3.4. In vitro drug release studies

In the *in vitro* release test using the dialysis bag technique NLC Nanojects (Fig. 2), showed initial burst in first 1 h. This could be explained by the fact that only 30% of the drug is present in encapsulated form in the crystallized solid lipid and rest is present in solubilized form in oil globules or solubilized in surfactant micelles. The initial burst may be due to release of this unencapsulated drug getting released first from the micelles then from the oil globules in the dissolution medium. Complete release occurred at the end of 24 h indicating that the drug encapsulated in the solid lipid core can also leach out even though at a slower rate. While the NE of ARM showed constant increments in release and 100% release occurred at the end of 6 h. This clearly indicates the influence of the nanostructure on the *in vitro* release of the drug. Since, the composition of the NE and NLC is almost similar (except the fact that NLC contains solid lipid), it can be inferred that the presence of solid lipid and the difference in the nanostructure arising due to its incorporation may both be responsible for the significantly sustained release of ARM observed with Nanoject of ARM. It should also be noted that in the Nanoject of ARM, in addition to NLC structure, nanoemulsion and mixed micelles could also be present and the low encapsulation efficiency of ARM in Nanoject supports this statement. We would be conducting appropriate spectroscopic studies in future work to identify the various nanostructures present in Nanoject.

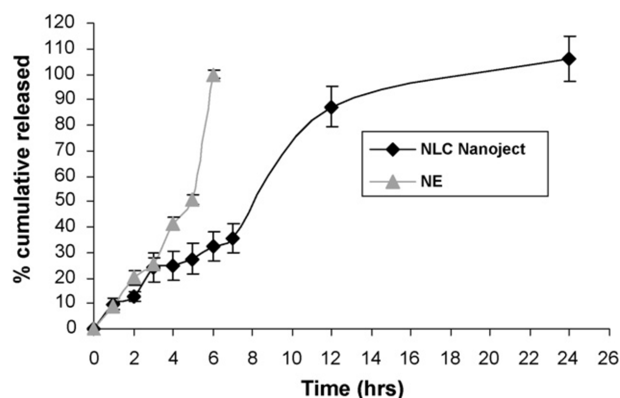

Fig. 2. *In vitro* release studies on Nanoject ($n = 3$).

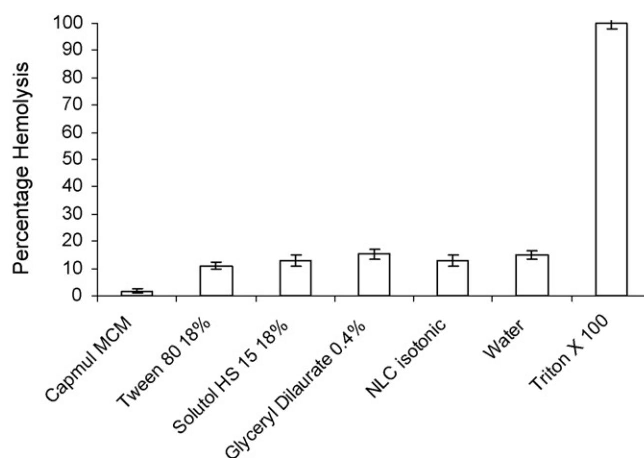

Fig. 3. Percentage hemolysis caused by Nanoject excipients and Nanoject ($n = 3$).

3.5. In vitro erythrocyte toxicity study

Triton X 100, a known hemolytic agent acted as a positive control in the study and showed 100% hemolysis of erythrocytes (Fig. 3); thus validating the experiment. Capmul MCM showed negligible hemolysis of the erythrocytes indicating its suitability in the parenteral formulations. Glyceryl dilaurate (7%), tween 80 (18%) and 18% Solutol HS 15 (all at the concentration represented by the black dot in Fig. 1) showed some degree of hemolysis. Interestingly, Nanoject which contained Capmul MCM, 7% glyceryl dilaurate, 18% tween 80 and 18% Solutol HS 15 showed hemolysis that was higher than the Capmul MCM and lesser than the hemolysis caused by the other components ($P < 0.05$). It was expected that the combination of all these components would give an additive effect resulting in considerably higher hemolysis. However, it was not observed in this study. It can be inferred that the assembling of aforementioned components into a NLC structure changes their mode and degree of interaction with the erythrocytes and hence the combination of all these components does not show any additive effect on the hemolysis of erythrocytes. Similar observations have been reported for the lipid emulsions which corroborate this observation (Jumaa et al., 1999). It should also be noted that the observations of the *in vitro* hemolytic study do not correlate with *in vivo* observations but it gives some idea about the haemolytic potential. Also, in the present study, test samples were contacted with erythrocytes for 1 h which is unlikely to happen *in vivo*.

3.6. Pharmacodynamic evaluation: in vivo antimalarial efficacy testing in P. berghei infected mice

In vivo antimalarial efficacy (Fig. 4) of the Nanoject at two different doses of ARM (ND-I and ND-II where ND-I was greater than ND-II by 20-fold), Nanoject vehicle (NV) and marketed formulation

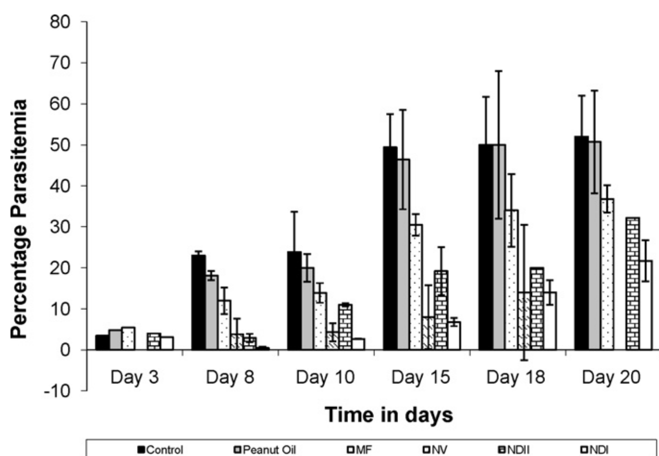

Fig. 4. Percent parasitemia observed in *P. berghei* infected mice ($n = 5$).

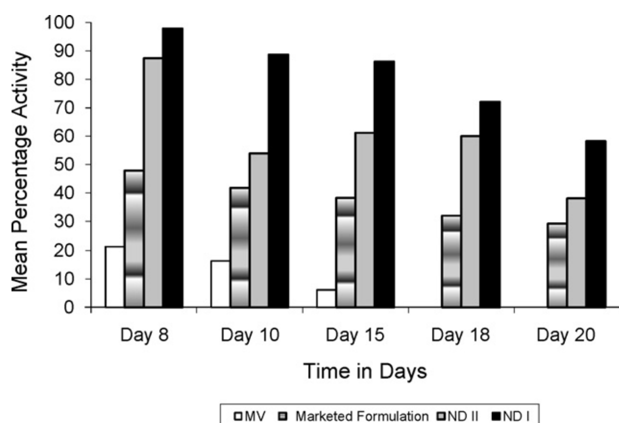

Fig. 5. Antimalarial activity of various formulations in *P. berghei* infected mice ($n = 5$).

(MF) with respect to reduction in percent parasitemia and antimalarial activity is depicted in Fig. 5. As expected, the control group (no treatment) showed highest parasitemia (Fig. 4). As compared to the control group, all the other groups showed reduction in the parasitemia during the course of study ($P < 0.05$). ND-I showed highest reduction in the parasitemia (or highest antimalarial activity) as compared to all other groups ($P < 0.005$). The antimalarial activity of ND-I was 2.5-fold higher as compared to the marketed formulation (Larither®) at same dose level of ARM. The ND-I resulted in almost complete clearance of the parasite from the animals (Figs. 5 and 6) whereas the marketed formulation (Larither®) showed ~45% activity on the 8th day. Interestingly, NV, the blank NLC, also showed considerable reduction in the parasitemia as compared to control and marketed formulation on day 8th day which was an unexpected outcome of the study. The antimalarial activity of ND-II and NV was as high as 85% on 8th day. The ND-I continued to show efficient antimalarial activity till 15th day whereas the antimalarial activity by ND-II has been considerably declined by then. On 20th day, ND-I still showed around 60% antimalarial activity which was 2-fold and 1.5-fold higher than that of marketed formulation (Larither®). The results of the long-term survival studies are shown in Table 5. ND-I showed 60% survival whereas ND-II showed 20% survival at the end of 31 days. MF and NV showed 100% mortality at the end of the study indicating that they are not able to offer long-term protection.

Table 5
Results of 31-day survival study

Group ($n = 5$)	Long-term survivors
MV	0/5
MF	0/5
NV	0/5
ND I	3/5
ND II	1/5
Ctrl	0/5

4. Discussion

The novel delivery strategies have a significant scope in improving the delivery of ARM as the current injectable ARM formulations cannot offer optimal therapy. Researchers have explored the potential of liposomes in improving the antimalarial efficacy of ARM on parenteral administration. The liposomes of ARM demonstrated significantly higher and long-term antimalarial activity of the ARM as compared to conventional injectable formulations (Chimanuka et al., 2002). However, liposomes suffer from several limitations such as poor stability and high excipient cost. Additionally, they are complicated to manufacture and difficult to scale-up. NLC have evolved as second-generation lipid nanocarriers which retain advantages of liposomes and unlike liposomes they exhibit good physical stability and are easy to manufacture and scale-up. In

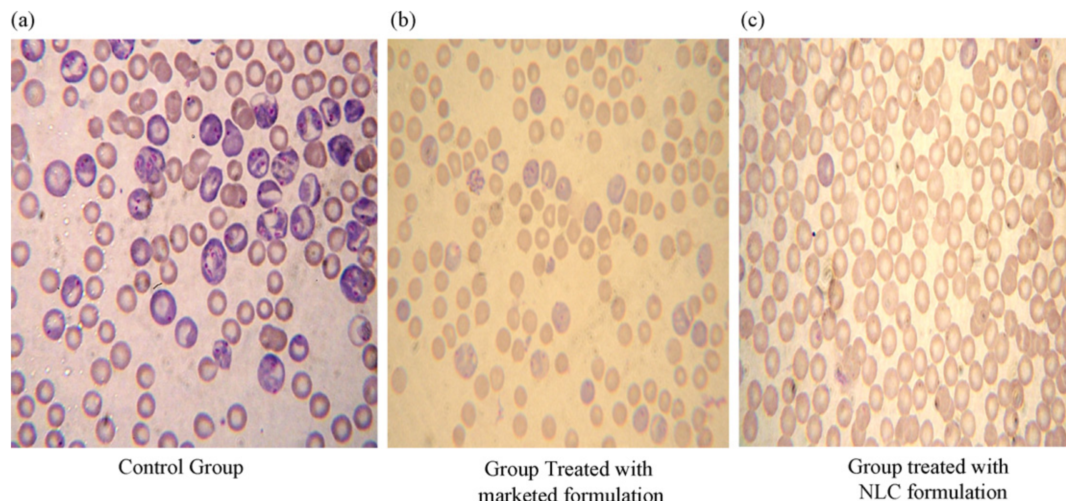

Fig. 6. Photomicrograph of the blood smears of various treatment groups on 8th day at 100× magnification under oil immersion.

view of this, NLC of ARM (Nanoject) were fabricated by employing a commercially feasible microemulsion template technique. The components used for the fabrication of Nanoject are biocompatible. The microemulsion template technique successfully yielded lipid nanocarriers with particle size of 63 nm. The developed Nanoject showed compatibility with the IV fluids and were autoclavable (data not shown) which indicated their suitability for IV route. For evaluation of antimalarial activity, Nanoject were administered at two dose levels (ND I and ND II) in order to understand whether Nanoject can offer reduction in ARM dose. ND II was 20 times lesser than that of ND I with respect to ARM dose. It should be noted that all formulations namely, ND I, ND II, Nanoject vehicle (NV) and Marketed formulation were administered by intraperitoneal route instead of IV route. Since marketed formulation cannot be administered by IV route, it would be difficult to compare the marketed formulation with the Nanoject. Hence, to obtain comparative evaluation, intraperitoneal route was employed. Future studies would be focused on the antimalarial efficacy of Nanoject on IV administration.

In vivo studies clearly demonstrated that both the doses of Nanoject (ND I and ND II) were significantly more effective as compared to the marketed formulation (Larither®). This clearly indicated the advantage of nanostructure and aqua base of the formulation in the delivery of ARM. ND I showed quick onset of action (∼95% antimalarial activity) as compared to marketed formulation (∼45% antimalarial activity) on 8th day which could be due to the unentrapped (but solubilized) ARM in Nanoject and aqueous nature of the formulation. At the same time, ND I showed antimalarial activity for a longer duration indicating that Nanoject may be long-circulating *in vivo* and that ARM entrapped in Nanoject may be released in a sustained manner. The results of survival studies also corroborate this hypothesis. However, to validate the hypothesis pharmacokinetic studies should be carried out which would be done in the future.

The most striking and unexpected observation of the investigation is the antimalarial activity of Nanoject vehicle (NV), as well as ND II which contains 20-fold less ARM as compared to ND I. Both NV and ND II demonstrated higher antimalarial activity as compared to marketed formulation at all points of evaluation. The antimalarial activity of NV and ND II was similar on 8th day and later on ND II showed slightly higher activity. The survival studies corroborate this statement. Since the concentration of all the excipients in NV and ND II was same, it may be assumed that the excipients of Nanoject may have antimalarial activity. However, to date, there are no such reports on the antimalarial activity of the excipients used in the Nanoject. Capmul MCM (a mixture of glyceryl caprylate and caprate) is reported to have bacteriostatic potential (as per the manufacturer's information) but there are no *in vivo* reports on either antibacterial or antimalarial activity of Capmul MCM. Glyceryl dilaurate on the other hand has shown antimicrobial potential *in vitro* in earlier studies from our group (Pattani et al., 2006). However, its antimalarial potential is still unknown. The surfactants used in the Nanoject are not reported to have any antibacterial or antimalarial activity. Literature indicates that oily phases such as fatty acids can have antimalarial action. In 1992, Kumaratilake et al., reported that fatty acids and their methyl esters can kill *P. falciparum* by interfering with the fatty acid biosynthetic pathway of the parasite (Kumaratilake et al., 1992). Subsequently, Krugliak et al. (1995) reported antiplasmodial effect of a series of C_{18} fatty acids against the FCR3 strain of *P. falciparum*. The excipients used for the fabrication of Nanoject (including the surfactants) are fatty acid derivatives or esters and they would be hydrolyzed to their respective fatty acid esters by serum esterases over a period of time. Though none of the Nanoject excipients and their respective fatty acid esters has been explored by Kumaratilake et al. (1992) and Krugliak et al. (1995), it is possible that fatty acid esters may exhibit antimalarial activity. It may also be possible that the lipidic excipients of Nanoject have a stimulating effect on the immunological system as reported earlier by Chimanuka et al. (2002) for blank liposomes. It may be possible that the lipidic components of Nanoject may enhance the immunological response mediated by $CD4^{+}T$ cells of the Th_2 type. Also there are similar reports in the literature (Todd et al., 1998) which demonstrate the inception of immunological cascade caused by injection of particulate carriers like emulsions causing damage at the site of injection leading to nonspecific inflammation, attraction of macrophages, etc. The particulate nature of NLC may also increase the presentation of the antigen by macrophages and dentritic cells and, also major histocompatibility complex (MHC) class I molecules resulting in cytotoxic T-lymphocyte (CTL) induction (Schneerson et al., 1991). The *in vitro* and *in vivo* antimalarial activity of the individual components of Nanoject and the possible mechanism of antimalarial activity of the vehicle remains to be elucidated. Nonetheless, this investigation demonstrates that NLC based Nanoject offers several advantages over the currently marketed oily intramuscular formulations (Larither®). With an increase in efficiency and sustained release of ARM using Nanoject, perhaps the current dosage of ARM can be reduced, providing comparative relief to the malaria patients.

5. Conclusion

The feasibility of microemulsion template technique in the fabrication of nanostructured lipid carriers (NLC) of ARM was successfully established. NLC of ARM (Nanoject) offer significant improvement in the antimalarial activity and duration of action of ARM as compared to the conventional injectable formulation. Nanoject can be considered as a viable alternative to the current injectable IM formulation.

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