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Solid microemulsion preconcentrate (NanOsorb) of artemether for effective treatment of malaria

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ABSTRACT

A microemulsion preconcentrate was formulated on the basis of solubility of artemether (ARM) in the various oily phases and surfactants and phase diagrams. Various solid adsorbents were evaluated for their ability yield solid microemulsion preconcentrates (NanOsorb-ARM). NanOsorb-ARM on dilution yielded microemulsion with average globule size of 183 nm and polydispersity index of 0.498 when determined using photon correlation spectroscopy. The antimalarial activity of NanOsorb-ARM, ARM solution and marketed ARM formulation (Larither®) was evaluated in *Plasmodium berghei* infected mice as per Peter's four day protocol. The acute lethal dose and the subacute toxicity of NanOsorb-ARM were determined as per the method suggested in Organization for Economic Cooperation and Development (OECD) guidelines. The NanOsorb-ARM exhibited significantly higher antimalarial activity ($P < 0.05$) as compared to the marketed formulation of artemether (Larither®). Surprisingly, placebo NanOsorb also showed significantly higher antimalarial activity as compared to Larither® indicating that excipients used for the formulation of NanOsorb may have antimalarial activity. Subacute toxicity studies demonstrated that NanOsorb-ARM is comparatively safer than artemether oily solution with respect to survival, gross pathology, hematology and serum biochemistry in mice of both the genders.

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 species of parasites: *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae* and *Plasmodium ovale*. Amongst these, the most severe malaria is caused by blood-borne Apicomplexan parasite *P. falciparum* which is responsible for almost all malaria related deaths. Existing treatments for malaria include a limited number of clinically effective antimalarial agents. However, the clinical utility of the most of the antimalarial agents is hampered due to problems such as poor oral bioavailability and the emergence of drug-resistant parasite strains. Paradoxically, due to lack of economic incentives, there are not many initiatives for the development of new anti-malarial agents. On the other hand, despite numerous efforts and investigations, there is no effective and promising malaria vaccine on the horizon till date. This scenario has enforced the smart and effective utilization of the current antimalarial agents with the help of novel drug delivery systems. Researchers have explored different novel approaches such as solid dispersions (Abdul-Fattah and Bhargava, 2002), liposomes (Stensrud et al., 2000; Date et al., 2007), immunoliposomes (Date et al., 2007; Owais et al., 1995), polymeric nanoparticles (Rodrigues et al., 1995) and dendrimers (Bhadra et al., 2005) or different routes such as transdermal route (Jeans and Heard, 1999) and rectal route (Karunajeewa et al., 2006) with the objective to improve the efficacy of the antimalarial agents. Although these approaches have shown some success to improve the efficacy of the antimalarial agents, none of them have reached the clinics due to problems such as high cost of manufacture (in case of liposomes and immunoliposomes), high cost of excipients (in case of liposomes, polymeric nanoparticles) difficulty in industrial scale up (in case of dendrimers, polymeric nanoparticles) and patient non-compliance (in case of rectal route).

Since last decade, microemulsions have gained a great interest as a commercially feasible novel lipid-based delivery system and they have shown capability to improve oral bioavailability and therapeutic efficacy of several therapeutic agents. Microemulsions are thermodynamically stable, transparent, isotropic, low-viscosity

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colloidal dispersions consisting of microdomains of oil and/or water stabilized by an interfacial film of alternating surfactant and cosurfactant molecules (Lawrence and Rees, 2000). The various attractive advantages of micromeulsions such as nanosize (<200 nm), ease of scale-up and manufacturing, long shelf life, ability to improve dissolution rate and lymphatic transport of hydrophobic drugs give them an edge over other novel delivery systems such as liposomes, dendrimers and polymeric nanoparticles. Thus, microemulsions can be considered as a vehicle of choice for oral drug delivery and the successful commercialization of microemulsion preconcentrates of cyclosporine A (Sandimmune® Neoral) is sufficient to highlight their potential. In recent years, there is a growing trend to formulate solid microemulsion preconcentrates by adsorbing liquid microemulsion preconcentrates onto suitable solid carriers (Ito et al., 2005). Such solid microemulsion systems can be easily filled in capsules and on oral administration, they readily form microemulsion *in vivo*; presenting the drug in nanosized and 'ready to absorb' form.

The objective of the present investigation was to formulate solid microemulsion preconcentrate (NanOsorb) of a widely used antimalarial agent artemether (ARM) and to evaluate it for the antimalarial efficacy and safety. ARM is a potent and rapidly acting antimalarial agent available for the treatment of severe multiresistant malaria and is included in WHO list of essential medicines (WHO web site). It is active against *P. vivax* as well as chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* and is also indicated in the treatment of cerebral malaria. However, the therapeutic potential of ARM is considerably hampered due to its low oral bioavailability (~40%) (Karbwang et al., 1997). The low bioavailability of ARM stems from its poor aqueous solubility. Literature indicates that bioavailability of ARM increases with the administration of fatty meals (Lefèvre and Thomsen, 1999). In view of this, the lipid-based delivery systems such as microemulsions are likely to have good potential in improving oral bioavailability and in turn the therapeutic efficacy of ARM. The main objective of the investigation was to design and test the efficacy of a novel and commercially feasible oral delivery strategy for ARM, NanOsorb. Microemulsion preconcentrates or self-microemulsifying drug delivery systems have gained a great interest in the drug delivery research due to their ease of commercialization and also due to their ability to improve oral bioavailability of numerous hydrophobic drugs such as cyclosporine (Lawrence and Rees, 2000). Solid microemulsion preconcentrates have recently been described and they have exhibited more commercial potential and patient acceptability as compared to microemulsion preconcentrates (Ito et al., 2005). With this rationale, we formulated solid microemulsion preconcentrate (NanOsorb) of ARM and evaluated its potential in improving the antimalarial efficacy of ARM by means of a suitable *in vivo* model.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Artemether (ARM) was kindly provided as a gift sample by Ipca Laboratories Ltd. (Mumbai, India). Capmul® MCM (Glyceryl Mono-, di-caprylate) and Captex 200 (Propylene Glycol Dicaprylate/Dicaprate), was received as a gift sample from Abitech Corp., USA through Indchem International (Mumbai, India). Capryol 90 (Propylene glycol monocaprylate), Labrasol (Caprylocaproyl macrogol-8 glycerides), Gelucire 44/14® (Lauroyl macrogol glycerides), Plurol Oleic CC 497 (Polyglyceryl 6-dioleate) and Labrafil M 1944 CS (Oleoyl macrogol-6 glycerides) were obtained as a gift sample from Gattefosse', France through Colorcon Asia Ltd., (Goa, India). Miglyol 812 (Caprylic/capric triglyceride) was received as a gift sample from Sasol GmBH through S. Zhaveri and Co. (Mumbai, India). Cremophor EL (PEG-35-castor oil), Cremophor RH 40 (PEG-35-hydrogenated castor oil) and Solutol HS 15 (PEG-660- 12-hydroxystearate) were kindly provided by BASF India Ltd. (Mumbai, India). Neusilin UFL2® (magnesium aluminium metasilicate) was obtained as a gift sample from Fujii Chemicals Ltd., Japan through Gangwal Chemicals, (Mumbai, India). Transparent empty hard gelatine capsules were obtained as a gift sample from Associated Capsules Ltd. (Mumbai, India). Pharmaceutical grade peanut oil was obtained as a gift sample from Kamani oil Industries, (Mumbai, India). All other chemicals and solvents were purchased from Merck International and s. d. fine chemicals Ltd., Mumbai, India. All the materials were used as received without any further modifications.

2.1.2. Parasite

Plasmodium berghei ANKA strain was used for *in vivo* evaluation of antimalarial activity. The strain was found to be free of contamination with *Eperythrozoon coccoides* after examination. 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. It is sensitive to all antimalarial agents that are used currently.

2.1.3. 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, 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 $22 \pm 3\,^{\circ}C$ and 65% relative humidity, were fed a standard mouse diet and provided with clean drinking water *ad libitum* throughout the experiments.

2.2. Solubility studies

The solubility of ARM in different oils and surfactants was determined by using shake flask method. The surfactants were melted before subjecting to solubility studies wherever required. 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 $25 \pm 2\,^{\circ}C$. After 24 h, each sample was centrifuged at 5000 rpm for 10 min. The supernatant (0.5 ml) was diluted suitably and the amount of ARM present in the supernatant was analyzed by HPLC.

2.3. HPLC analysis of ARM

The quantity of ARM solubilized in various vehicles was determined by using HPLC method reported 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 (250×4 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.

2.4. Pseudo-ternary phase diagram

The first step towards the formulation development was to determine the feasibility of the microemulsion formation. The

boundaries of the microemulsion domains were determined by plotting pseudoternary phase diagrams for the components short listed from solubility studies. The pseudoternary phase diagram of oil (Capmul® MCM), surfactant mixture (Gelucire 44/14®+ Labrasol® at the ratio of 1:1) and doubled distilled water was plotted using water titration method. Briefly, mixtures of the oil (Capmul® MCM) and surfactants (Gelucire 44/14®+ Labrasol® at the ratio of 1:1) 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. To the resultant mixtures, water was added dropwise till the first sign of turbidity in order to identify the end point and after equilibrium, if the system became clear then the water addition was continued. After complete equilibrium was reached, the mixtures were checked visually for transparency and by visualizing through crossed polarizers (fabricated in house using polarizing lenses, Nikkon, Japan) for the optical isotropicity. The systems which appeared black when visualized through the crossed polarizers were deemed to be within the microemulsion region. No attempts were made to identify the other regions of the phase diagrams. The amount of each of the component (%w/w) for a particular composition was determined with the help of pseudo-ternary phase diagram.

2.5. Formulation of solid microemulsion preconcentrate of ARM (NanOsorb-ARM)

The microemulsion preconcentrate of ARM was prepared by mixing the required amounts of Gelucire 44/14®, Labrasol® and Capmul® MCM. The composition of the microemulsion preconcentrate is highlighted by a dot in the phase diagram (Fig. 1) and it can hold 160 mg of ARM which is 4-fold higher than the amount of ARM present in the currently marketed formulation (Larither®, Icpa Laboratories, India; a capsule formulation that contains 40 mg ARM, suitable diluents such as lactose and flow enhancers such as Aerosil). For the preparation of solid microemulsion preconcentrate, 0.29 g of ARM microemulsion preconcentrate (equivalent to 40 mg of ARM) was mixed with various solid carriers namely dibasic calcium phosphate, anhydrous lactose, microcrystalline cellulose, calcium carbonate, magnesium carbonate, Aerosil 200 and Neusulin UFL2, in various ratios (2:1, 1:1, 1:2 and 1:4). Briefly, the microemulsion preconcentrate was added dropwise over the solid adsorbent contained in a broad bottom beaker. After each addition, the mixture was homogenized using glass rod to ensure uniform distribution of the droplet. The adsorbent that was required in a small amount to give a free flowing solid microemulsion preconcentrate with high bulk density was chosen for the further studies.

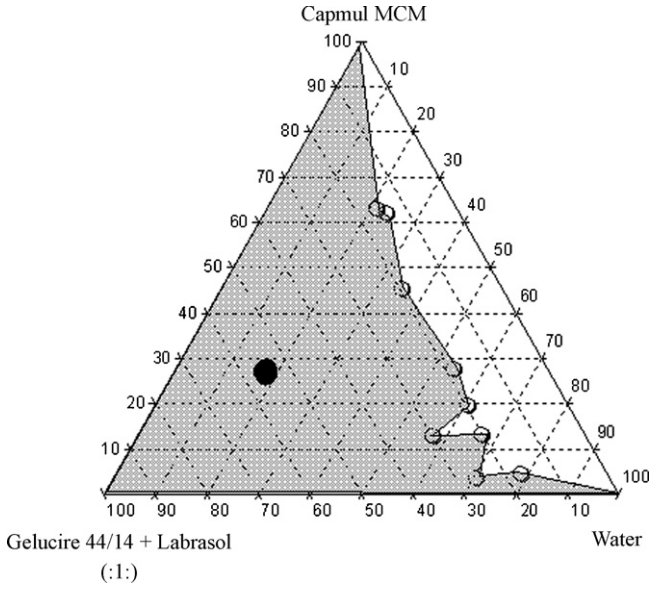

Fig. 1. Pseudo-ternary phase diagram for Gelucire 44/14®+ Labrasol-Capmul® MCM-water.

2.6. Determination of globule size and polydispersity index

The globule size and polydispersity index of ARM microemulsion preconcentrate and NanOsorb-ARM was determined by photon correlation spectroscopy (PCS; Beckman N4 plus submicron particle size analyzer, Wipro, India). Both the formulations were diluted with double distilled water to ensure that the light scattering intensity (between 6e+004 and 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 the measurements were carried out in triplicate at a temperature of 25 ± 2 °C and at an angle of 90° to the incident beam. All data obtained were analyzed by *Contin* program.

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

The protocol for animal studies was approved by the Institutional ethical committee of the Tata Institute of Fundamental Research, (TIFR). The protocol was designed on the basis of "Peters four day suppressive test" described by Peters et al. (1975). The lethal strain of *P. berghei* was used for the experiments. In-house bred mycoplasma free male Swiss mice (weighing around 30 g each) were infected by intraperitoneal inoculation of donor mouse blood diluted in acid citrate dextrose (ACD) buffer containing approximately 10^6 *P. berghei* infected RBCs on day '0'. The mice were randomly divided into various groups (n = 10 per group) as depicted in Table 1. Starting from day '0' to day '3' post infection, the different groups were given 4 mg/kg of ARM by oral gavage. On fourth day, the blood was withdrawn from tail vein and the bloodsmears were prepared. Bloodsmears were fixed with methanol and stained with Giemsa stain and the parasites were counted. Parasitemia is the quantitative content of parasites in the blood. Parasitemia was reported as percentage parasitemia after counting 250 RBCs from each slide. Activity of different ARM formulations was calculated by the following formula suggested in the standard protocol by Fidock et al. (2004).

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

The animals in all the groups were also monitored for the survival till 10 days.

2.8. 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 $P < 0.05$.

2.9. Acute toxicity studies on NanOsorb-ARM

The protocol for acute toxicity studies was approved by the Institutional ethical committee of the Tata Institute of Fundamental Research (TIFR), Mumbai. Swiss male mice (n = 3) weighing 35 ± 5 g were given different doses of NanOsorb-ARM and ARM dissolved in pharmaceutical grade pea nut oil (ARM-PO) ranging from

Table 1
Summary of the different groups and their treatment regiment used for *in vivo* antimalarial efficacy studies

No.	Group ($n = 10$)	Infection	Treatment
1	Marketed formulation (Larither®)	+	Larither® equivalent to 4 mg/kg of the ARM
2	NanOsorb-ARM	+	NanOsorb-ARM equivalent to 4 mg/kg of ARM
3	ARM solution (ARM dissolved in 15% DMSO)	+	Solution equivalent to 4 mg/kg of ARM
4	Placebo NanOsorb (NanOsorb-ARM control)	+	Placebo NanOsorb diluted to similar extent as that of NanOsorb-ARM
5	Control	+	No treatment

ARM: Artemether; distilled water was used as a diluent for all the formulations.

700 mg/kg to 2000 mg/kg by oral gavage. The doses were decided as per the main test specified in (Organization for Economic Cooperation and Development) guidelines (OECD web site). Toxic effects and number of deaths resulting after the administration of different doses of ARM-PO and NanOsorb were observed over the period of 24 h.

The main test consists of a single ordered dose progression in which animals were dosed, one at a time, at a minimum of 48 h intervals. The first animal receives a dose a step below the level of the best estimate of the LD_{50}. If the animal survives, the dose for the next animal is increased by [a factor of] 3.2 times the original dose; if it dies, the dose for the next animal is decreased by a similar dose progression. The dose of 1000 mg/kg was taken as the LD_{50} of ARM.

2.10. Determination of subacute toxicity

Swiss male and female mice ($n = 6$) were used for the determination of subacute toxicity study. Marketed formulation of ARM, Placebo NanOsorb and NanOsorb-ARM were administered orally at 3-dose levels (doses equivalent to 1 mg/kg, 2 mg/kg, and 4 mg/kg of ARM) for 28 days. On 28th day, blood from all the animals was collected by retro orbital bleeding and various biochemical and hematological parameters were determined. Mice were sacrificed on 28th day and the organs were examined for any gross pathological changes. Animals were monitored for behavioral abnormalities or mortality during the course of the study. In case of any death during the course of the study (if any), the mouse was immediately subjected to autopsy. Analysis of biochemical parameters was carried out at Department of Pathology, Bombay Veterinary College, Parel, Mumbai.

3. Results and discussion

3.1. Solubility studies

Solubility studies were carried out to identify potential ingredients for the formulation of microemulsion. Amongst the various oils that were screened, Capmul® MCM exhibited highest solubilizing potential for ARM and it was selected as an oily phase for further studies (Table 2). Amongst the various surfactants, Cremophor EL and Cremophor RH 40 exhibited highest solubilizing potential for ARM (Table 3). However, Cremphores were not selected for further studies due to the adverse effects such as allergic reactions associated with their long-term use (Ten Tije et al., 2003). Gelucire 44/14® and Labrasol® were preferred to fabricate microemulsion as they have been reported to improve bioavailability of various hydrophobic drugs such as piroxicam (Yuksel et al., 2003) and vitamin E (Barker et al., 2003). Gelucire 44/14® has good emulsifying properties but is semisolid at room temperature. Hence, it is usually not preferred to be used alone for the fabrication of microemulsion preconcentrates as it may not lead to formation of homogenous liquid preconcentrate. Labrasol is liquid at room temperature but has shown to have relatively poor emulsifying properties as compared to Gelucire 44/14® when evaluated by method reported by Date and Nagarsenker (2007). However, preliminary studies indicated that a mixture of Gelucire 44/14® and Labrasol® (at the ratio of 1:1) has liquid consistency at room temperature and also better emulsifying properties when evaluated by method reported by Date and Nagarsenker (2007). Hence, for the further studies, a mixture of Gelucire 44/14® and Labrasol® at the ratio of 1:1 was used.

Table 2
Solubility of ARM in different oils ($n = 3$; ±S.D.)

Oil	Solubility (mg/g)
Capmul® MCM	316 ± 7.5
Capryol 90	42 ± 4.7
Captex 200	126 ± 9.8
Oleic acid	29 ± 2.4
Plurol Oleic CC 497	3 ± 8.4
Labrafil M 1944 CS	7 ± 9.4
Lauroglycol 90	116 ± 3.4
Miglyol 812	34 ± 1.4

3.2. Pseudoternary phase diagram

The region of microemulsion existence for Gelucire 44/14®+ Labrasol®-Capmul® MCM-water systems was determined by plotting pseudo-ternary phase diagram (Fig. 1). The shaded area in the phase diagram indicates the microemulsion existence region. The black dot in the Fig. 1 represents the composition of the microemulsion selected for the further studies. In this composition, the ratio of oil (Capmul® MCM) to surfactants (Labrasol®+ Gelucire 44/14®) was 2:3. The selected microemulsion could withstand accelerated stress tests such as freeze thaw cycling and centrifugation at 5000 rpm; confirming the thermodynamic stability of the microemulsion. The microemulsion was found to be optically isotropic when viewed through crossed polarizer fabricated in house. For the further studies, microemulsion without water (microemulsion preconcentrate) was used. This preconcentrate would readily form microemulsion in the body on dilution with physiological fluids.

3.3. Formulation of solid microemulsion preconcentrate of ARM (NanOsorb-ARM)

It was observed that solid adsorbents such as dibasic calcium phosphate, lactose, microcrystalline cellulose, magnesium carbonate and calcium carbonate did not have good adsorption capacity to

Table 3
Solubility of ARM in different surfactants ($n = 3$; ±S.D.)

Surfactant	Solubility (mg/g)
Tween 20	50 ± 2.4
Tween 80	12 ± 9.4
Cremophor EL	280 ± 1.4
Solutol HS 15	11 ± 6.9
Cremophor RH 40	143 ± 2.7
Labrasol®	21 ± 2.1
Gelucire 44/14®	44 ± 1.3

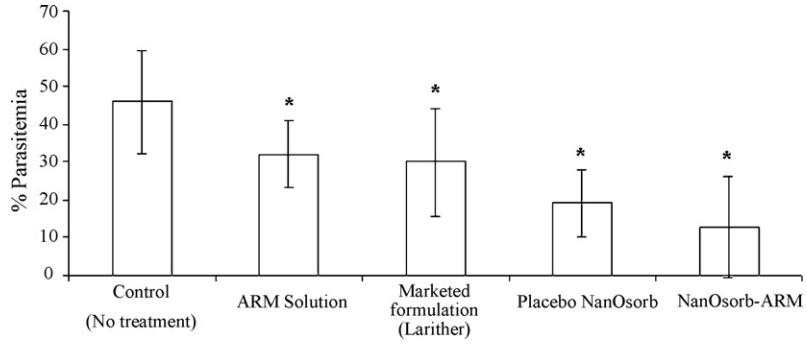

Fig. 2. Percent parasitemia observed in different treatment groups on day 4 ($n = 10$); (*)Significantly lesser than the control; NanOsrb-ARM showed significantly less parasitemia as compared to Larither® ($P < 0.05$).

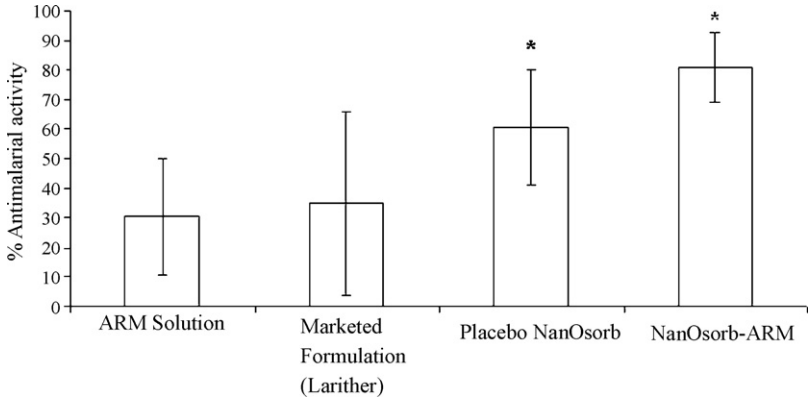

Fig. 3. Antimalarial activity of various ARM formulations on day 4 ($n = 10$); (*) Significantly higher than the ARM solution and Larither® ($P < 0.05$).

Table 4
Number of survival observed with different groups

Group	No. of survivals on 11th day
Control	3/10
ARM solution (ARM dissolved in 15% DMSO)	2/10
Marketed formulation (Larither®)	2/10
NanOsorb-ARM	5/10
Placebo NanOsorb	5/10

yield a free flowing solid microemulsion preconcentrate whereas Aerosil 200 and Neusulin® UFL2 exhibited good adsorption capacity to yield free flowing solid microemulsion preconcentrate at the ratio of 2:1 (preconcentrate:Neusilin®). Although, both, Aerosil 200 and Neusulin® UFL2 could give free flowing solid preconcentrates, Neusilin UFL2® was selected as it could give free flowing powders with higher bulk density (data not shown). Furthermore, antacid properties of Neusilin UFL2® (Neusilin UFL2® literature available on Fuji chemical web site) would be of further advantage as ARM and other artemisinin derivatives are known to degrade in acidic environment of gastrointestinal tract. It was found that 0.29 g of solid microemulsion preconcentrate could hold 40 mg of ARM and can be easily filled in size '0' hard gelatine capsule.

3.4. Determination of globule size and polydispersity index

The ARM microemulsion preconcentrate on dilution yielded microemulsions with the globule size of 167 nm and polydispersity index of 0.598 whereas NanOsorb-ARM on dilution yielded

Table 5
Results of the subacute toxicity studies: hematology ($n = 6$)

Treatment groups	Hemoglobin (12–16%)[a]		Plateletes (3–20 × 10^3)[a]		Hematocrit (37–55%)[a]		RBCs (5.5–8.5 × 10^6)[a]	
	Males	Females	Males	Females	Males	Females	Males	Females
NanOsorb-ARM equivalent to 1 mg/kg of ARM	13.2–16.4	12.6–12.8	9.64–10.4	5.32–16.94	33–64	39–41	5.3–6.88	6.6–7.46
Placebo NanOsorb[b]	11.7–13.1	6.6–13.3	4.60–11.19	4.07–10.41	34–42	17–40	7.7–7.77	5.27–6.7
NanOsorb-ARM equivalent to 2 mg/kg of ARM	13.3–14.2	12.3–12.7	11.0–17.3	7.11–20.62	43–46	40–41	6.13–6.89	5.67–7.46
Placebo NanOsorb[c]	12.4–14.4	13.3–14.3	5.71–17.02	10.38–15.20	42–50	42–45	8.5–8.63	6.59–8.3
NanOsorb-ARM equivalent to 4 mg/kg of ARM	10.1–14.1	13.8–14.1	14.19–16.94	2.08–6.2	33–47	43–46	5.9–7.6	5.42–7.46
Placebo NanOsorb[d]	12.6–13.2	12.1–13.5	2.60–10.63	1.99–4.42	41–43	40–41	6.13–6.89	5.67–7.46
Marketed formulation equivalent to 1 mg/kg of ARM	12.5–14.4	13.1–16.5	7.38–9.21	7.81–11.52	38–43	40–52	6.3–7.08	6.12–8.87
Marketed formulation equivalent to 2 mg/kg of ARM	7.3–14.3	12.4–13.6	6.51–8.92	8.03–10.06	24–50	42–52	4.59–7.6	6.6–9.88
Marketed formulation equivalent to 4 mg/kg of ARM	10.4–13.4	8.2–14.3	10.10–13.19	8.78–10.07	35–44	30–46	5.58–6.67	3.84–7.61

[a] Values in parentheses indicate normal values.
[b] Placebo NanOsorb diluted to the same extent as that of NanOsorb-ARM equivalent to 1 mg/kg of ARM.
[c] Placebo NanOsorb diluted to the same extent as that of NanOsorb-ARM equivalent to 2 mg/kg of ARM.
[d] Placebo NanOsorb diluted to the same extent as that of NanOsorb-ARM equivalent to 4 mg/kg of ARM.

microemulsion with globule size of 183 nm with a polydispersity index of 0.498. Although the globule size of the microemulsion increased after adsorption of ARM preconcentrate on Neusilin UFL2®, the difference is not significant.

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

In vivo antimalarial efficacy of the various ARM formulations with respect to antimalarial activity and reduction in percent parasitemia is depicted in Figs. 2 and 3. As depicted in Fig. 2, the control group (no treatment) on day 4 showed highest parasitemia, validating the animal model used for the study. The animals treated with ARM solution and marketed formulation (Larither®) showed reduction in the percent parasitemia as compared to that of control group. The animals treated with ARM NanOsorb showed highest reduction in the percent parasitemia as compared to all other groups ($P < 0.05$) showing the utility of the microemulsion approach in improving the delivery of ARM (Fig. 2). The antimalarial activity of ARM NanOsorb was highest amongst all the groups that were studied (Fig. 3). The antimalarial activity of NanOsorb-ARM was 2.6-fold, 2.3-fold higher than that of the ARM solution and ARM marketed formulation (Larither®). This clearly demonstrates the effectiveness of the microemulsion in improving the therapeutic efficacy of ARM. It is also noteworthy that the animals treated with NanOsorb-ARM showed higher survival rate than that of ARM solution and ARM marketed formulation (Table 4).

However, the most striking and unexpected observation of the investigation is the antimalarial activity of placebo NanOsorb (NanOsorb without ARM). Placebo NanOsorb demonstrated a significant ($P < 0.05$) reduction in the percent parasitemia as compared to that of ARM solution and marketed formulation (Larither®). The antimalarial activity may be attributed to the components of the placebo NanOsorb (Capmul® MCM, Gelucire 44/14®, Labrasol® and Neusilin UFL2®) though none of them are reported to have any such activity. Neusilin UFL2® is a magnesium aluminium metasilicate and is reported to be inert on oral administration. Hence, the antimalarial activity could be due to the other components. Chemically, Capmul® MCM is a mixture of glyceryl caprylate and caprate. Labrasol® is polyethoxylated caprylic and capric acid derivative whereas Gelucire 44/14® is a mixture of polyethoxylated stearic acid derivatives. Capmul® MCM 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. However, literature indicates that oily phases such as fatty acids can have antimalarial action. Kumaratilake et al. (1992) reported that fatty acids and their methyl esters can kill *P. falciparum* by interfering with the fatty acid biosynthetic pathway of the parasite. Subsequently, Krugliak et al. (1995) reported antiplasmodial effect of a series of C_{18} fatty acids against the FCR3 strain of *P. falciparu*. As mentioned earlier, all the components of placebo NanOsorb are derivatives of fatty acids which on oral administration would be hydrolyzed to various fatty acids such as caprylic acid, capric acid and stearic acid and may demonstrate the antimalarial action. Our future work would be focused on the exploration of the *in vitro* and *in vivo* antimalarial activity of the individual components of placebo NanOsorb to identify the most active component and the possible mechanism of antimalarial activity.

Interestingly, placebo NanOsorb, showed higher survival rate as compared to that of ARM solution and ARM marketed formulation (Table 4), indicating that the components of NanOsorb may have antimalarial action. Finally, the results of this pharmacodynamic activity also suggest that with the help of microemulsion approach, the therapeutic dose of ARM can be significantly reduced which

Table 6
Results of the subacute toxicity studies: serum biochemistry ($n = 6$)

Treatment groups	Albumin (2.6–4.3 g/dl)[a]		Globulin (1.3–2.5 g/dl)[a]		Total protein (4.5–7.8 g/dl)[a]		Urea nitrogen (7–24 mg/dl)[a]		Creatinine (0.4–1.8 mg/dl)[a]	
	Males	Females	Males	Females	Females	Males	Males	Females	Males	Females
Control	2.00–2.98	2.34–2.88	1.38–2.35	0.91–2.61	3.69–4.96	3.47–4.04	12.58–19.63	15.12–18.28	1.05–1.42	0.98–1.60
NanOsorb-ARM equivalent to 1 mg/kg of ARM	1.51–2.13	2.10–3.51	1.35–2.55	0.66–1.69	2.87–4.34	3.52–5.20	12.21–20.50	23.25–32.08	1.58–1.28	0.67–1.23
Placebo NanOsorb[b]	1.51–2.56	2.10–3.51	0.91–2.55	1.58–2.21	2.87–3.47	3.68–5.20	21–30	19.00–32.08	1.17–1.76	1.23–1.97
NanOsorb-ARM equivalent to 2 mg/kg of ARM	1.87–3.42	2.00–2.88	1.42–2.25	1.75–2.61	2.39–4.94	3.75–4.75	16.95–19.88	24.20–28.28	1.71–1.96	1.79–1.98
Placebo NanOsorb[c]	1.90–3.42	1.51–1.88	0.66–1.42	1.36–2.55	3.15–4.44	2.87–3.39	14.25–19.52	19.88–21.21	0.96–1.88	1.58–1.76
NanOsorb-ARM equivalent to 4 mg/kg of ARM	1.88–2.56	1.88–2.88	0.91–2.55	0.91–2.51	3.39–3.47	3.39–4.74	25.12–30.87	25.12–30.87	1.17–1.76	1.17–1.85
Placebo NanOsorb[d]	1.51–2.88	1.51–1.88	0.91–1.86	1.36–2.55	2.87–4.74	2.87–3.39	19.21–28.12	31.87–33.23	1.58–2.01	1.66–1.76
Marketed formulation equivalent to 1 mg/kg of ARM	1.88–3.42	1.51–1.88	0.91–2.55	1.36–2.55	3.39–4.47	2.87–3.39	29.12–36.52	32.88–37.87	1.17–1.88	1.76–1.99
Marketed formulation equivalent to 2 mg/kg of ARM	1.78–2.56	2.10–3.51	0.91–2.55	1.42–1.69	3.39–3.69	3.68–5.20	31.87–36.12	31.25–33.99	1.76–1.88	1.23–1.88
Marketed formulation equivalent to 4 mg/kg of ARM	1.90–3.42	1.87–2.34	0.66–2.23	1.45–2.61	3.52–4.44	2.89–4.95	26.25–31.25	24.20–27.80	1.20–1.96	1.71–1.88

[a] Values in parentheses indicate normal values.
[b] Placebo NanOsorb diluted to the same extent as that of NanOsorb-ARM equivalent to 1 mg/kg of ARM.
[c] Placebo NanOsorb diluted to the same extent as that of NanOsorb-ARM equivalent to 2 mg/kg of ARM.
[d] Placebo NanOsorb diluted to the same extent as that of NanOsorb-ARM equivalent to 4 mg/kg of ARM.

will be advantageous in reducing the dose related toxicity and dose related resistance issues associated with ARM.

The 2.6-fold, 2.3-fold higher antimalarial activity of NanOsorb-ARM is a combined result of the nanosize of the microemulsion, instantaneous dissolution of ARM which would facilitate quick absorption, probable enhancement in bioavailability due to lipidic nature of the drug, protection of ARM from acidic microenvironment of stomach due to the presence of Neusilin UFL2® and antimalarial effect of the excipients of the NanOsorb.

The pharmacokinetic studies on NanOsorb-ARM are in progress to determine the increase in the bioavailability and the preliminary studies in rabbits have shown promising results.

3.6. Acute and subacute toxicity of NanOsorb-ARM

LD_{50} (median lethal oral dose), is a statistically derived single dose of a substance that can be expected to cause death in 50% of animals when administered by the oral route. The LD_{50} value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg). In the acute toxicity studies, no death was observed in the groups receiving 700 mg/kg and 1000 mg/kg dose of both ARM-PO and NanOsorb-ARM after 48 h. At 1500 mg/kg casualty was seen in ARM-PO group but the group receiving equivalent amount of NanOsorb-ARM was alive. The animals from the groups receiving 2000 mg/kg of ARM-PO and NanOsorb-ARM were dead. This indicates that the LD_{50} value of NanOsorb-ARM was between 1500 mg/kg and 2000 mg/kg and that of ARM- PO was between 1000 mg/kg and 1500 mg/kg suggesting higher safety of NanOsorb-ARM.

During the subacute toxicity studies, no mortality was observed in case of NanOsorb-ARM, placebo NanOsorb and marketed formulation at all the dose levels. No pathological alterations were observed in any organs on gross examination in all the groups. The effect of NanOsorb-ARM, placebo NanOsorb and marketed formulation on various hematological parameters such as hemoglobin content, number of platelets, hematocrit and number of RBCs and biochemical parameters such as serum albumin, serum globumin, total protein, urea-nitrogen and creatinine was studied at 3-dose levels. The values of various biochemical and hematological parameters are as depicted in Tables 5 and 6. All the parameters were within the normal range indicating that ARM NanOsorb is safer on oral administration in mice of both the genders.

4. Conclusion

The solid microemulsion preconcentrate (NanOsorb) significantly improved the therapeutic efficacy of artemether demonstrating the utility of the novel drug delivery strategies for antimalarial agents. *In vivo* studies clearly demonstrated that NanOsorb-ARM has significantly higher antimalarial activity as compared to the ARM solution and marketed formulation (Larither®). Thus, the utility of microemulsion preconcentrates for enhancing the therapeutic efficacy of ARM is established in this investigation. The acute and subacute toxicity studies successfully established the safety of the NanOsorb in the animals. NanOsorb-ARM can be a viable alternative to the existing artemether formulations and the ease of commercialization associated with solid microemulsion preconcentrates suggest that NanOsorb-ARM could have a tremendous market potential.

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