ORIGINAL RESEARCH

TARGETED SOLID LIPID NANOPARTICLES: A NOVEL APPROACH AGAINST MALARIA

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ABSTRACT:

Malaria is the most prevalent parasitic disease in the world with approximately 250 million clinical episodes annually to global health.In 2015, there were roughly 214 million malaria cases and an estimated 4, 38,000 malaria deaths. About 3.2 billion people, which are nearly half of the world's population, are at risk of malaria. The disease also causes a high economic burden, mainly affecting developing countries. In the current study, a targeted drug delivery system was developed using solid lipid nanoparticles loaded with artemether to target infected erythrocytes infected by malaria parasite and having characteristics of prolonged circulation time in blood and controlled release for increasing efficacy and effectiveness of antimalarial therapy and overcoming certain limitations of conventional drug delivery systems and further characterization was done.

KEYWORDS: Malaria, Antimalarial therapy, Targeted drug delivery, Controlled release

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1.INTRODUCTION

Malaria is the most prevalent parasitic disease of human beings causing tremendous health burden especially in tropical areas accounting for 1 to 2 million deaths round the globe yearly.[1] It is transmitted by female mosquito of Anopheles genus and five species of the apicomplex protozoan of genus Plasmodium are responsible for malarial infections in human beings namely: *P. falciparum, P. vivax, P. ovale, P. malariae,* and in some parts of south East Asia by the monkey malaria *P. knowlesi*.[2] There are various challenges faced by antimalarial therapy like; complexicity of parasitic life cycle and dosage regimens, appearance of multidrug resistant strains, non-availability of less toxic drugs, side effects, drug interactions, non-specificity of drug delivery systems affecting other organs and tissues, requirement of high minimum effective dose due to lack of spatial characteristics of drug delivery systems used preventing spread of disease and higher cost of treatment.[3]

Nano-carriers like Solid lipid nanoparticles (SLNs) have generated a great deal of interest in controlled and targeteddrug delivery. Solid lipid nanoparticles have several advantages over the conventional nanocarriers such as:

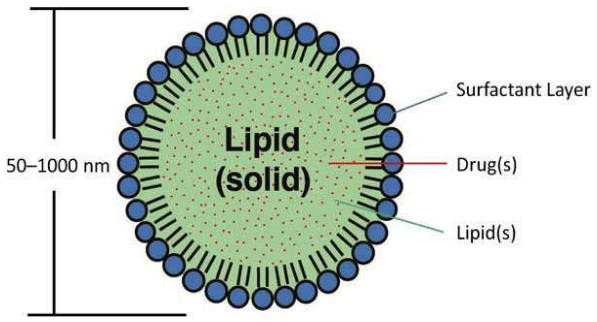


Fig. 1: Structure of solid lipid nanoparticle[4]

- Small size and narrow size distribution
- Controlled release of active drug over a long period
- Protection of drug from chemical degradation
- Biocompatible with no toxic metabolites
- Stable, cheaper and easy to scale up[5]

Artemether was the drug of choice for loading SLNs. It involves interaction of the peroxide-containing drug with heme, hemoglobin degradation by product, derived from proteolysis of hemoglobin. This interaction is believed to result in the formation of a range of potentially toxic oxygen and carbon- centred radicals.Targets all the asexual stages, blood schizonts especially and effective against all five species of parasite, multiple mechanism of actions and is well tolerated with fewer side effects.Produce rapid clearance of parasitemia and rapid resolution of symptoms- reduces parasite number 100-1000 times per cycle. Artemether is used in combination with Lumefantrine (orally) for treatment of malaria and is considered the best current treatment for uncomplicated malaria & active against multi-drug resistant P. falciparum malaria. The oral drug delivery of artemether has limitation of low bioavailability (<40%) due to degradation in acidic environment. Artemether is also available as injectable in oily vehicles. Oil based artemether intramuscular injection causes muscle pain on IM injection and muscle stiffness after injection. The novel drug delivery systems have significant scope in improving the delivery of drug and achieving target specificity as the current injectable artemether formulations cannot provide optimal therapy. It has been demonstrated that IV delivery of drug provides highest bioavailability to body as compared to other routes of drug delivery. However, currently no product is available that enables IV delivery of drug. Thus there is a need for a water based formulation of artemether which provides good

bioavailability as well as prevents pain on administration as in case of intramuscular formulations.

Heparin was used as a targeting ligandfortargeted drug delivery system:

- To prolong, localise, target
- Have a protected drug interaction with the target
- Site specificity
- Decreased drug dosage
- Reduced side effects
- More uniform drug effect[6]

It has been demonstrated to have antimalarial activity by inhibiting aggregation of erythrocytes (anti-rosetting activity) and having specific binding affinity for Plasmodium-infected red blood cells (pRBCs) vs. non-infected erythrocytes. [7] It forms a hydrophilic stealth coating having ability to inhibit complement activation thus evading reticuloendothelial system uptake and showing a longer systemic circulation [8] .Heparin also inactivates merozoites and prevents further invasion of erythrocytes.[9]

In the current study, it was investigated to develop a targeted drug delivery system using solid lipid nanoparticles loaded with artemether to target infected erythrocytes infected by malaria parasite and having characterstics of prolonged circulation time in blood and controlled release for increasing efficacy and effectiveness of antimalarial therapy and overcoming certain limitations of conventional drug delivery systems

2.MATERIALS AND METHOD:

Artemether was purchasedfrom Tokyo Chemical Industry Co. Ltd., Tokyo, Japan. Heparin Sodium and EDC.HCl were purchased fromSiscoResearchLaboratories Pvt. Ltd., Mumbai. Tristearin, Phosphatidylcholine and stearylamine were obtained from Central Drug House, Mumbai.

2.1.Preformulation studies:

Preformulation study was started from Physical examination in which drug was found to be white, hygroscopic and crystalline powder. Chemical identification test according to Indian Pharmacopoeia-2010 was done to identify artemether by its iodine liberating tendency from potassium iodide after it forms Dihydroartemisinin.Identification of Artemether was continued using HPLC analysis according to procedure given in Indian Pharmacopoeia-2010 on the basis of retention time matching method and further purity was determined quantitatively. [10]FTIR spectral analysis was further carried out to identify the drug as well as other excipients. Melting point determination was further done for identification of drug using melting point apparatus.Solubility studies were conducted using solvents like Distilled water DMF, DMSO, ethanol, methanol, Acetonitrile and chloroform[9]. Partition coefficient determining study was done based on n-octanol/ distilled water and n-octanol/ Phosphate buffer saline pH-7.4 systems[9]. UV spectrophotometric characterization of drug was done by derivatization of artemether to Dihydroartemisinin by heating artemether with 1N HCl at 80ºC for 20 minutes to derivatize artemether to Dihydroartemisinin and to solubalize dihydroartemisinin and absorption maxima was determined and calibration curve was prepared[12]. Drug excipient compatibility determination was done to detect any significant drug-excipient interaction UV spectrophotometrically.

2.2.Preparation ofArtemether loaded Solid Lipid Nanoparticles:

Initially, lipid is melted 5ºC above melting point of lipid. Artemether was dissolved in methanol and this was added to previousely melted lipid with heating to

evaporate the methanol. After complete dissolution the lipid melts containing Artemether was added to hot aqueous surfactant solution preheated at 10ºC above lipid's melting point and homogenized to yield primary emulsion. Primary emulsion was further homogenized at temperature 10ºC above melting point of lipid. The nanoemulsion produced was than cooled at room temperature and was further sonicated.[13]

$$\% \text{ Hemolysis} = \frac{\text{Absorbance of Sample-Absorbance at 0\% Hemolysis}}{\text{Absorbance at 100\% Hemolysis-Absorbance of Sample}} \times 100$$

2.3. STABILITY STUDIES:

Stability study of the optimized formulation was carried out according to ICH and WHO guidelines. The formulation was filled in amber colored bottles and kept in the stability chamber (REMI Environmental Test Chamber, India) maintained at 27˚± 2˚ C and 4˚± 2˚ C (RH-65±5%), for 10, 20, 30 and 45 days. The samples were analyzed for the % residual drug content and change in particle size at different time intervals. Initial drug content was taken as 100%.[9]

3.RESULTS AND DISCUSSION:

3.1.Preformulation studies:

The current work was started from preformulation studies. Artemether was found to be white, hygroscopic and crystalline powder. In chemical identification test, yellow colour due to liberation of iodine was clearly observed. HPLC analysis assisted in identification of artemether based on retention time matching method and purity was determined to be 98.11%. FTIR spectral analysis of drug and other excipients further helped in identification. Characterstics peaks in standard FTIR spectra were matched of with that of FTIR spectra of test samples of drug and excipients and all the results were in compliance. The observed melting point range of 85º-90º C was in compliance with reported value of 89ºC given in standard literature. The drug was observed to be slightly soluble in distilled water and soluble in other solvents under study. In Partition coefficient determination studiesvalues observed of Log P were 3.25 and 3.65 for n-octanol/ distilled water and n-octanol/ Phosphate buffer saline pH-7.4 systems respectively, showing lipophilic nature of the drug. The results were in compliance with value of Log P (3.06-3.53)given in standard literature.Determination of absorption maxima (ʎmax) was done by scanning approapriate dilution in Ultraviolet range from 200-800 nm and was found to be at 256nm. Using this as scanning wavelength calibration curve was prepared with value of correlation coefficient, R^2=0.9966. in drug-excipient interaction determination study, there was no significant deviation in value of absorbance from pure drug sample was found in case of values for drug-excipient mixtures.

Fig. 3: Chemical identification test for artemether

Table 1: Showing protocol for HPLC analysis and results (I.P.-2010)

Column characterstics	C18 (250 x 4.6 mm i.d. ; 5 µm particle size) maintained at 30 °C
Mobile phase	Acetonitrile : water in the ratio 62 : 38
Flow rate	1 ml /min
Run time	15 minutes
Sample	4 mg/ml
Injection volume	20 µL
Detection	216nm
Standard value	Retention time-5.34 minutes
Observed value	Retention time-5.20 minutes
% Purity	98.11

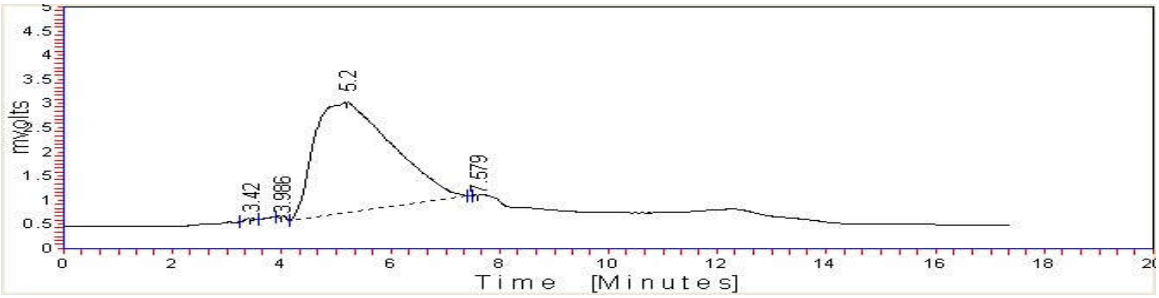
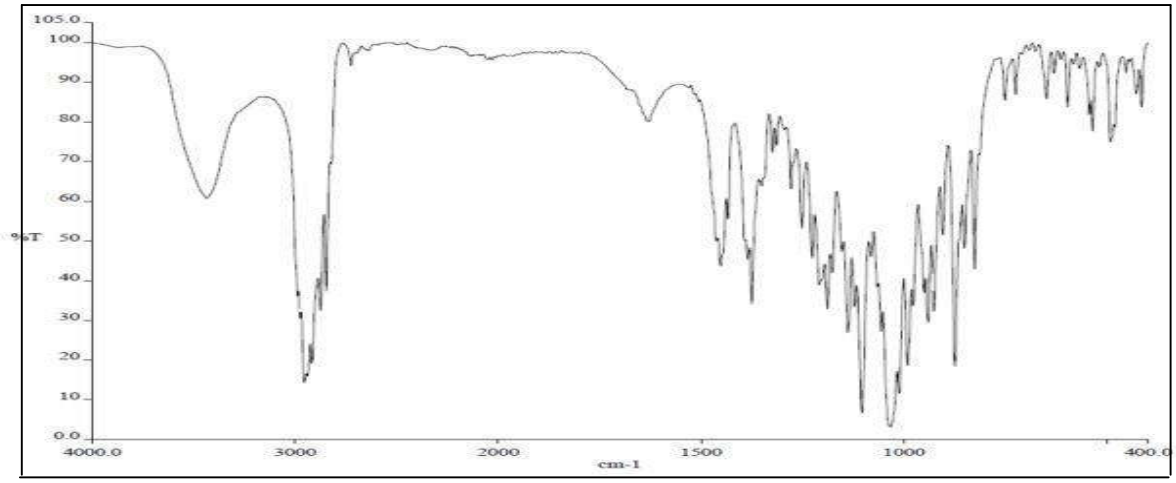


Fig. 4: Showing peak for artemether in HPLC chromatogram



Wavenumber (cm^{-1})

Fig. 5: FTIR Spectra of reference standard for Artemether[x]

Table 2: Characterstics peaks in FTIR spectra of Artemether

Peak position (cm^{-1})	Reason (stretching/ bending)	Groups involved
3462.34	O-H stretching	Alcohols and phenol
2956.97	C-H stretching	Alkanes
1433.51	C-H bending	Alkanes

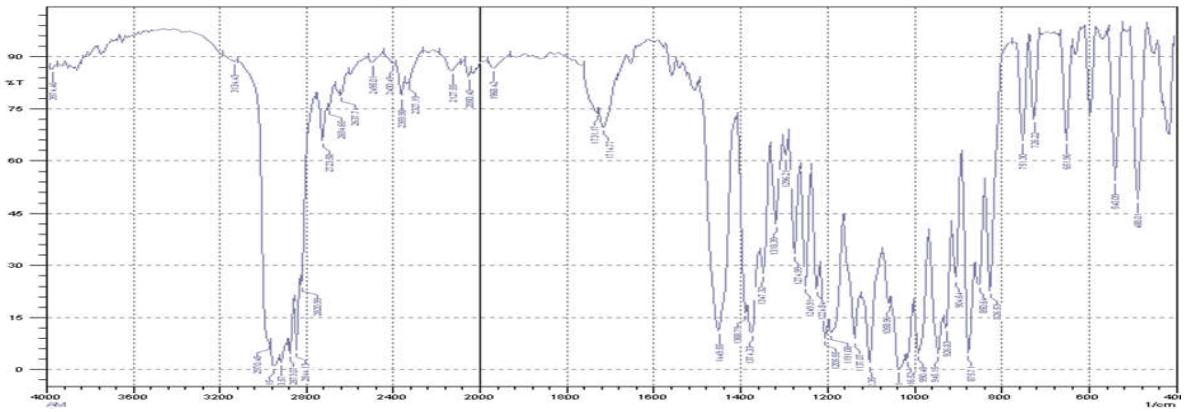


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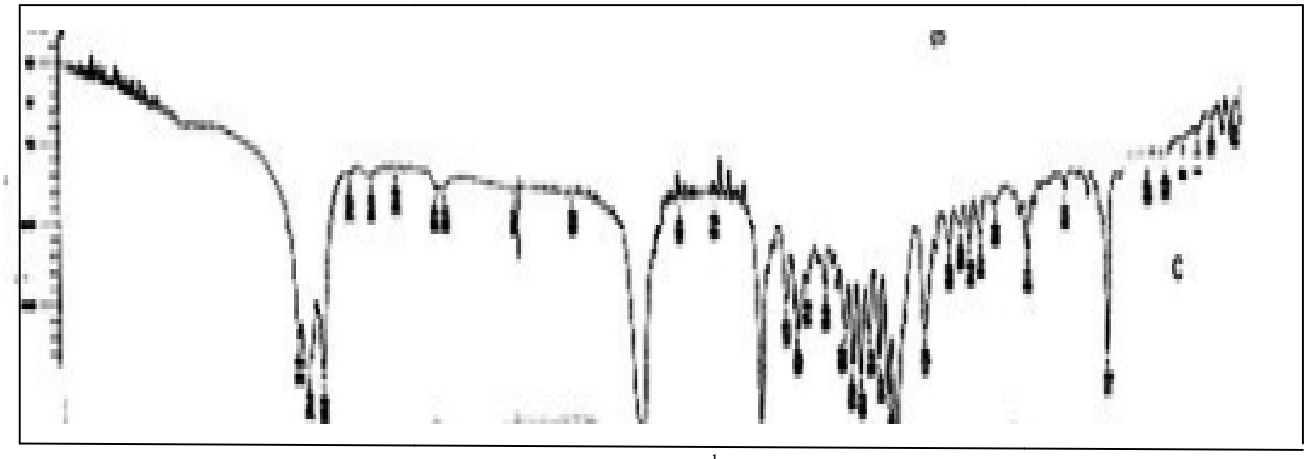
Fig. 6: FTIR Spectra of test sample of artemether



Wavenumber (cm^{-1})

Fig. 7: FTIR Spectra of reference standard of tristearin [xi]

Table 3: Characterstics peaks for Tristearin

Peak position (cm^{-1})	Reason (stretching/ bending)	Groups involved
1735	C=0 stretching	Ester
2854-2920	C-H stretching	Aliphatic groups

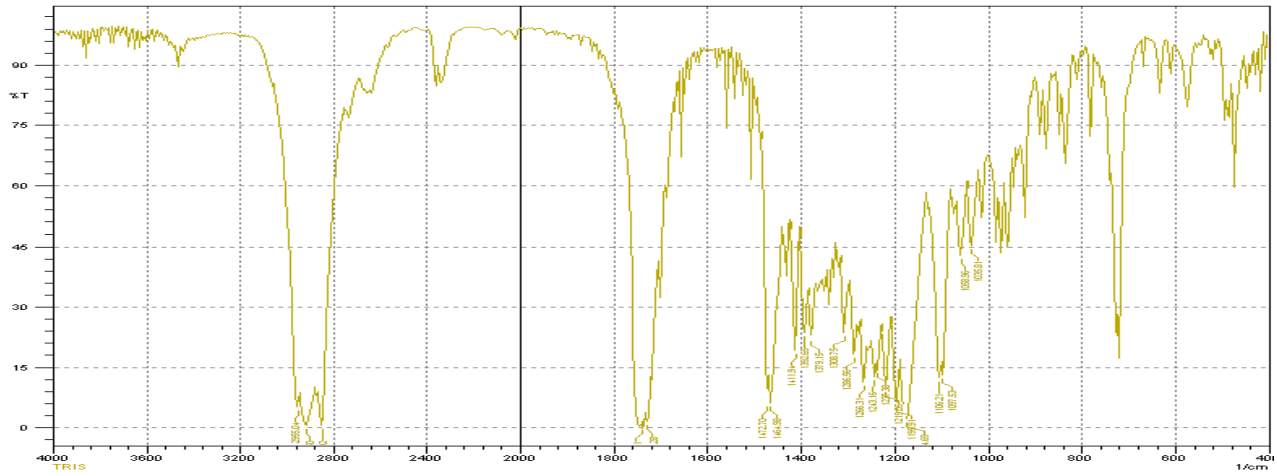


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Fig. 8: FTIR Spectra of sample of Tristearin

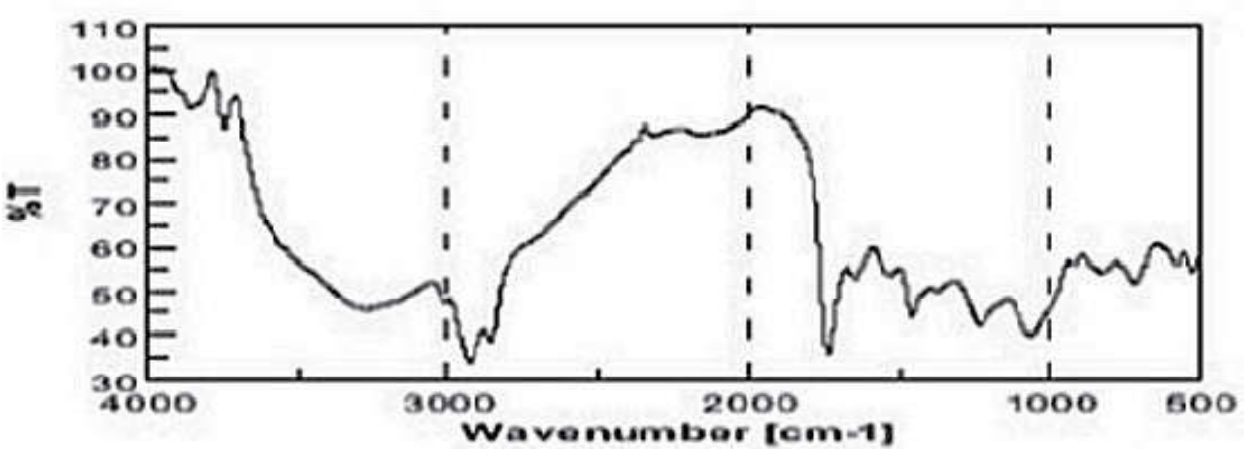
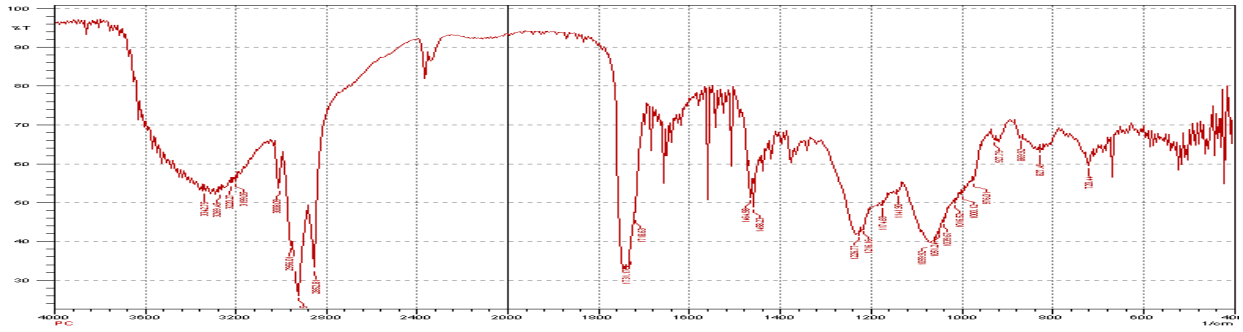


Fig. 9: FTIR spectra of Phosphatidyl Choline standard [xii]

Table 4: Characterstics peaks for Phosphatidylcholine

Peak position (cm^{-1})	Reason (stretching/ bending)	Groups involved
3710	N-H stretching	Amino
3400	O-H stretching	Hydroxyl group
2960-2860	C-H stretching	Aliphatic group



Wavenumber (cm^{-1})

Fig. 10: FTIR spectra of test sample of Phosphatidyl Choline

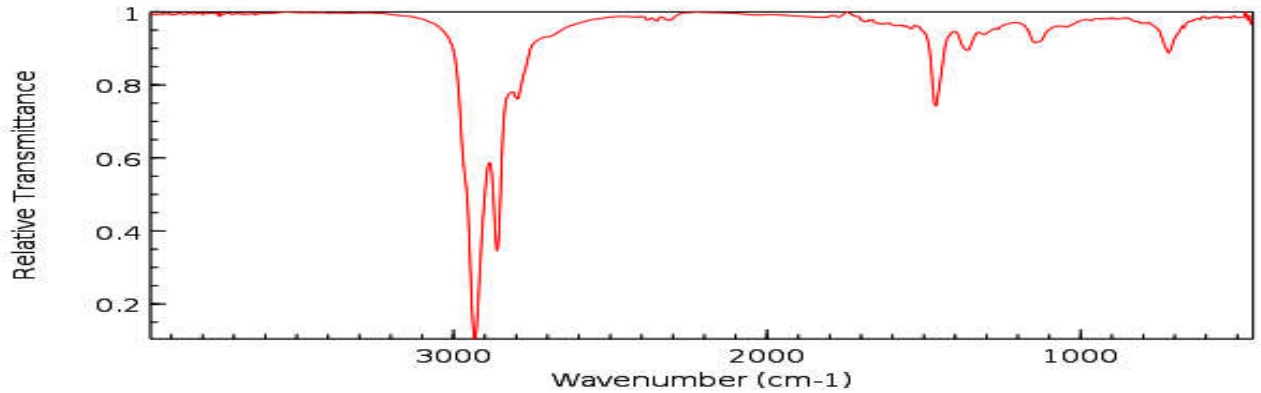
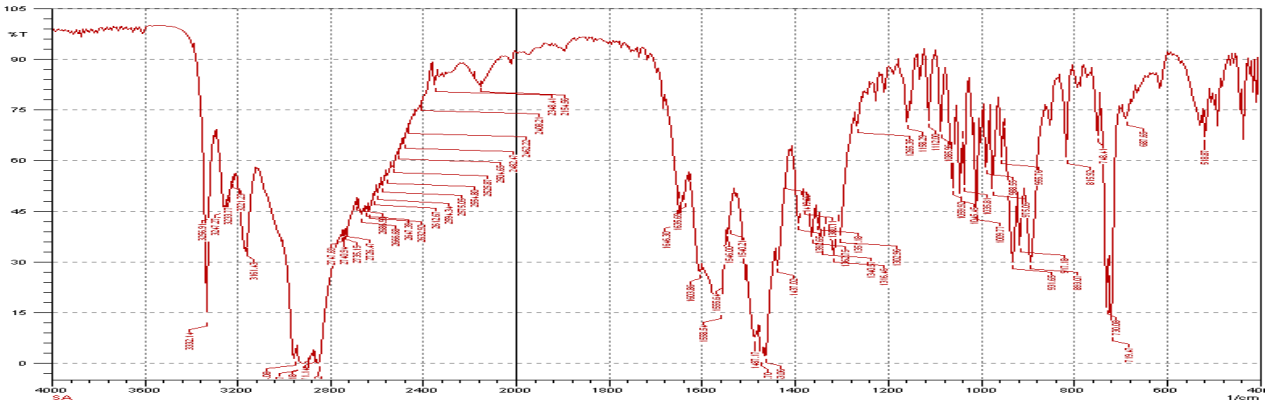


Fig. 11: FTIR Spectra of Stearylamine standard[xiii]

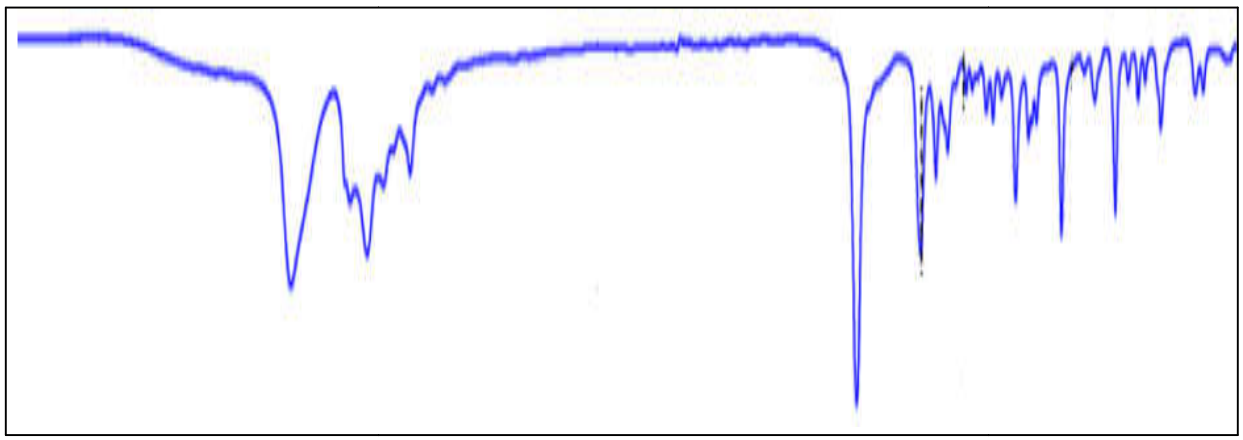
Table 5: Characterstics peaks for Stearylamine

Peak position (cm^{-1})	Reason (stretching/ bending)	Groups involved
2 peaks 2850-3000	C-H stretching	Aliphatic group



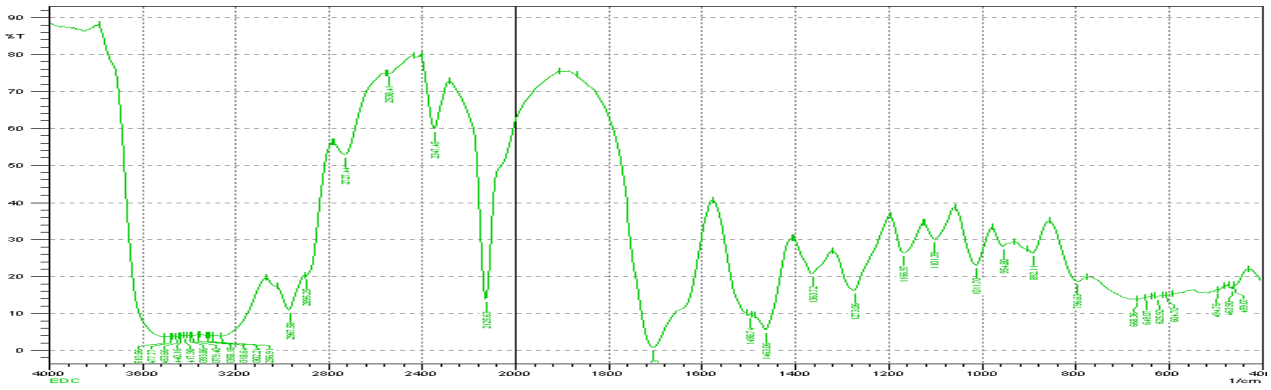
Wavenumber (cm^{-1})

Fig. 12: FTIR Spectra of test sample of stearyamine



Wavenumber (cm^{-1})

Fig. 13: FTIR Spectra of EDC. HCl standard[xiv]



Wavenumber (cm^{-1})

Fig. 14: FTIR Spectra of EDC. HCl test sample

Table 6: Calibration curve of artemether

S.No	Concentration (in µg/ml)	Absorbance
1.	5	0.0956
2.	10	0.1888
3.	15	0.2711
4.	20	0.3910
5.	25	0.4464
6.	30	0.5375
7.	35	0.6230

8.	40	0.6967
9.	45	0.7362
10.	50	0.8575

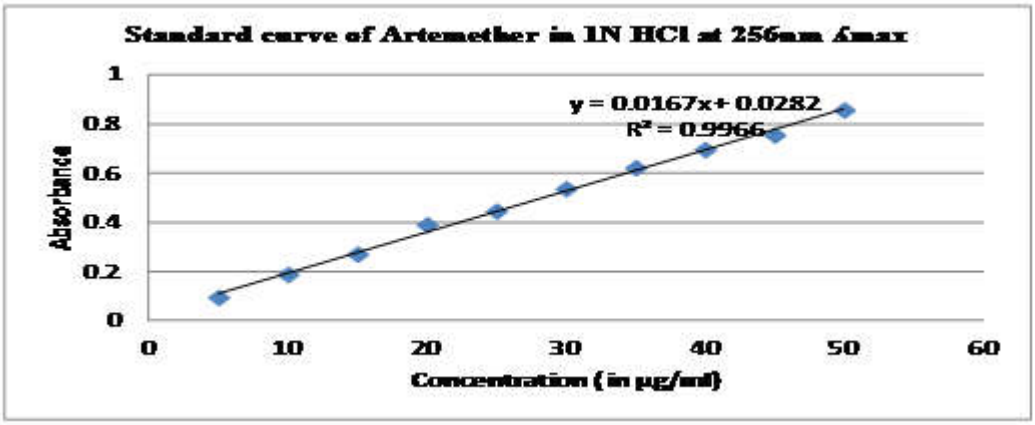


Fig.15: Showing calibration curve for artemether in 1N HCl at 256 nm

Optimization of SLN formulation was further done. It was observed that as the concenteration of tristearin increases particle size decreases.As the drug concenteration increases, % drug entrapment increases than becomes constant showing saturation of lipids with drug molecules. On increasing the surfactant concentration it was observed that average particle size decreased. But, due to leaching out of drug, % drug entrapment decreased.It was found that on increasing concenteration of stearylamine average particle size also increases. Stearylamine provides positive charge to the surface of SLNs which leads to their repulsion and hence, particles with higher average particle size are obtained. It was observed that by increasing stirring speed of laboratory homogenizer, particles with smaller average particle size were obtainedbut, at higher speeds bigger particles results with lower % drug entrapment. It was observed that at longer stirring times % drug entrapment decreases with lesser impact on average particle size.

Amide linkage was detected by Nessler's reagent test in which yellowish precipitate was observed which turned light brownish when kept for some time. This provided evidence for the presence of amide linkage. Peak for amide linkage in the UV spectrophotometric range of 210-220 nm was detected and further confirmation was done by determining the presence of peak for amide linkage in FTIR spectra of targeted preparation. Conjugation efficiency was determined UV spectrophotometrically to be 20.78% by preparing calibration curve of heparin and then determining the amount of heparin conjugated with solid lipid nanoparticles.

3.2.Characterization of Optimized SLN formulation:

The average particles size for optimized SLN formulation was found to be 241.5±0.06. The value of polydispersity index was found to be 0.179±1.26.The value of zeta potential was found to be -23.9 mV. Transmission Electron Microscope (TEM) images of solid lipid nanoparticles (optimized SLN formulation) showed that particles were spherical in shape and do not show considerable change in shape. SEM images of lyophilized sample of SLN formulation showed smooth surfaces with some disturbances in spherical structure of SLN due to heat treatment before SEM analysis. % drug entrapment was found to be 68.51±0.02. Drug loading capacity was determined to determine amount of drug per gram of Solid lipid nanoparticles. Drug loading capacity was found to be 11.17%.

The optimized Artemether loaded SLNs and Targeted Artemether loaded SLNs formulations were subjected to *in-vitro* drug release studies using Laboratory Magnetic stirrer at 37±0.5ºC for 48 hours. It was observed that the Artemether loaded SLNs showed 55.87±1.03% release in 48 hours at pH-7.4 and Targeted Artemether loaded SLNs showed 53.81±0.09% drug release in 48 hours at pH-7.4.This showed that the SLN formulations were found to be successful in providing sustained drug release which along with longer circulation time and site specific characterstics can increase the efficacy of anti-malarial therapy.

Hemolytic toxicity study of optimized SLN formulation was done and the SLN formulation was found to have lesser hemolytic toxicity as compared to Artemether drug solution.

Table 7: Optimized values of formulation variables and processing variables for Artemether loaded SLN formulation

Lecithin: Lipid	1:1
Drug : Lipid	10 : 100
Drug : Stearylamine	100 : 1.5
Surfactant concentration (% v/v)	1.%
Stirring speed (in rpm)	3000
Stirring time (in min)	40
Sonication time (in min)	3

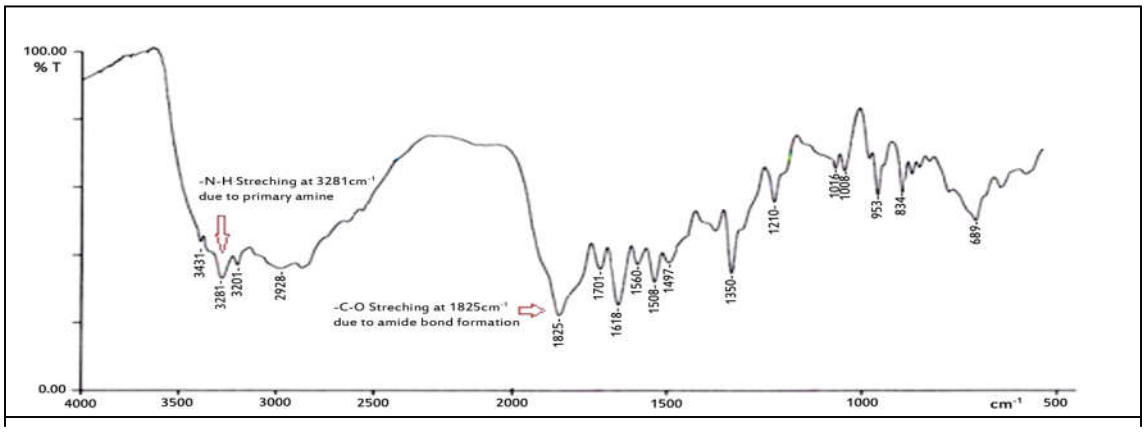


Fig. 16: Showing standard spectra showing peak for amide linkage [xv]

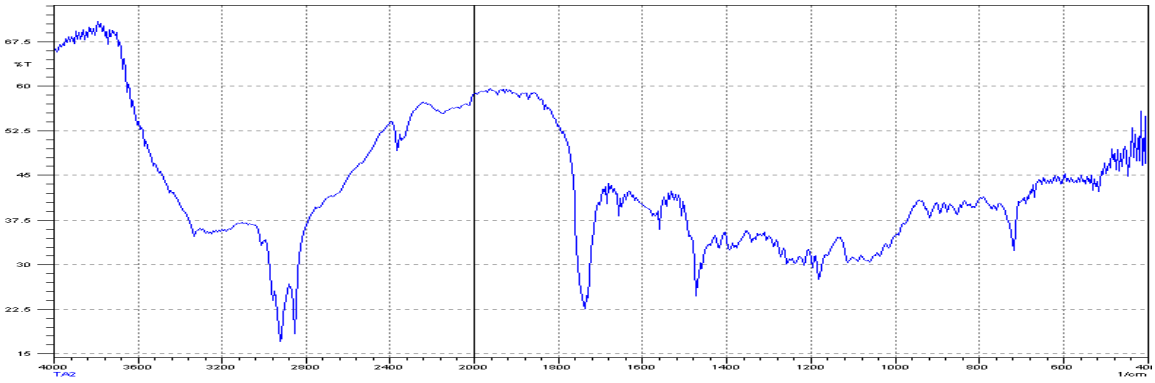


Fig. 17: Showing FTIR spectra of Heparin conjugated Solid lipid nanoparticles

Table 8: Calibration curve of heparin Sodium in 0.1N HCl at 232λmax by degrading heparin at 60ºC

S.No.	Concentration (in µg/ml)	Absorbance
1.	5	0.1318
2.	10	0.2557
3.	15	0.3671
4.	20	0.4757
5.	25	0.5513
6.	30	0.6649
7.	35	0.7401
8.	40	0.8400
9.	45	0.9091
10.	50	0.9990

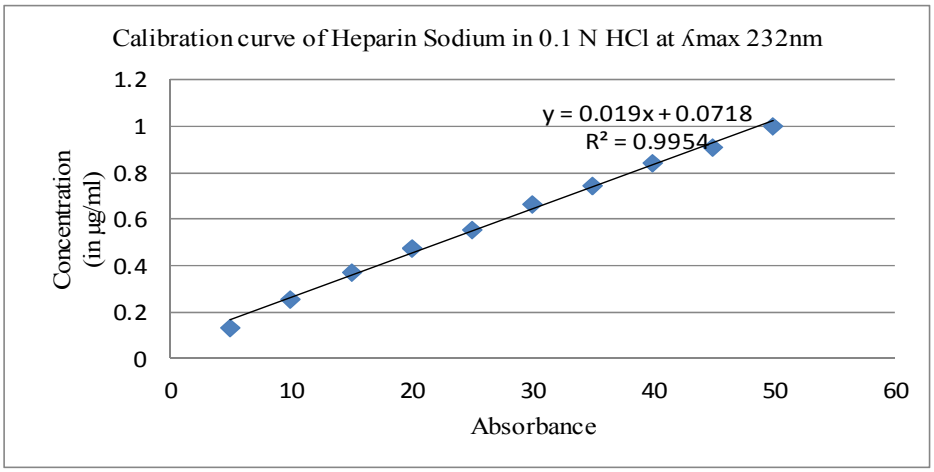


Fig. 18: Showing Calibration curve of Heparin Sodium in 0.1 N HCl at 232 nm λmax

Table 9: Average particle size, PDI, Zeta potential values for optimized SLN formulation

Formulation	Targeted Artemether Loaded SLN formulation
Average particle size (nm)	241.5±0.79
PDI	0.179±0.06
Zeta potential (mV)	-23.9

Fig. 19: Transmission Electron Microscope photomicrographs of Solid lipid nanoparticles

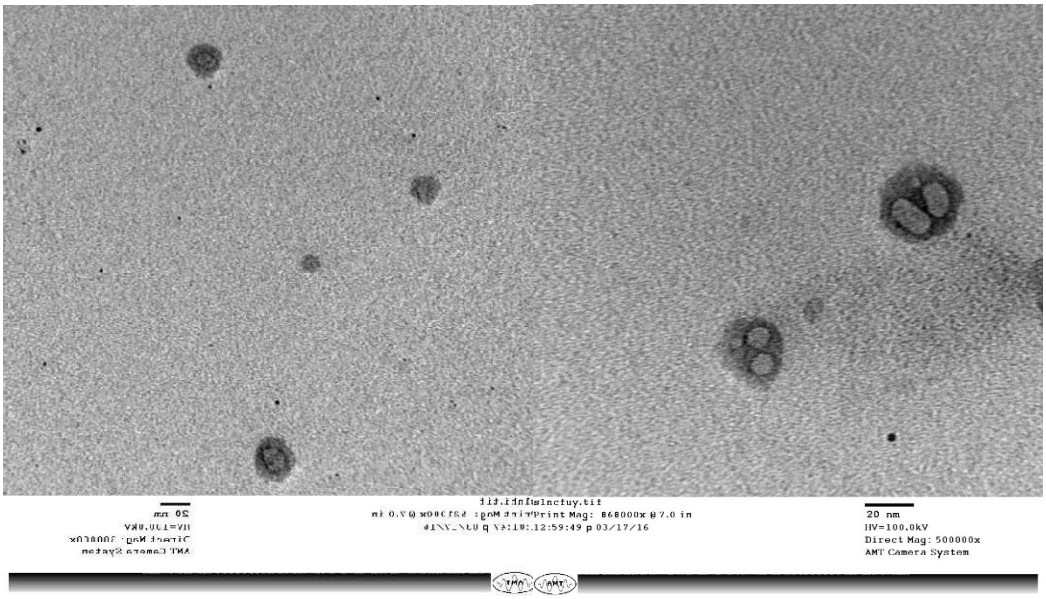


Fig. 20: Scanning Electron Microscopy images of Solid lipid nanoparticles

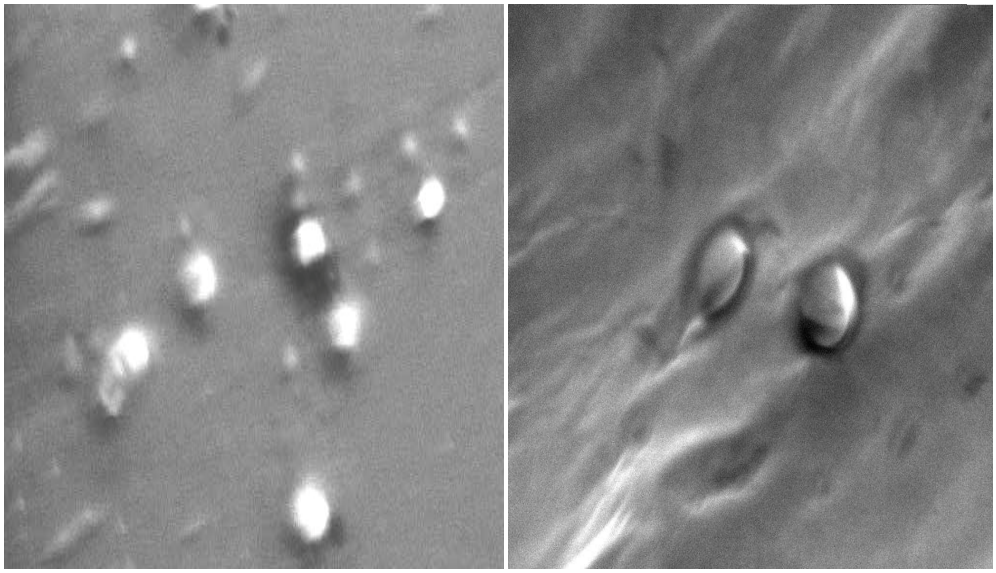


Table 10: Percentage Drug Release Profile from Artemether loaded solid lipid nanoparticles in Phosphate Buffer pH 7.4

S.No.	Time (in hours)	% Drug release
1.	1	11.76±1.32
2.	3	16.24±2.11
3.	5	17.43±0.27
4.	7	51.86±0.03
5.	24	54.92±1.19
6.	48	55.87±1.03

S.D. ± Mean, n=3

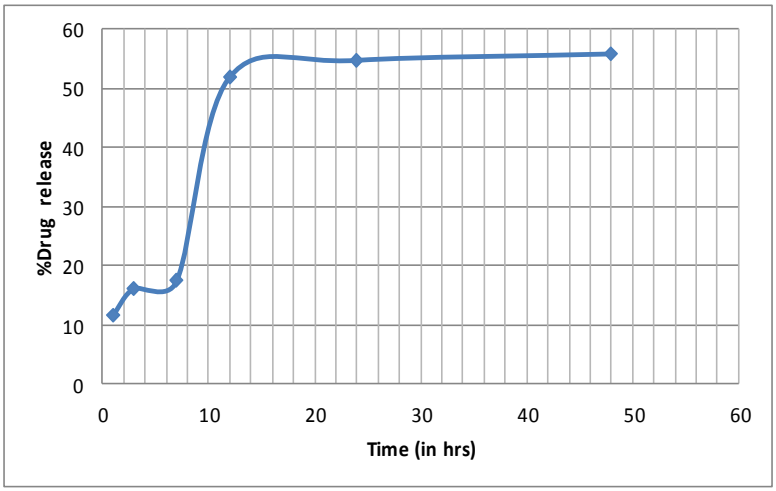


Fig. 21: Percentage in-vitro drug release profile from Artemether loaded SLNs in phosphate buffer pH 7.4.

Table 11: Percentage drug release profile from Targeted Artemether loaded SLNs in PBS pH-7.4

S.No.	Time (in hours)	% Drug release
1.	1	9.17±0.33
2.	3	13.69±1.29
3.	5	18.96±0.04
4.	7	41.08±0.89
5.	24	47.50±1.17
6.	48	53.81±0.91

S.D. ± Mean, n=3

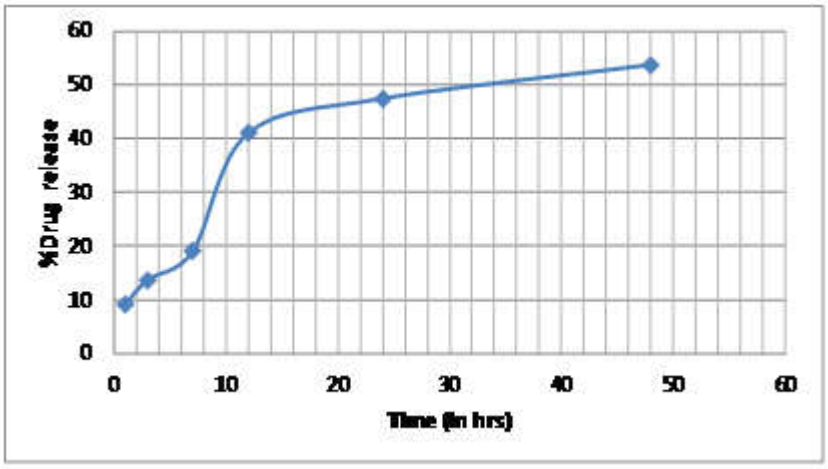


Fig. 22: Percentage in-vitro drug release profile from Targeted Solid Lipid Nanoparticles loaded with Artemether

Drug Loading Capacity: It helps in dealing with solid lipid nanoparticles after their separation from the medium and to know the drug content. It is calculated using the formula:

% Drug loading capacity= entrapped drug/nanoparticles weight×100

Entrapped drug= 6.85mg

Total weight of solid lipid nanoparticles=61.271mg

6.85/61.271.271×100=11.17%

I.e. 10mg of solid lipid nanoparticles contain 1.17 mg of artemether.

Table 12: Showing values of % Hemolysis for artemether solution and SLN-preparation

S.No.	Sample	% Hemolysis
1.	Artemether solution	13.01±1.25

2.	Targeted Artemether Loaded SLN-preparation	12.18±0.76

3.3.Stability studies:

The optimized formulation was stored at 27˚ ± 2˚C & 4˚± 2˚. The changes in % drug content of formulations was observed at different time intervals for 45 days considering initial % drug content as 100%.. The % drug content decreased to 78.08% at 27˚ ± 2˚C which was a significant reduction in % drug content. The % drug content decreased to 89.61% at 4˚± 2˚C which was not a significant decrease in drug content indicating a more stable formulation at 4˚± 2˚C. The formulation was further evaluated for change in particle size at different time intervals for 45 days. The formulation kept at 27˚± 2˚C showed greaterincrease in particle size from 241.5±0.51 to 319.6±0.11. the formulation kept at 4˚ ± 2˚C showed lesser increase in size from initial value of 241.5±0.51 to 281.1±0.94 Thus, it can be concluded that Artemether loaded targeted solid lipid nanoparticles were more stable at storage condition 4˚ ± 2˚C considering % residual drug content and particle size.

Table 13: Effect of storage temperature on % Drug content at 27˚ ± 2˚C & 4˚ ± 2˚C

S.No.	Storage Conditions	% Drug content Initial	% Drug content After 10 days	% Drug content After 20 days	% Drug content After 30 days	% Drug contents After 45 days
1.	27˚± 2˚ C	100	96.26	95.09	82.36	78.08
2.	4˚± 2˚ C	100	99.57	95.83	92.04	89.61

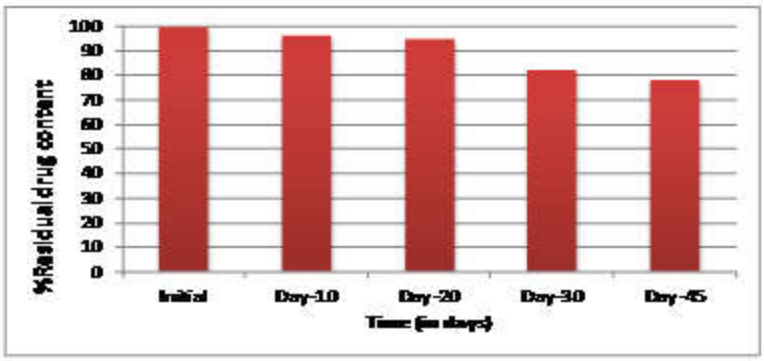


Fig. 23: Showing effect of storage temperature on % drug content at 27˚± 2˚ C

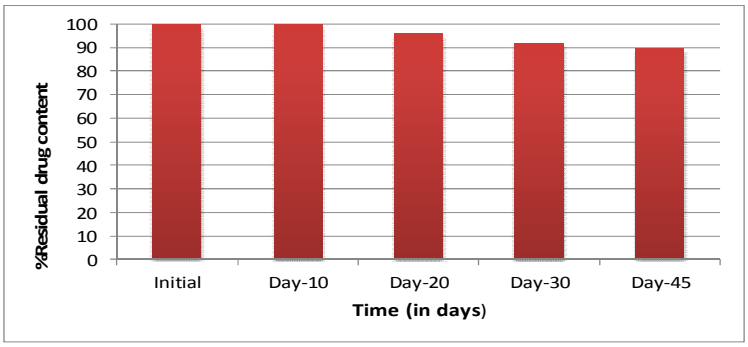
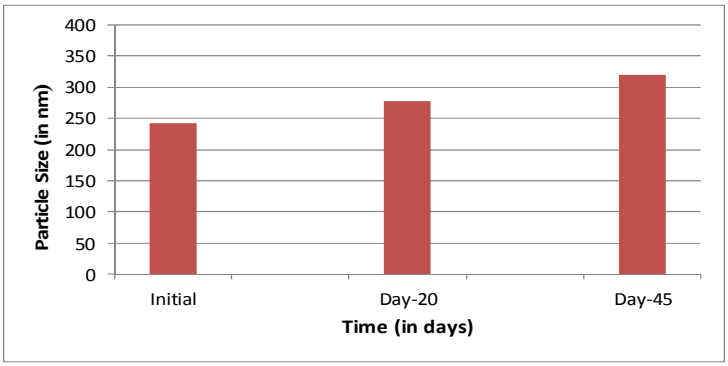


Fig. 24: Showing effect of storage temperature on % drug content at 4˚± 2˚ C

Table 14: Effect of storage temperature on particle size at 27˚ ± 2˚C & 4˚ ± 2˚C

S.No.	Storage conditions	Initial Particle size (nm)	Particle size on day 20 (nm)	Particle size on day 45 (nm)
1.	27˚ ± 2˚C	241.5±0.51	277.7±0.88	319.6±0.11
2.	4˚ ± 2˚C	241.5±0.51	265.8±1.40	281.1±0.94



Fig, 25: Showing effect of storage temperature on particle size at 27˚± 2˚ C

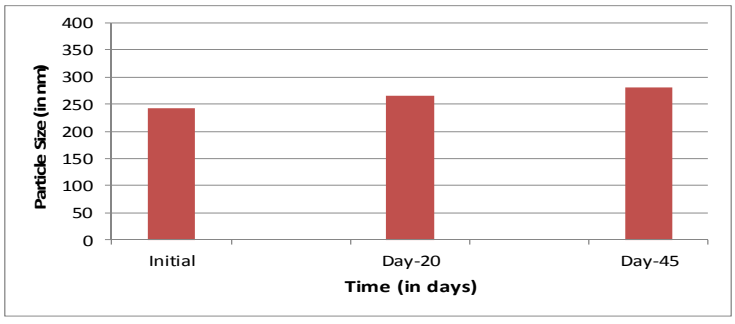


Fig. 26: Showing effect of storage temperature on particle size at 4˚± 2˚ C

4. CONCLUSION:

The feasibility of modified hot melt method in the preparation of Artemether loaded Solid Lipid Nanoparticles and conjugation of heparin with the surface of SLNs was successfully established. Artemether loaded SLNs offer significant improvement in relation to toxicity and controlled release characterstics are concerned as compared to conventional injectable. There was no sign of behavioural change noticed throughout the whole study, confirming that the optimized formulation is safe and stable. Further in-vivo studies need to be carried out to demonstrate the complete efficacy of hypothesis given here.

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CONFLICT OF INTERESTREPORTED: NIL; SOURCE OF FUNDING: NONEREPORTED