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DEVELOPMENT OF NANOSTRUCTURED LIQUID CRYSTALLINE FORMULATION OF ANTIMALARIAL AGENTS ARTEMETHER AND LUMEFANTRINE

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ABSTRACT

The main objective of the research work was to formulate a lipid based delivery system that may be useful for increasing the bioavailability of BCS class II drugs. Such type of lipid based drug carriers may keep the drug in dissolved state until the drug is completely absorbed. The present work mainly focused to formulate liquid crystalline nanoparticles in combination to improve the solubility of both ARTM and LMF which could probably improve absorption of ARTM and LMF drugs and may circumvent the drawback of poor bioavailability. In-vitro dissolution study for OF1 (F9) was carried out and it had been found that 98.00% Artemether and 89.00% Lumefantrine released within 72 hrs. OF1 was further evaluated for various parameter including organoleptic evaluation and physical stability and all are found within the acceptable ranges over a period of 3 months.

KEYWORD: Bioavailability, Artemether, Lumefantrine, LiquidCrystals, Nanoparticles, Organoleptic etc.

INDRODUCTION

Malaria is most prevalent health problem in various countries, where transmission occurs frequently and even areas, where transmission has been controlled or eliminated. Near of 300 to 500 million cases of malaria are reported annually, therefore it becomes one of the most common infectious disease in world. According to WHO more than 90% of the 1.5 to 2 million deaths accounted for malaria each year in African children, about 1.5 million cases of malaria are reported annually in India out of which 40-50% are associated with Plasmodium

falciparum infestation.[1]

1.1.1Causative agents

There are 5 types of Plasmodium species which cause acute infectious disease Malaria.Plasmodium falciparum,Plasmodium vivax,Plasmodium ovale,Plasmodium malariae,Plasmodium knowlesi. The vast majority of deaths are caused by P. falciparum and P.vivax, whereas P.malariae cause a milder form of malaria that is rarely fatal.[2] The zoonotic species P.knowlesi prevalent in Southeast Asia causes malaria in macaques but can also cause severe infections in humans.[3] Cerebral malaria is defined as a severe P.

falciparum-malaria presenting with neurological symptoms, including coma (with a Glass Gow coma scale less than 11, or a Blantyre coma scale greater than 3), or with a coma that lasts longer than 30 minutes after a seizure.[4]

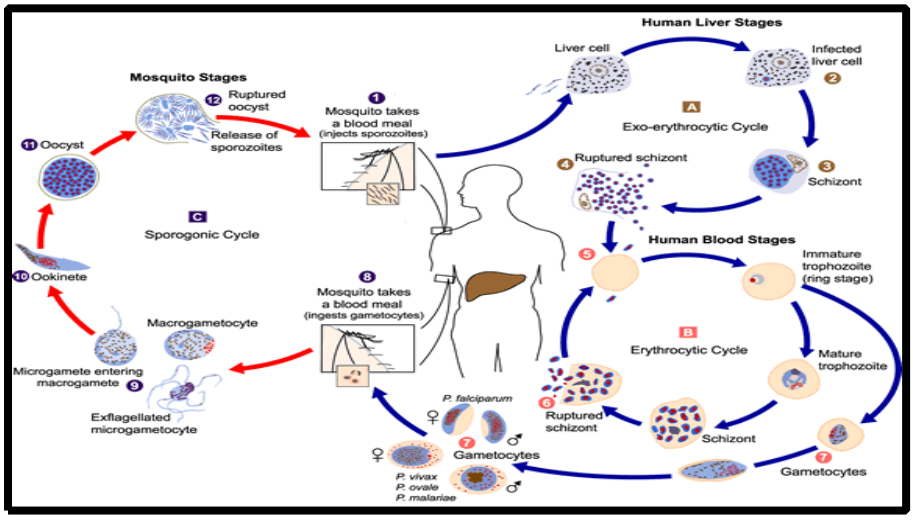


Fig 1.1: A diagram showing the life cycle of the malaria parasite, detailing the A. Exo-erythrocytic cycle, B. Erythrocytic cycle and C. Sporogonic cycle. (Source:http://dpd.cdc.gov/dpdx/html/Malaria.htm)

MATERIAL AND METHODS

1.1.2 Preformulation study

1.1.3 Melting point determination

Melting point of Artemether and Lumefantrine was determined by capillary rise method using digital melting point apparatus. Practically determined melting points were compared with the literature values.[12]

1.1.4 Solubility studies

1.1.5 Solubility study in water

Solubility of Artemether and Lumefantrine was determined in distilled water. The practical values of the parameters were compared with values given in literature.

1.2.1 Solubility study in different solvents

Solubility of Artemether and Lumefantrine was determined in different solvents like methanol, ethanol, ethyl acetate, chloroform and distilled water. The initial solubility of ART and LMF was determined by weighing out 10 mg (or other suitable amount) of ART and LMF. To this add 10 µl of solvent of interest. If the compound doesn't dissolved, a further 40 µl of solvent was added and its effect noted. Successive amount of the solvent was then added until the compounds were dissolved. This method give an approximate value of solubility.[5]

1.2.2 Partition coefficient[6]

Partition coefficient of Artemether and Lumefantrine was determined by shake flask method. Two separate conical flasks of 50 ml were taken, into which 10 ml of water and 10 ml of n-octanol taken separately and allowed to shake over wrist shaker for 24 hrs at 37oC to achieve pre-saturation of both phases, allow the phases to separate. In the each flask add 10 mg of Artemether and Lumefantrine and allowed to shake the flask over the wrist shaker for 24 hrs. The solutions were allowed to stand at room temperature for 30 min. Two phases of octanol and water samples were separated by separating funnel. Aqueous phase containing Artemether and Lumefantrine were filtered using Whattmann's filter paper grade number 41. The filtered aliquots were analyzed spectrophotometrically at 256 nm for Artemether and 342 nm for Lumefantrine.

1.2.3 Microscopic examination

1.2.4 Ultra-violet spectrophotometric analysis

1.2.5 Determination of λmax of Artemether

λmax of Artemether is determined in methanol, water, at buffer pH 6.8 and at pH 1.2 in the given sample of drug.

1.3.1 Determination of λmax of Lumefantrine.

10 mg of Lumefantrine accurately weighed and transferred to 100 ml volumetric flask. It was dissolved in 20 ml of methanol then sonicated for 15 min, and the volume was made up with methanol. The solution was scanned between 200-400 nm and obtained results were

compared with the reference values.[7]

1.3.2 Formulation and development

Liquid crystalline nanoparticles (cubosomes or hexosomes) containing Artemether and Lumefantrine were formulated by Hydrotropic dilution method. In this method ethanolic solution of GMO with drug and aqueous solution of poloxamer 407 were prepared by vortexing. Ethanol used to dissolve monoolein, oleic acid, Artemether, Lumefantrine and aqueous phase used to dissolve poloxamer 407. Water phase containing the poloxamer (10% w/v) added to the ethanolic phase drop wise with continuously vortexing resulting in the precipitation of the GMO. A milky suspension is formed which indicate the formation of liquid crystalline as described in Fig. 4.1.35

1.3.3 MATERIALS AND METHODS

Table 1.1: List of drugs and excipients

S. No.	Drugs and excipients
1	Artemether
2	Lumefantrine
3	Glyceryl monooleate
4	Poloxamer 407
5	Oleic Acid

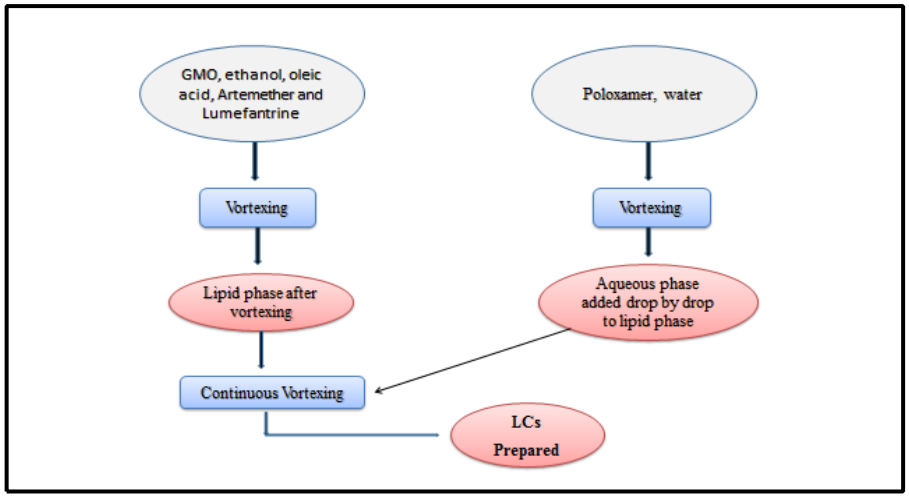


Fig1.2: Formulation chart for LCs of Artemether & Lumefantrine

1.3.4 Characterization of Artemether & Lumefantrine liquid crystalline dispersion

1.3.5 Particle size analysis

The mean particle size and polydispersity index was measured using laser diffraction on a Malvern Zetasizer Ver. 6.01r (Serial Number: MAL1027952, Malvern instruments Ltd.) at

2oC considering a viscosity of pure water 0.8872. The particle size was analysed by diluting the prepared formulations with distilled water.[8,9]

1.4.1 Entrapment efficiency[8,9]

Entrapment efficiency of Artemether and Lumefantrine was determined using the Nanosep device. The liquid crystalline dispersion of Artemether and Lumefantrine were centrifuged in cooling centrifuge (Remi India) using Nanosep device (MWCO: 2-3 KD, Pall Life Science; India). 0.5 ml of prepared LCN formulation was taken in Nanosep device and then placed in cooling centrifuge (Remi; India). The sample is then centrifuged at 13000 rpm for 30 min at 10ºC. Then aqueous phase was collected, and analysed for Artemether and Lumefantrine at 256 nm and 342 nm respectively by UV spectroscopy. The preliminary trials were performed for the optimization of centrifugation speed and time. Time and speed 13000 rpm for 30 min respectively were optimized to separate unetrapped drugs from the LCN .The encapsulation efficiency (EE) was determined using the following equation:

1.4.2 Evaluation of optimized formulation

The optimized formulation was identified based on constraints using design expert software (version 9.1.0, state ease Inc., Minneapolis, MN). The optimized formulation was formulated according to method given in 5.3.2 and evaluated for particle size, entrapment efficiency, in-vitro release, physical stability and chemical stability.

1.4.3 Physiochemical characterisation

1.4.4 Organoleptic evaluation

Drug studied on creaming, Discoloration.

b. Creaming

Optimised formulation “OF1” was analysed for creaming. Creaming involve the separation ofdispersed phase form the liquid crystalline dispersion on storage under normal condition at room temperature. The dispersion type OF1 of LCN was oil in water. The formed dispersion was visually assessed for creaming during the storage period at 25oC/60% RH on weekly basis up to three months.[10]

1.4.5 In-vitro release study[11]

In-vitro drug release study of Artemether and Lumefantrine was performed in simulated gastric fluid pH 1.2 containing 1% w/v BKC and phosphate buffer of pH 6.8 containing 0.5%

w/v SLS by using dialysis bag method, dialysis membrane having pore size 2.4 nm and a molecular weight cut off 12000-14000 Dalton (HiMedia, India) were used. The dialysis bag retains the nanoparticles and releases the free drug into the dissolution media. The dialysis membrane was pre-treated with sodium bicarbonate and EDTA solution and kept in diluted EDTA solution prior to use. The bag was washed with distilled water prior to use. 2 ml formulation of Artemether and Lumefantrine was placed in dialysis bag. Separate dialysis bags containing the formulation 2 ml in each were immersed in 200 ml simulated gastric fluid for Lumefantrine and intestinal fluid (Artemether) maintained at 37±0.5oC and stirred at 100 rpm.

$$\% \text{ Release of ARTM or LMF} = \frac{\text{Mass of ARTM or LMF in releasing media}}{\text{Ttotal Mass of ARTM or LMF}} * 100$$

1.5.1 Melting point

Table 1.2: Melting point of Artemether and Lumefantrine

Parameter	Drug	Observed	Reference
Melting point	Artemether	85±2°C	86-88°C
	Lumefantrine	130±2°C	128-132°C

1.5.2 Solubility studies

Solubility of ARTM and LMF was determined in different solvents including distilled water, methanol, ethanol, ethyl acetate and chloroform..

Table 1.3: Solubility of Artemether in different solvents

Solvent	Observed (mg/ml)	Reference (mg/ml)
Ethanol	14	16
DMSO	9	10
DMF	17	20
Distilled water	1.5	2

Table 1.4: Solubility of Lumefantrine in different solvents

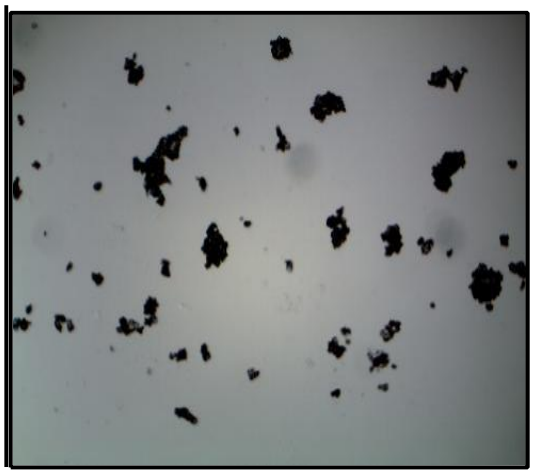
Solvent	Observed (mg/ml)	Reference (mg/ml)
Ethanol	2.2	2.8
DMSO	98	100
DMF	97	100
Oleic acid	156	158
Distilled water	0.05	0.1

1.5.3 Partition coefficient

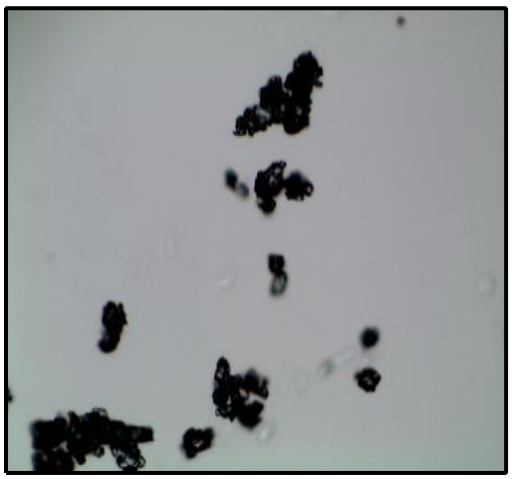
Partition coefficient of Artemether and Lumefantrine was determined by Shake flask method, it was found that the values of partition coefficients of ARTM and LMF were complied with the reference values represented in Table 1.5. Obtained result indicated that these drugs are highly lipophilic in nature and may possess good permeability across the cellular membrane.

Table 1.5: Partition coefficient

Parameter	Drug	Observed	Reference
log $P_{O/W}$	Artemether	3.26	3.06-3.53
log $P_{O/W}$	Lumefantrine	2.9	2.29-3.52

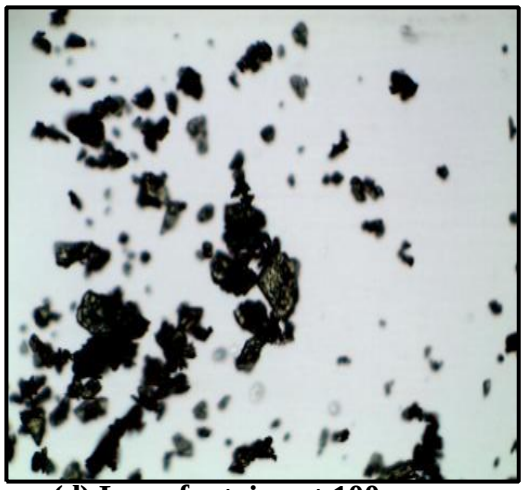


(a) Artemether at 40x



b) Artemether at 100x

(c) Lumefantrine at 40x



(d) Lumefantrine at 100x

Fig. 1.3: microscopicexamination of Artemether and Lumefantrine

1.5.4 Infra red analysis of drugs

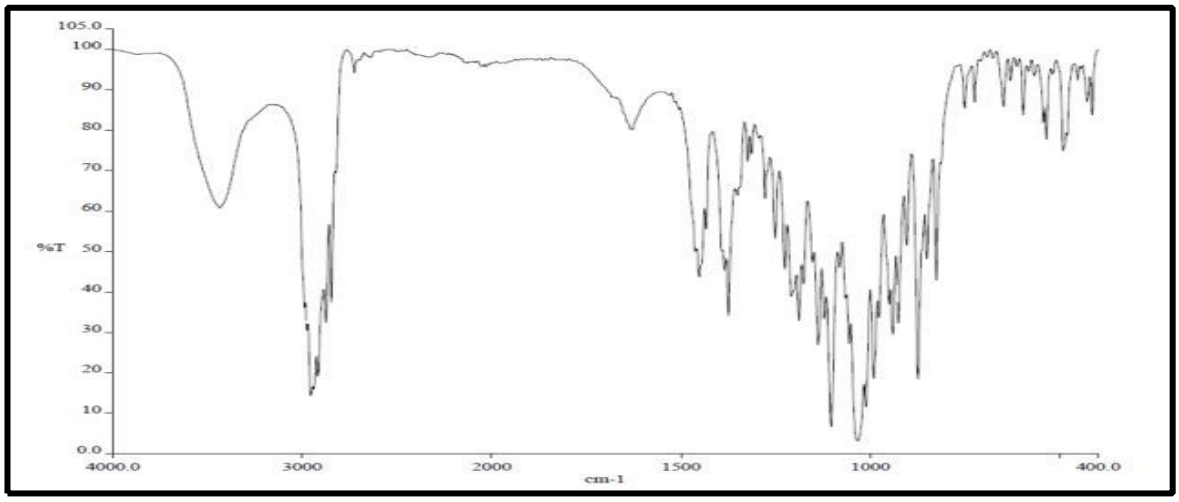


Fig. 1.4: Reference FTIR spectrum of Artemether

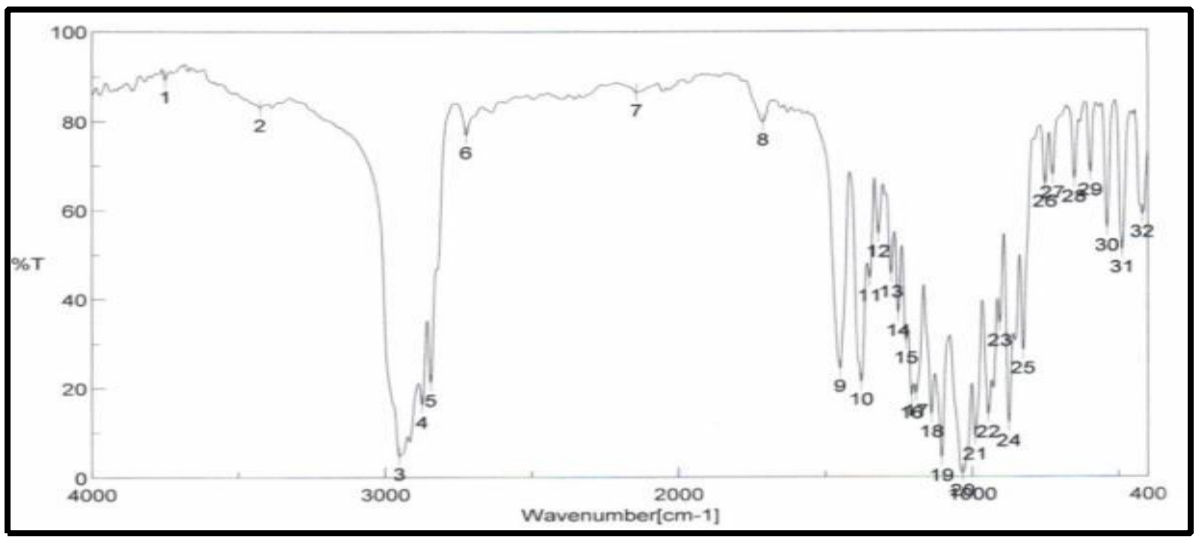


Fig. 1.5: FTIR spectrum of test sample of Artemether

sample spectra of Artemether respectively. On FTIR analysis, obtained spectra (Fig. 1.5) of test samples of drug matched with the reference spectra given in USP 2009. The peaks obtained in FTIR spectra of test sample were examined and found in accordance with the functional groups present in reference spectra of Artemether. From this study it was confirmed that procured drug sample was authentic.

Lumefantrine

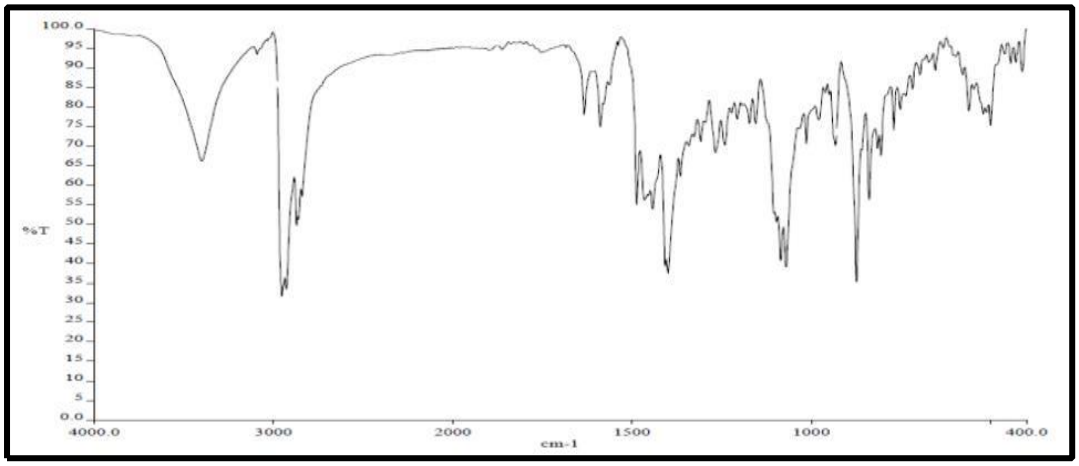


Fig. 1.6 Reference FTIR spectrum of Lumefantrine

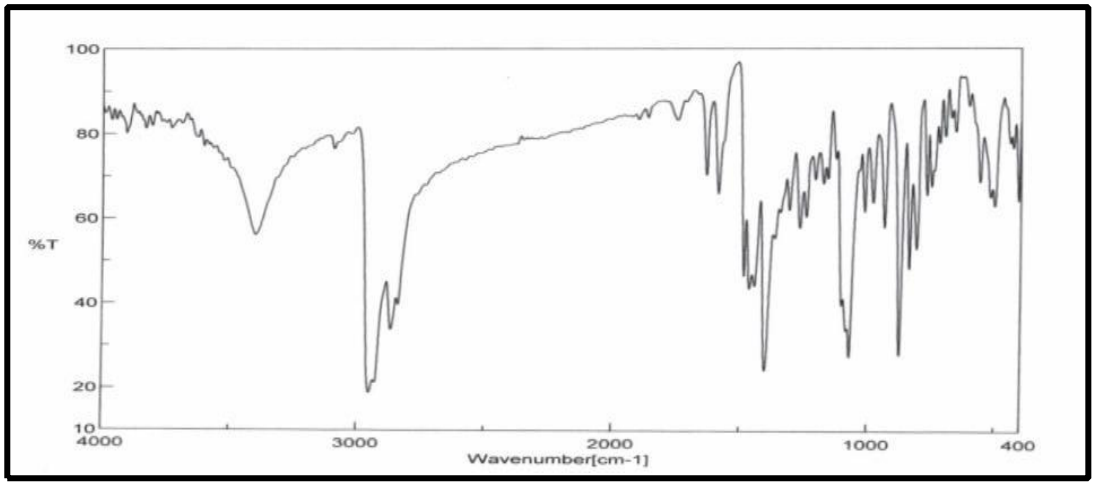


Fig. 1.7: FTIR spectra of test sample of Lumefantrine

1.5.5 Determination of λ_{max} of Artemether and Lumefantrine

- **Determination of λ_{max} of Artemether In buffer pH 1.2**

λ_{max} of Artemether in buffer pH 1.2 after derivatizing with 1N HCl was found 256 nm.

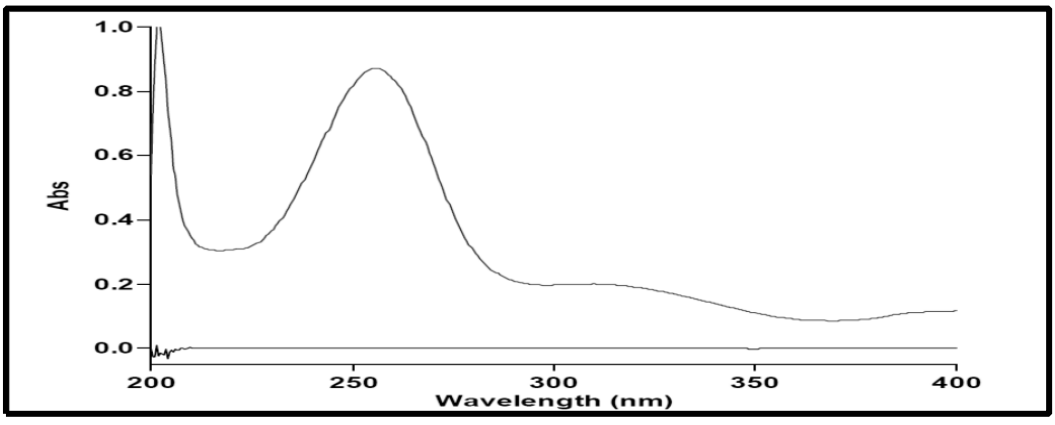


Fig. 1.8: Absorption maxima of Artemether in buffer pH 1.2

λ_{max} of Artemether in buffer after treating with 1N HCl was found 256

In phosphate buffer pH 6.8

λ_{max} of Artemether in phosphate buffer pH 6.8 was found 256 nm

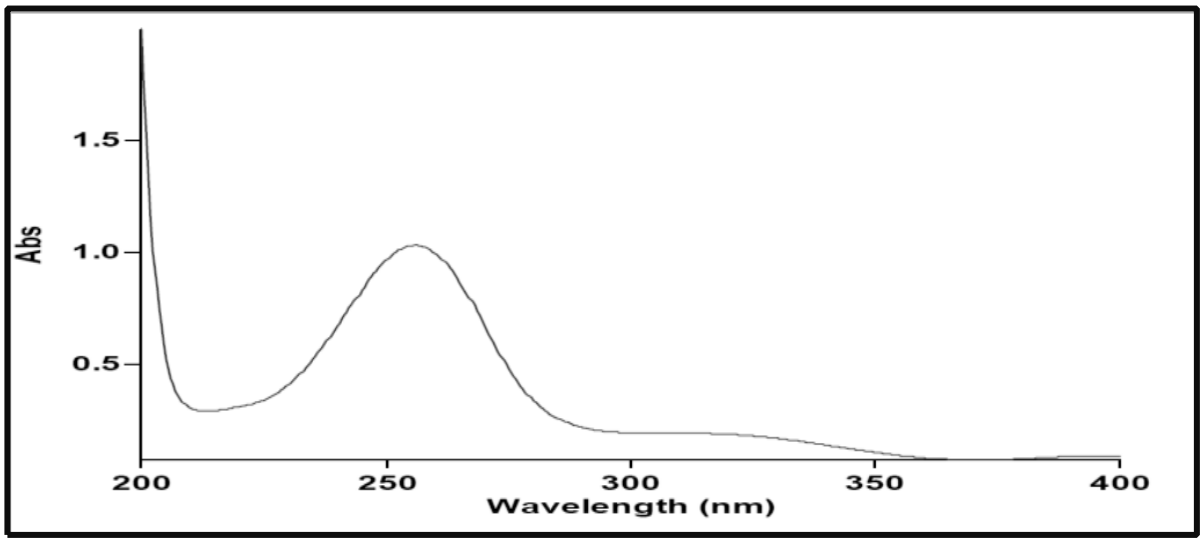
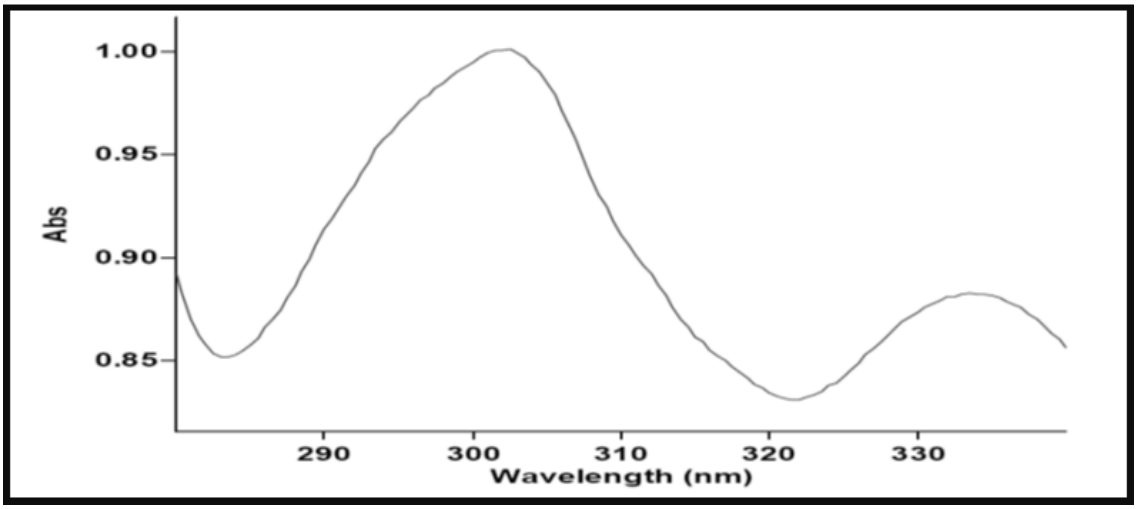


Fig. 1.9: Absorption maxima of Artemether in phosphate buffer pH 6.8

λ_{max} of Artemether in phosphate buffer pH 6.8 after treating with 1N HCl was found 256 nm and which compiled with the literature value 256 nm .

- **Determination of λ_{max} of Lumefantrine**



Fi.g. 1.10 Absorption maxima of Lumefantrine in 0.1 N HCl

λmax of Lumefantrine in 0.1N HCl was found 342 nm and obtained UV spectra of scanned sample of pure drug was depicted in Fig. 1.10

- **In buffer pH 1.2**

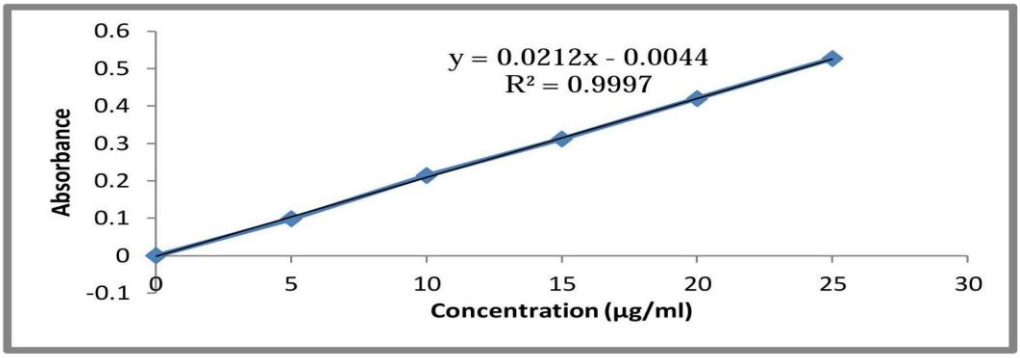


Fig. 1.11: Calibration curve of Artemether in buffer pH 1.2

Fig. 6.12 representing the calibration curve of LMF. The calibration equation for straight line was observed to be Y= 0.021X-0.0044 correlation coefficient (R^2) of 0.9997 which was used to calcu late concentration of samp les for dissolution study and other analytical purposes.

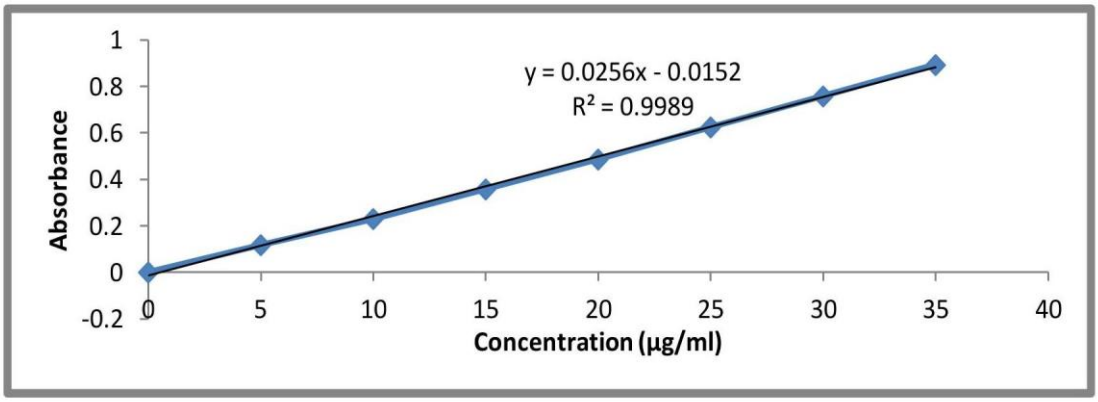


Fig. 1.12: Calibration curve of Artemether in phosphate buffer pH 6.8

In phosphate buffer pH 6.8

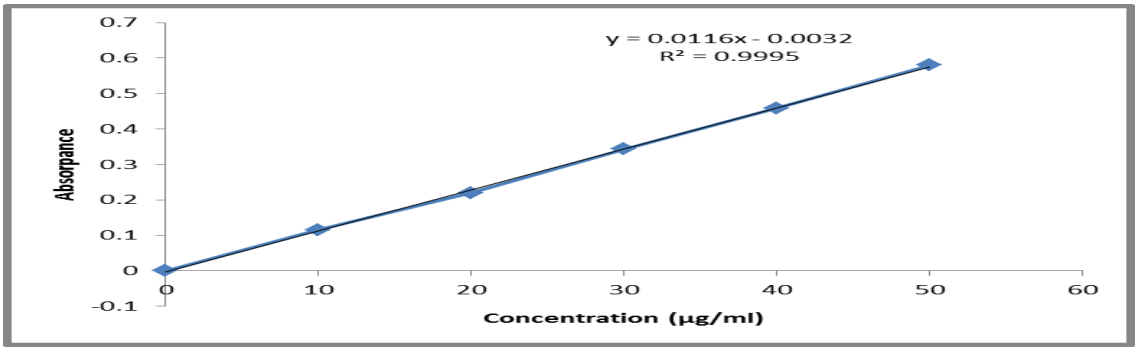


Fig.1.13 Calibration curve of Lumefantrine in 0.1 N HCl

1.2.1Formulation and Development

Table 1.6: Formulation variables of LCs of Artemether/Lumefantrine

S. NO.	Code	Independent variables
1	X_1	Artemether
2	X_2	Lumefantrine
3	X_3	Oleic acid

Table 1.7: Response variables LCs of Artemether/Lumefantrine

S. NO.	Code	Dependent variables
1	Y_1	Particle size
2	Y_2	Entrapment efficiency of Artemether
3	Y_3	Entrapment efficiency of Lumefantrine

Table 1.7: Actual and coded values of independent factors

*Every formulation contained 5 ml of liquid dispersion

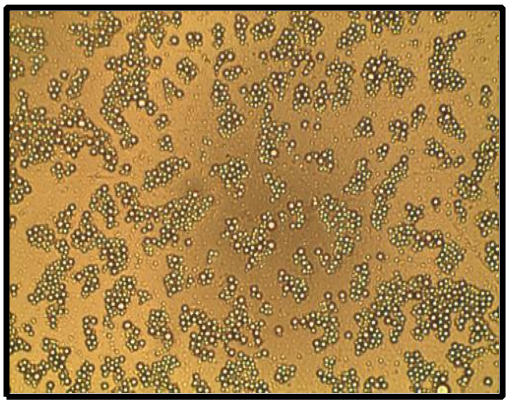
1.2.1 Evaluation of Artemether and Lumefantrine LCN

Table 1.8: Compositions and particle size of liquid crystalline nanoparticles of Artemether and Lumefantrine

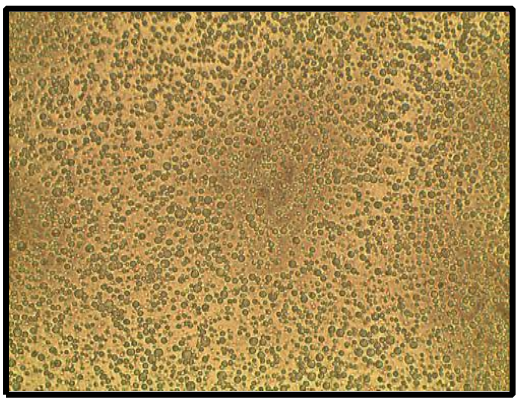
S. No.	Trial code	Composition			Particle size analysis	
		ARTM (mg)	LMF (mg)	Oleic acid (mg)	Z-average (d.nm)	PdI
1	F1	20	100	125	158	0.60
2	F2	15	75	125	176	0.50
3	F3	15	75	125	184	0.30
4	F4	15	75	125	162	0.40
5	F5	20	50	125	195	0.20
6	F6	10	75	150	165	0.22
7	F7	15	50	150	187	0.58
8	F8	15	50	100	200	0.48
9	F9	10	50	125	193.5	0.10
10	F10	15	100	150	180	0.45
11	F11	20	75	100	192	0.38
12	F12	10	100	125	164	0.41
13	F13	10	100	125	157	0.25
15	F15	15	75	125	190	0.22
16	F16	15	100	100	156	0.38
17	F17	20	75	150	189	0.43

1.2.2 Optical microscopic examination

I. Microscopic examination of F9 (LCs dispersion, PDI 0.6) at 40X



F9 dispersion after 2 days



F9 dispersion after 7 days

Fig. 1.14: microscopic examination of F9 formulation

Table 1.9: Microscopic examination of LCN of Artemether and Lumefantrine

Microscopic examination			
S. No.	**Formulation code**	**Uniformity of dispersion**	**Presence of oil drops**
1	F1	++	—
2	F2	++	—
3	F3	++	—
4	F4	++	—
5	F5	+++	—
6	F6	+++	—
7	F7	+++	+
8	F8	++	+
9	F9	+++	—
10	F10	+++	+
11	F11	++	—
12	F12	++	—
13	F13	++	—
14	F14	++	
15	F15	++	
16	F16	++	—
17	F17	++	—

+++ = Uniformity of dispersion + = Oil drops present

++ = Less Uniform — = Oil drops not present

1.2.3Optimized formulation of LCN of Artemether and Lumefantrine

Based on the analysis of all the batches, it has been found that experimentally determined results of "OF1" showed closeness between the predicted values and observed values for all responses. This formulation considered as optimized and further evaluated for physical

stability and *in vitro* drug release.

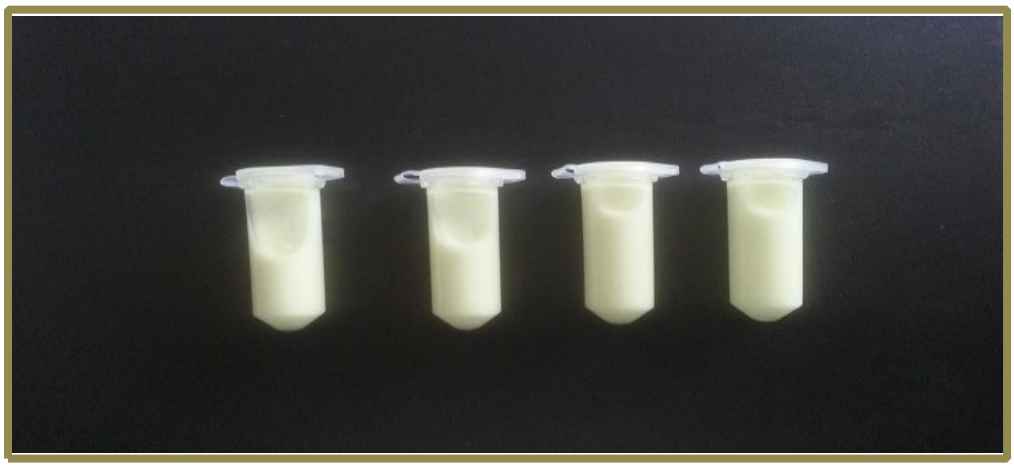


Fig. 1.15: Optimized formulation

1.2.4 Evaluation of optimized formulation "OF1"

1.2.5 Stability study of optimized formulation

a. Organoleptic evaluation

Optimized formulation was evaluated for phase separation, creaming and discoloration of product. Biweekly basis visual assessment of optimized formulation was conducted for 3 months at storage condition 25^{O}C/60% RH and obtained results were shown in table 1.11

Table 1.10: Result of physical stability study

S.no	Parameters	1st month		2nd month		3rd month	
		Weeks		Weeks		Weeks	
		2nd	4th	2nd	4th	2nd	4th
1	Phase separation	—	—	—	—	+	+
2	Creaming	—	—	—	—	—	—
3	Discoloration	—	—	—	—	—	—

Where:

+++ = unacceptable changes + = acceptable changes

++ = significant changes — = no change

Table 1.11: Physical stability Study at 25 ^{O}C/60% RH

S. no	Parameters	25^{O}C/60% RH			
		Initial	1st month	2nd month	3rd month
1	Particle size (nm)	193.50±0.50	195.4±0.60	205.50±0.50	215.00±0.35
2	EE of ARTM	85.50±0.50	84.33±1.52	81.60±0.40	78.40±0.60
3	EE of LMF	88.50±0.50	87.93±0.87	85.50±0.50	80.50±0.50

Each value represents mean ± standard deviation (n= 3) EE= entrapment efficiency

1.3.1 *In-vitro* release study of optimized formulation (OF1)

In-vitro dissolution study for Artemether was conducted for 72 hrs. In similar way Lumefantrine drug release was estimated for 72 hrs, separately in simulated gastric fluid using the dialysis membrane.

Table 1.12: *In-vitro* drug release of Artemether from optimized formulation in phosphate buffer pH 6.8

Time (Hrs)	Cumulative drug release (%)
1	0.20±0.10
2	0.53± 0.27
4	20.00±2.50
8	30.00±2.10
12	35.60±2.20
24	60.0±2.50
48	95.23±3.20
72	98.08±3.54

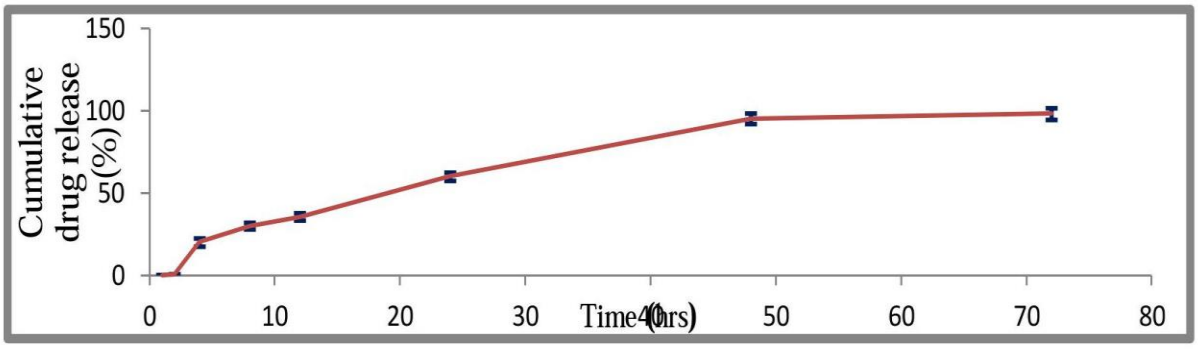


Fig. 1.16: *In-vitro* dissolution profile of Artemether from optimized formulation

Table 1.13: *In-vitro* drug release of Lumefantrine from optimized formulation in simulated gastric fluid pH 1.2

Times (Hrs)	Cumulative drug release (%)
1	0.20±0.10
2	
4	5.946±1.50
8	12.17±2.20
12	20.60±2.50
24	40.50±2.65
48	75.64±2.46
72	89.20±2.80

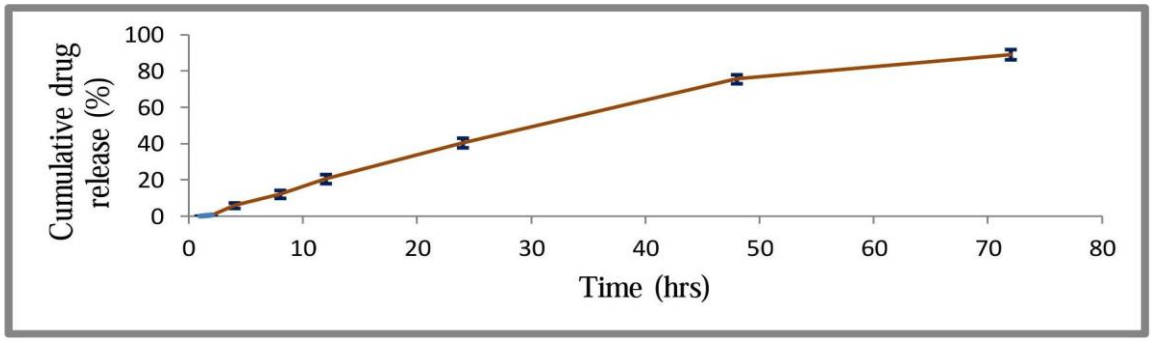


Fig. 1.17: *In-vitro* dissolution profile of Lumefantrine from optimized formulation

CONCLUSION

An attempt to enhance the solubility of Artemether and Lumefantrine was achieved by incorporating these drug candidates in a lipid carrier. Liquid crystalline nanoparticles of Artemether and Lumefantrine were prepared by hydrotropic dilution method using glyceryl monooleate, oleic acid, poloxamer, ethanol and water. The optimization of liquid crystalline nanoparticles was achieved by response surface methodology (BBD).

- Pure sample of Artemether and Lumefantrine were supplied and used throughout the experiments.
- Artemether and Lumefantrine were practically insoluble in water but soluble in ethanol, DMSO and DMF.
- Partition coefficient for Artemether and Lumefantrine was found 3.26 and 2.90 respectively.
- λ_{max} of Artemether and Lumefantrine was 256 nm and 342 nm, determined by UV visible spectroscopy.

- Out of 17 trials, trial F9 was suggested as an optimized formulation. The selection was made on the basis of particle size and EE of ARTM and LMF.
- Entrapment efficiency of ARTM and LMF was found 85.00% and 88.50% respectively.
- Drug release study of ARTM and LMF revealed that the drugs were released in a remarkably controlled manner up to 72 hrs.
- Stability study revealed that the OF1 (optimized formulation) stable over a period of three months.

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