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# Engineered chylomicron mimicking carrier emulsome for lymph targeted oral delivery of methotrexate

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ABSTRACT

The aim of the present study was to develop chylomicron mimicking carrier emulsome for oral lymphatic delivery of methotrexate (MTX), an anticancer drug. The compritol 888 ATO (CA) was used as lipid core and soya lecithin (PC) as stabilizer. The optimized emulsome (1:1.2 mole ratio of CA:PC) showed mean particle size of 160.3 ± 10.2 nm and with 72.8 ± 6.5% drug entrapment efficiency. The differential scanning calorimetric studies revealed a depression in endothermic onset for MTX loaded emulsome. The rapid burst release of the drug was observed in simulated gastric fluid (SGF pH 1.2) with significant increase in particle size of emulsome. However in simulated intestinal fluid (SIF, pH 7.4) a slow and consistent release of the drug was obtained over period of 24 h. Storage stability studies were performed at different temperatures (4 ± 1 and 25 ± 1 °C) for 3 months which suggested that EML remain more stable when stored at refrigerated condition. The *in vivo* studies were carried out on albino rats and response was estimated collecting blood and lymph both. The pharmacokinetic parameters $C_{max}$, $t_{max}$ and $AUC_{0\rightarrow 12\,h}$ after duodenal administration of optimized emulsomal formulation and plain MTX solution were 7.1 and 2.4 μg/mL, 4 and 1 h, 40.45 and 7.2 h μg/mL respectively. The relative bioavailability of MTX was enhanced nearly 5.7 times with optimized EML formulation when compared to plain MTX solution with higher uptake and longer residence time of MTX molecules in lymphatics. Thus, emulsome could be used as lymphotropic carrier for delivery of bioactive(s) and hence for bioavailability enhancement.



## 1. Introduction

Intestinal lymphatic system has gained renewed interest for delivery of hydrophobic drugs, macromolecules like peptides, proteins and vaccines (Vyas, 1997; Trevaskis et al., 2008). It offer several advantages including avoidance of first pass metabolism, selective treatment of diseases and infections of the mesenteric lymphatics, enhancement of the absorption of the large molecules such as peptides/particulates and inhibition of cancer cell metastasis (O'Driscoll, 2002). A major function of the intestinal lymphatics is to facilitate the absorption of long chain fatty acids via re-esterification and reassembling them into chylomicrons within the enterocytes. Exogenous compounds absorbed via the intestinal lymph are generally transported in association with the lipid core of intestinal lipoproteins thereby requiring co-administered lipid to stimulate lipoprotein formation and to deliver the content in the systemic circulation through well-known transcellular mechanism of lipid transport (Porter and Charman, 2001).

Several approaches including prodrug synthesis, use of permeation enhancers, surface modification, complex formation and more recently colloidal lipid carrier based strategies have been developed for the delivery of drugs to intestinal lymphatics (Ueda et al., 1983; Charman and Porter, 1996; Vyas et al., 1999; Khoo et al., 2001). The later one is popular in terms of means and mechanism associated with it. Previously, solid lipid nanoparticles have been developed and utilized successfully for this purpose (Rai et al., 2008; Paliwal et al., 2009) by our group. Emulsome (EML) represents lipid-cored carriers stabilized with high percentage of soya lecithin (5–10% by weight) suitable for entrapment of both the hydrophobic and hydrophilic drugs (Amselem et al., 1994; Vyas et al., 2006). EML may possess structural resemblance with the chylomicrons (natural lipoprotein of the body) and so expected to mimic these lipoproteins in behavior. These mini lipids like particles are repeatedly taken by endogenous lipid absorption mechanism through enterocytes of GIT tract. Subsequently the route through regional lymphatic system, where through appropriate ligands they may be trapped by lymph nodes, is to deliver the drug specifically to lymph nodes and so to the lymphatic sarcomas (Porter and Charman, 2001). Methotrexate (MTX), an anticancer drug, is a drug of choice in carcinomas of intestinal lymphatic region. However, drug suffers with the problem of low bioavailability and poor lymphatic drainage on oral administration using conventional carrier systems. It is desirable to deliver the drug in sufficient high concentration in the intestinal lymphatic region.

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In this sequence we previously evaluated performance of different lipid core material on the characteristics of solid lipid nanoparticles specifically designed for oral lymphatic delivery of MTX. As a result Compritol ATO® (CA, Glycerol behenate-$CH_3(CH_2)_{20}COOCH_2CH(OH)CH_2OH$) was found to be appropriate core material with encouraging results in comparison to others (Paliwal et al., 2009). This study was aimed to develop, CA cored MTX loaded EML which could solubilize and retain MTX within multiple bilayers of lecithin purposely specific for treatment of intestinal lymphosarcoma. The MTX-loaded EML were optimized and studied for their particle size and shape to correlate structural resemblance with chylomicrons, drug entrapment efficiency, *in vitro* release in simulated pH conditions of gastrointestinal tract to assess its potential as oral formulation and storage stability at various temperatures. The thermal analysis was performed to observe modifications in crystalline behavior of lipid. The *in vivo* study protocol was designed for estimating both blood plasma profile and lymphatic concentration of the MTX after intradudonal administration.

## 2. Materials and methods

### 2.1. Materials

MTX and CA were kindly supplied as a gift from Dabur Research Foundation (Ghaziabad, India) and Colorcon Asia Ltd. (Goa, India) respectively and were used without further purification. Soya lecithin (PC), Sephadex G-50 and 6-carboxyfluroscein (6-CF) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4) were prepared according to the official methods of US Pharmacopoeia. All other chemicals and reagents were of analytical grade if not specified.

### 2.2. Preparation of carrier system

MTX loaded EML was prepared following the previously reported method of Amselem et al. (1994) with slight modification as per laboratory setup. The scheme of preparation of EML is shown in Fig. 1. Briefly, CA and PC in different molar ratio were taken in a round-bottom flask and dissolved in minimum amount of chloroform. The chloroform was then evaporated until complete dryness under reduced pressure using rotary flash evaporator (Strike 102, Italy) to form a thin lipid film on walls of the round-bottom flask. The dried film was hydrated with 10 mL phosphate buffered saline (PBS pH 7.4) containing 10 mg MTX and homogenized by ultasonication (Soniweld, India) for 10 min at 40% frequency to obtain nano-sized EML. The free unentrapped drug was removed by passing the dispersion through a sephadex G-50 column (Fry et al., 1978). The so obtained EML dispersion was lyophilized using 100 mg sucrose as cryoprotectant in 1 mL dispersion stored at −50 °C for 5 h and then kept under vaccum for 36 h. The lyophilized formulations were stored at refrigerated temperature till further use.

### 2.3. Characterization of carrier system

#### 2.3.1. Optimization of parameters

*2.3.1.1. Optimization of EML/liposome ratio.* In order to optimize the EML/liposome ratio in prepared dispersions, different CA/PC ratio of 1.0:0.3 to 1.0:1.2 (w/w) was used to encapsulate 6-CF as highly hydrophilic marker. The other parameters were kept constant. These formulations were subjected to recovery study for optimizing maximum EML/Liposome ratio with help of Sephadex G-50 column.

*2.3.1.2. Optimization of sonication time.* EML dispersion was subjected to probe sonication (Soniweld, INDIA) for further reduction in particle size. The sonication time was optimized from 5 to 20 min with regard to particle size and entrapment efficiency.

#### 2.3.2. Entrapment efficiency

The entrapment efficiency of MTX in EML was calculated as reported previously (Vyas et al., 2006). Briefly, EML were disrupted using 50% (v/v) n-propanol in PBS (pH 7.4) and the liberated drug was estimated by spectrophotometrically with UV-spectrophotometer (Cintra, 10, Japan) at 259 nm.

#### 2.3.3. Size and zeta potential

The average particle size and zeta potential were measured by photon correlation spectroscopy (ZS90 zeta sizer Malvern Instruments, UK) at 25 °C. The samples were kept in polystyrene cuvettes and observations were made at 90° fixed angle every time.

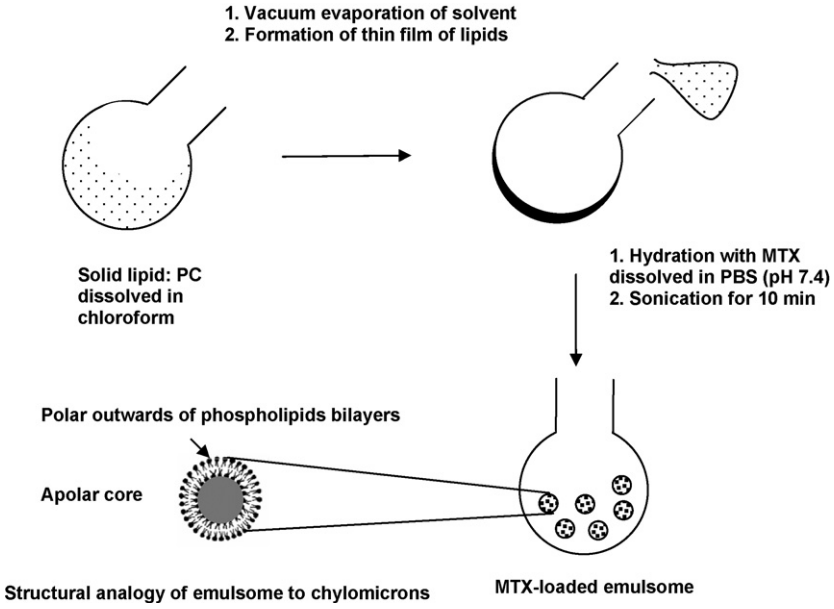


**Fig. 1.** Scheme of preparation of MTX loaded emulsome by film hydration method.

*2.3.4. Transmission electron microscopy*

Prepared EML were characterized for morphology by transmission electron microscope (JEOL, Japan) using copper grid coated with carbon film. The phosphotungstic acid (PTA, 1%, w/v) was used as a negative stain.

*2.3.5. Differential scanning calorimetry*

DSC analysis was performed using Mettler Toledo DSC 822e (Columbus, OH). The scans were recorded at a heating rate of 10 °C/min in the range of 30–550 °C. Analysis was performed under a nitrogen purge (80 mL/min). An empty aluminum sample pan was used as reference. About 10 mg sample was taken for analysis every time. The analysis were carried out with pure MTX, pure CA, CA after evaporation of chloroform, EML without MTX and MTX loaded EML.

*2.3.6. In vitro release studies*

*In vitro* release studies of MTX loaded EML were performed using dialysis method with cellophane membrane of MWCO 10 kDa (Sigma, MO, USA). The formulation (2 mL) was placed in the donor compartment and the receiver compartment was filled with 100 mL dialysis medium (SGF pH 1.2 and SIF pH 7.4) stirred continuously at 100 rpm using magnetic stirrer (Remi, India) at 37 ± 1 °C. After regular time intervals samples (1 mL) were withdrawn from receiver compartment. After each withdrawal of sampling, equal volume of dialysis medium was added in the receiver compartment so as to maintain equal volume level each time. All samples were withdrawn in triplicate. Samples were analyzed for amount of MTX released with UV-spectrophotometer (Cintra 10, Japan) at 259 nm in all pH conditions.

*2.3.7. Storage stability studies*

The formulations were also studied for storage stability various temperatures (4 ± 1 and 25 ± 1 °C) for 3 months and pH conditions (SGF pH 1.2 and SIF pH 7.4) at corresponding GIT transit time. These formulations were examined in regular time interval for any change in particle size and entrapment efficiency.

*2.3.8. In vivo studies*

The *in vivo* studies were carried out under the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal, Ministry of Culture, Government of India) and all the study protocols were approved by the local institutional Animal Ethics Committee. Among various formulations we selected optimized EML formulations for *in vivo* study. Albino rats of either sex were weighed (250–350 g) and divided into five groups of six animals each. The animals were kept for overnight fasting but given free access to water. All the animals of group I was given 10 mg/kg dose of plain MTX intraduodnaly (solution in PBS pH 7.4): the animals of group II, III and IV were given MTX-EML-C, MTX-EML-D and MTX-EML-E respectively in equivalent dose of MTX. The group V received PBS buffer solution pH 7.4 without drug and serve as control. All the animals were anesthetized by urethane injection (1.2 g/kg). After that animals were dissected at abdominal site and mesenteric lymph duct was cannulated as described previously (Warshaw, 1972). Simultaneously, jugular vein was also cannulated for collection of blood samples. All the animals were kept in supine position during experiment and infused continuously with physiological saline solution at the rate of 4 mL/h/kg. Lymph and blood samples were collected periodically and analyzed for MTX content using High Performance Liquid Chromatography (HPLC) (Moghbel et al., 2003). Briefly, samples were deprotienized using trichloroacetic acid before injection and were passed through membrane filter pore size 0.45 μm. The supernatant was injected into a 250 mm × 4.6 mm octadecylsilane column. Mobile phase was consisted of TRIS–phosphate buffer (pH 5.7):methanol:acetonitrile (82:11:7) with a flow rate of 1.8 mL/min. MTX was detected at 313 nm by UV and at ambient temperature. Paraaminoacetophenone was used as internal standard. Methotrexate and internal standard were having retention times 4.4 and 6.5 min respectively.

*2.3.9. Pharmacokinetic analysis*

The peak plasma concentration ($C_{max}$) and the time to reach peak concentration levels ($t_{max}$) were directly obtained from the curve of time versus plasma drug concentration. Area under the curve (AUC) was estimated by linear trapezoidal rule method. The other pharmacokinetic parameters were calculated using WinNonlin software Version 5.2.

*2.3.10. Statistical analysis*

The results were expressed as mean ± standard deviation. In some cases statistical analysis was carried out by using Student's *t*-test and statistical significance was designated as $p < 0.05$. One-way analysis of variance followed by post hoc test (Tukey's test) was applied for multiple comparison ($n = 6$).

## 3. Results

*3.1. Optimization of EML/liposome ratio*

EML was developed using reported method of Amselem et al. (1994). The molar ratio of CA and PC was varied from 1.0:0.3 to 1.0:1.5 for optimization. Initially it was anticipated that the use of higher concentration of PC as stabilizer may develop the chances of formation of liposome and other similar vesicles along with EML. Hence 6-CF was used as hydrophilic marker to prepare EML at different CA:PC ratio. The entrapment of 6-CF was taken as measurement to optimize EML/liposome ratio. The amount of percent 6-CF recovered was calculated after passing dispersions through Sephadex G-50 column. When CA:PC molar ratio was 1:0.3 (EML-A) amount of 6-CF recovered was 85.64 ± 3.12%. Whereas for formulations of 1:0.6 (EML-B), 1:0.9 (EML-C), 1:1.2 (EML-D) CA:PC mole ratio, the amount of 6-CF recovered were 83.15 ± 6.08%, 82.46 ± 4.41% and 78.15 ± 7.82% respectively (Table 1). As CA:PC ratio was further increased to 1:1.5 (EML-E), a significant decrease in amount of 6-CF recovered, i.e. 40.25 ± 7.82% was noticed. This may be due to formation of aqueous compartment enclosing vesicles like liposome. Hence from these results, it was concluded that EML-D was optimized formulation containing higher EML/liposome ratio.

*3.2. Characterization of emulsome*

The optimization of particle size and entrapment efficiency was done on the basis of PC to lipid ratio. It was noted that as the concentration of PC increased from 0.3 to 1.2 moles (with respect to 1 mole of CA) the particle size of the EML decreased from 426.5 ± 13.3 to 352.7 ± 15.6 nm (Table 1). However, further increase in concentration of PC to 1.5 moles as compared to 1 mole of CA sudden increase in the particle size was observed to 452.6 ± 8.9 nm. This might be due to possible formation of liposome in excessive concentration of PC. The various EML formulations showed zeta potential in the range of −18.3 ± 2.6 to −25.1 ± 4.1 mV suggesting stability of dispersion. The entrapment efficiency of MTX-EML-A, MTX-EML-B and MTX-EML-C was found to be 59.6 ± 5.6%, 61.0 ± 3.8% and 66.4 ± 6.5%, while in case of MTX-EML-D and MTX-EML-E; it was 72.6 ± 7.2% and 73.8 ± 8.8% respectively. This showed that as concentration of PC increase from 0.9 to 1.2 moles (compare formulation MTX-EML-C and MTX-EML-D) high drug loading was observed. However, further increase in concentration of PC to 1.5 moles showed nearly the same drug loading as per the cost of increased particle size of the carrier as discussed above. These results again suggested that CA:PC mole ratio of 1.0:1.2 was optimum for the formulation. However, to observe any other effect of

**Table 1**
Optimization and characterization parameters of MTX-loaded EM formulations at different mole ratio of CA:PC (sonication time was kept constant to 5 min); results are expressed as mean ± S.D. ($n = 6$).

| S. No. | Formulation code | CA:PC mole ratio | 6-CF Recovered (%) | Particle size (nm) | Entrapment efficiency (%) | Zeta potential (mV) |
|---|---|---|---|---|---|---|
| 1 | EML-A | 1.0:0.3 | 85.64 ± 3.12 | 426.5 ± 13.3 | 59.6 ± 5.6 | −18.3 ± 2.6 |
| 2 | EML-B | 1.0:0.6 | 83.15 ± 6.08 | 380.2 ± 10.2 | 61.0 ± 3.8 | −19.1 ± 3.1 |
| 3 | EML-C | 1.0:0.9 | 82.46 ± 4.41 | 366.4 ± 11.3 | 66.4 ± 6.5 | −20.8 ± 3.5 |
| 4 | EML-D | 1.0:1.2 | 78.15 ± 3.88 | 352.7 ± 15.6 | 72.6 ± 7.2 | −21.4 ± 3.4 |
| 5 | EML-E | 1.0:1.5 | 40.25 ± 7.82[a] | 452.6 ± 8.9 | 73.8 ± 8.8 | −25.1 ± 4.1 |

[a] Statistically significant difference ($p < 0.001$) between EML-E vs. EML-A, EML-B, EML-C and EML-D.

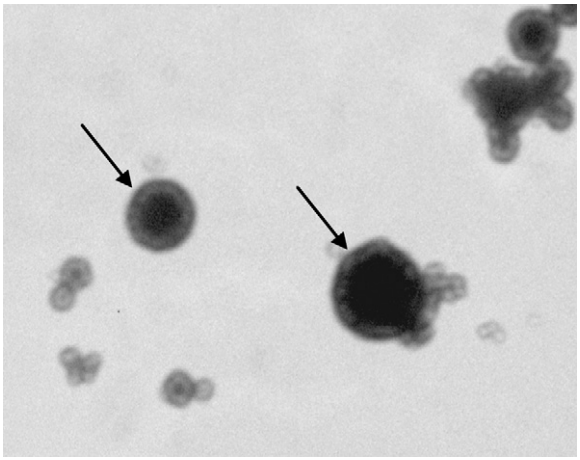

**Fig. 2.** Representative transmission electron microscopic photograph of MTX- EML-D (arrow indicates multiple bilayers of PC around the solid lipid core of emulsome which differentiate it from solid lipid nanoparticles).

PC and/or developed liposome if any, on *in vivo* performance we considered formulations MTX-EML-C, MTX-EML-D and MTX-EML-E optimum for further studies. Fig. 2 shows TEM photographs of MTX-loaded EML. It is apparent from the TEM photograph that particles were almost spherical with consistent appearance of lecithin bilayers around solid lipid core.

### 3.3. Optimization of sonication time

Optimized formulation MTX-EML-D was subjected to sonication for particle size reduction purpose. The sonication time may influence the entrapment of the MTX in bilayers of PC around the core. Hence, sonication time was optimized in terms of particle size and entrapment efficiency both (Table 2). It was observed that sonication of the carrier up to 5 min results into particles of 352.7 ± 15.6 nm with entrapment efficiency of 72.6 ± 7.2% whereas sonication up to 10 min produced particles of 160.3 ± 10.2 nm particle size and 72.8 ± 6.5% entrapment efficiency. Reduction of the particle size may be due to high input of energy during sonication and increased entrapment of MTX possibly may be due to moving and packing of the PC bilayers. Further increment in sonication time to 15 and 20 min showed insignificant ($p < 0.05$) reduction in particle size along with remarkable decrease of drug entrapment efficiency. Finally, sonication up to 10 min was considered optimum.

**Table 2**
Optimization of sonication time with respect to particle size and entrapment efficiency; results are expressed as mean ± S.D. ($n = 6$).

| Sonication time (min) | Particle size (nm) | Entrapment efficiency (%) |
|---|---|---|
| 5 | 352.7 ± 15.6 | 72.6 ± 7.2 |
| 10 | 160.3 ± 10.2[a] | 72.8 ± 6.5 |
| 15 | 140.3 ± 8.9[a,b] | 54.5 ± 5.1[d] |
| 20 | 138.2 ± 6.5[a,b,c] | 50.2 ± 8.3 |

[a] Statistically significant difference ($p < 0.001$) in particle size reduction between sonication time at 5 min and higher sonication time.
[b] Statistically significant difference ($p < 0.05$) in particle size reduction between sonication time at 10 min and higher sonication time.
[c] Insignificant difference ($p > 0.05$) in particle size reduction between sonication time at 15 and 20 min.
[d] Statistically significant difference ($p < 0.001$) in entrapment efficiency between sonication after 10 and 15 min.

### 3.4. DSC studies

Fig. 3 shows the DSC thermograms of pure MTX (A); pure CA (B); CA dissolved in chloroform followed by its complete removal (C); EML without MTX (D) and MTX loaded EML (E) respectively. In case of pure MTX two superimposed peaks between 120 and 170 °C were observed. It may be possible that one at lower temperature corresponds to melting and the other can be due to decomposition of the drug. For pure CA, melting started at 70.6 °C attaining maximum peak at 73.2 °C with difference of 2.5 °C. A sharp peak was obtained (Fig. 3B) suggesting crystalline nature of pure CA. Whereas, in case of CA with solvent evaporation onset and maximum peak were 70.4 and 78.3 °C with a difference of 7.8 °C with a broad peak (Fig. 3C). The CA in the form of EML without MTX showed endothermic onset at 70.5 °C and maximum peak at 77.8 °C with difference of 7.2 °C. The peak was broadening again in this case (Fig. 3D). Here, difference between onset and maximum peak are taken as a measure for the width of the peak. In last two cases width of the peak was more, i.e. 7.8 and 7.2 °C in comparison to pure CA which was 2.5 °C. The difference between the onset and the temperature of the maximum for these forms of the CA may be pinpointing for massive crystal order disturbance, i.e. lattice defects. However, the endothermic onset was nearly the same in all these cases. Since, development of

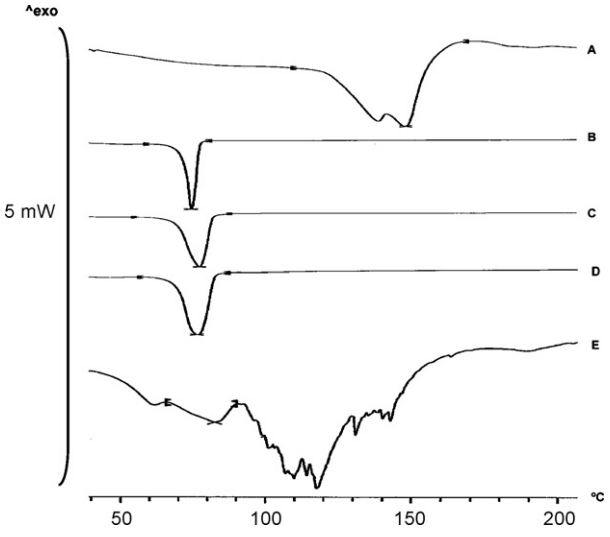


**Fig. 3.** DSC Thermograms: [A] MTX; [B] Pure CA; [C] CA after solvent (chloroform) evaporation; [D] EML of CA without MTX; [E] MTX-EML-D.

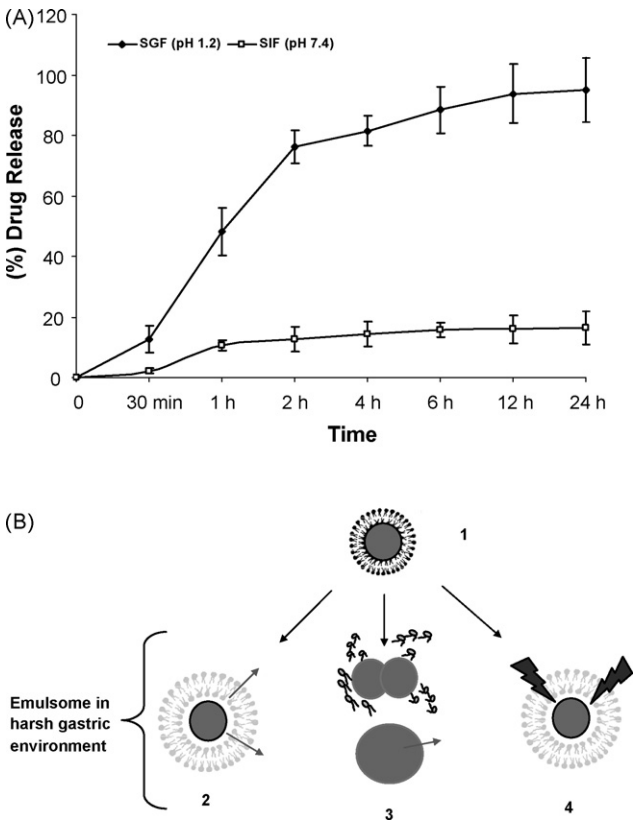


**Fig. 4.** (A) *In vitro* release studies in different time intervals of optimized formulation (MTX-EML-D, CA:PC ratio were 1.0:1.2 mole ratio) in different pH medium [A] SGF, pH 1.2; [B] SIF. pH 7.4 (results are expressed as mean ± S.D., $n = 3$). (B) Possible mode and means of burst release of MTX from EML [1] Morphology of MTX-loaded EML formulation; [2] drug release due to increase in particle size and thickness of diffusion layer; [3] destabilization of PC bilayers and aggregation of EML results in loss of drug entrapment; [4] enzymatic degradation of lipids enclosed core and coat.

the EML by film hydration method was non-thermal process and so there was very less probability for any possible polymorphic changes in the crystals of CA. A remarkable depression in onset and maximum peak was observed with MTX-EML-D, i.e. 69.8 and 84.3 °C respectively. Here, the difference between onset and maximum peak was highest, i.e. 14.8 °C, which was also indicated in thermogram in the form of broaden peak. This may be probably due to intermolecular interactions of drug and lipids (Fig. 3E).

### 3.5. In vitro release

*In vitro* release study was carried out by dialysis tube method with dialysis membrane of cut off size 10,000 kDa. Dialysis membrane retained EML and passed the MTX molecules released over time into the receiver compartment of the model. Fig. 4 A shows the percentage release of the MTX in SGF pH 1.2 and SIF pH 7.4 as a function of time up to 24 h. These results revealed that the amount of MTX released in 24 h in SGF was more than the 90% of entrapped drug. The release of the MTX in SIF was slow and steady and was found to be nearly 16.5% in 24 h.

### 3.6. Storage stability studies

Storage stability was evaluated for MTX-loaded EML formulations at different temperatures (4 ± 1 and 25 ± 1 °C) and also on different pH conditions (SGF pH 1.2 and SIF pH 7.4). It was observed that MTX-loaded EML, when stored for 3 months at refrigerated temperature, i.e. 4 ± 1 °C, were more stable in terms of change in particle size and entrapment efficiency, in comparison to room temperature storage (Table 3). A remarkable effect of pH on stability of MTX-loaded EML was also observed (Table 4). The formulations were kept for 2 h in SGF (pH 1.2). An increase in particle size along with high drug release was observed. An irreversible instability of formulation in low pH medium may be due to possible aggregation of the particles. However, formulations were stable enough in terms of both particle size and drug entrapment in SIF (pH 7.4) even after 6 h storage.

### 3.7. In vivo studies and pharmacokinetic analysis

The *in vivo* studies were conducted on five groups of albino rats of either sex. The animals were given an initial oral dose of soybean oil (1 mL to each animal of all group 1 h prior to study) in order to swell the lymphatic duct to make it accessible for cannulation. The animals were anesthetized and after surgical procedure formulations were administered via intradudonal route. Plasma and lymph samples were collected periodically and were estimated for MTX content using HPLC. Fig. 5 shows the plasma concentration versus time profile. The pharmacokinetic study was performed to observe the changes in pharmacokinetic parameters of MTX when administered in emulsomal formulation. The data obtained were subjected to appropriate statistical treatment and estimation of bioavailability was carried out by computing the area under the curve (AUC) from the plasma profile using trapezoidal rule (Table 5). A significant difference ($p < 0.05$) in $t_{max}$ value of plain MTX solution with that of MTX-EML formulations was observed. As $t_{max}$ for EML formulations were 4 h whereas in case of plain MTX solution it was found to be 1 h. Peak plasma concentrations were in the following order: MTX-EML-D > MTX-EML-E > MTX-EML-C ≫ MTX plain solution. The $AUC_{0\rightarrow\infty}$ recorded for the formulations MTX plain solution in PBS pH 7.4, MTX-EML-C, MTX-EML-D and MTX-EML-E were 7.67 ± 1.20, 28.9 ± 5.60, 45.93 ± 4.50 and 39.92 ± 5.1 μg h/mL respectively. A significantly higher bioavailability (nearly 5.7 times) of MTX was obtained when administered in the form of EML as compared to plain MTX solution. Similarly, the bioavailability estimated for MTX-EML-D was significantly higher ($p < 0.05$) than MTX-EML-C and MTX-EML-E.

**Table 3**
Stability studies of various MTX-loaded EML formulations at 4 ± 1 and 25 ± 1 °C after 3 months storage; results are expressed as mean ± S.D. ($n = 6$).

| Formulation code | Particle size (nm) | | | Entrapment efficiency (%) | | |
|---|---|---|---|---|---|---|
| | 0 day | After 90 days | | 0 day | After 90 days | |
| | RT | 4 ± 1 °C | 25 ± 1 °C | RT | 4 ± 1 °C | 25 ± 1 °C |
| MTX-EML-C | 178.4 ± 11.3 | 212.1 ± 5.2[a] | 604.5 ± 15.3[a,b] | 66.5 ± 8.5 | 64.2 ± 2.4[c] | 45.6 ± 5.3[a,b] |
| MTX-EML-D | 160.3 ± 10.2 | 180.4 ± 3.5[c] | 654.1 ± 21.2[a,b] | 72.8 ± 6.5 | 70.1 ± 2.1[c] | 50.6 ± 4.5[a,b] |
| MTX-EML-E | 252.6 ± 8.9 | 338.4 ± 14.6[a] | 828.9 ± 22.6[a,b] | 73.2 ± 5.8 | 68.2 ± 3.4[c] | 46.3 ± 5.8[a,b] |

RT = Room temperature (25 ± 2 °C).
[a] Statistically significant difference ($p < 0.001$) between results at room temperature and storage temperature conditions.
[b] Statistically significant difference ($p < 0.001$) between results at 4 ± 1 and 25 ± 1 °C.
[c] Statistically insignificant difference ($p > 0.05$).

**Table 4**
Stability studies of various MTX-loaded EML formulations at different pH conditions (in SGF pH 1.2 for 2 h and in SIF pH 7.4 for 6 h), results are expressed as mean ± S.D. ($n = 6$).

| Formulation code | Particle size (nm) | | | Entrapment efficiency (%) | | |
|---|---|---|---|---|---|---|
| | 0 h Initially | 2 h SGF (pH 1.2) | 6 h SIF (pH 7.4) | 0 h Initially | 2 h SGF (pH 1.2) | 6 h SIF (pH 7.4) |
| EML-C | 178.4 ± 11.3 | 788.6 ± 16.8[a] | 401.3 ± 6.3[a,b] | 66.5 ± 8.5 | 25.6 ± 2.3[a] | 51.2 ± 3.4[a,b] |
| EML-D | 160.3 ± 10.2 | 801.2 ± 18.2[a] | 412.8 ± 8.3[a,b] | 72.8 ± 6.5 | 26.3 ± 6.5[a] | 56.2 ± 4.2[a,b] |
| EML-E | 252.6 ± 8.9 | 958.6 ± 14.5[a] | 521.2 ± 6.8[a,b] | 73.2 ± 5.8 | 20.1 ± 2.1[a] | 58.3 ± 6.1[a,b] |

[a] Statistically significant difference ($p < 0.001$) between results in dispersion form and SGF.
[b] Statistically significant difference ($p < 0.001$) between results in SGF and SIF.

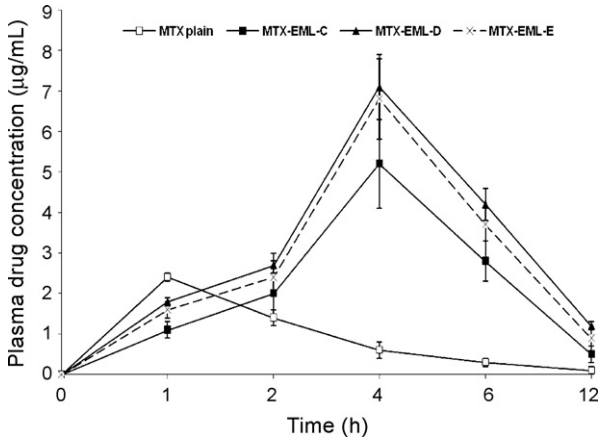


**Fig. 5.** Plasma profile of different MTX-loaded EML formulations. Results are presented as mean ± S.D. ($n = 6$).

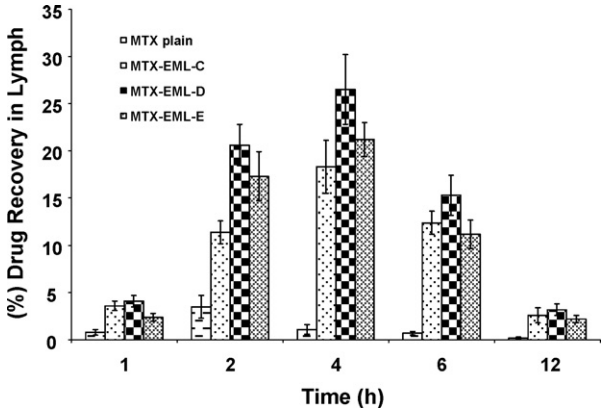


**Fig. 6.** *In vivo* lymphatic uptake of different MTX loaded EML formulations. Results are presented as mean ± S.D. ($n = 6$).

The percent lymphatic uptake of MTX at various time intervals was recorded and was found to be highest after 4 h of administration. The comparative performance of different formulations after 4 h of duodenal administration (MTX solution in PBS pH 7.4, MTX-EML-C, MTX-EML-D, and MTX-EML-E) were 1.1 ± 0.5%, 18.3 ± 2.8%, 26.5 ± 3.7% and 21.2 ± 1.8% respectively (Fig. 6).

## 4. Discussion

MTX is first line recommended drug for the treatment of sarcoma of intestinal lymphatic region. MTX when administered orally in higher dose suffers with the problems of low and variable bioavailability, high excretion rate and toxicity problems like gastric bleeding. A great challenge facing the pharmaceutical scientist is making such molecules into orally administered formulations with sufficient bioavailability. One of the most popular approaches to improve the oral bioavailability of such drug candidates is the utilization of a lipid based drug delivery system (Dahan and Hoffman, 2008). Lipid cored carriers have shown promising results for enhancing the bioavailability of several lipophilic drugs (O'Driscoll et al., 1991; Jenning et al., 2000). In the present study, it was hypothesized that nano-sized lipid based carrier may reduce the dose related side effects of MTX along with passive targeting in intestinal lymphatic region. In order to achieve maximum potential of the MTX, EML was developed that possessed structural analogy to one of the endogenously synthesized lipoproteins of the biological origin, i.e. chylomicrons. It was anticipated that drug-loaded carrier will be taken up by the mucus associated lymphoid tissue of the intestine and/or will be recycled through the enterocytes thus transported to the intestinal lymphatics (Vyas, 1997; Zara et al., 2002). During this moving and packing of the carriers the drug will be bypassed from the liver and so probably increased the concentration of the drug in lymphatic region and blood plasma as well.

The EML were developed by simple cast film method followed by sonication for particle size reduction purpose. The solid lipid to PC molar ratio was also optimized in order to get high lipid content stabilized as per the minimum use of PC. The sonication time may influence the entrapment of the MTX particularly disrupting the bilayers of PC around the core hence 10 min sonication time was found to be optimum. Overall results indicated that EML were of controlled size in nanometric range with narrow size distribution having optimum drug entrapment efficiency and they were suitable

**Table 5**
Pharmacokinetic parameters of different EML formulations, results are expressed as mean ± S.D. ($n = 6$).

| Formulation code | $C_{max}$ (µg/mL) | $t_{max}$ (h) | $AUC_{0\rightarrow 12\,h}$ (h µg/mL) | $AUC_{0\rightarrow\infty}$ (h µg/mL) | $MRT_{0\rightarrow 12\,h}$ (h) |
|---|---|---|---|---|---|
| MTX plain | 2.4 ± 1.1 | 1.0 ± 0.0 | 7.2 ± 2.7 | 7.67 ± 1.32 | 3.0833 ± 1.0212 |
| MTX-EML-C | 5.2 ± 2.3[ns] | 4.0 ± 0.0 | 27.2 ± 10.1[a] | 28.91 ± 6.34[b] | 4.9228 ± 1.8740 |
| MTX-EML-D | 7.1 ± 2.5[b] | 4.0 ± 0.0 | 40.45 ± 15.6[c] | 45.93 ± 10.32[c] | 5.2089 ± 2.1638 |
| MTX-EML-E | 6.8 ± 1.8[b] | 4.0 ± 0.0 | 36.30 ± 11.2[c] | 39.92 ± 12.10[c] | 5.0799 ± 2.6524 |

ns: Data insignificant from MTX plain.
[a] Significant differences ($p < 0.05$) of results from MTX plain.
[b] $p < 0.01$.
[c] $p < 0.001$.

for oral administration of MTX. It was clear from the TEM photograph of EML that particle were surrounded by multiple bilayers of phospholipids. This differentiates EML as carrier from solid lipid nanoparticles in terms of morphology. DSC is a well-known and widely employed technique for observing drug-excipient incompatibility and polymorphism. A decline in onset peak was observed with emulsomal formulation which may be due to small particle size of EML, high specific surface area and the presence of surfactant (Venkateswarlu and Manjunath, 2004). A burst release of the MTX in low pH medium may be attributed due to instability of PC in low pH gastric media and thus disruption of protective bilayers which ultimately resulted in the burst release of the contents (Paliwal et al., 2009; Venkateswarlu and Manjunath, 2004)). Possible mode and mechanism of burst release of PC stabilized formulations are shown in Fig. 4B. As an outcome of the result slow release of MTX in intestinal fluid medium may be due to entrapment of the drug in apolar core and also due to matrix and/or multilayer barrier characteristics of EML.

Storage stability of EML was considered to be best at refrigerated temperature condition and in SIF medium. To observe the effect of concentration of stabilizers used in EML formulations, we used different concentration of PC for different formulations. However, high concentration of PC did not provided stability to the formulations in low pH medium. These results were in accordance with our previous reports (Paliwal et al., 2009). We concluded from above results not to administer the formulation from oral route and to bypass the harsh gastric environment duodenal route was preferred during *in vivo* studies. Plasma profile revealed a higher drug concentration of MTX when given in form of EML. From pharmacokinetic study it was noticeable that bioavailability of MTX with MTX-EML-D and MTX-EML-E was approximately 1.5 times higher than MTX-EML-C. It showed that lecithin ratio with respect to solid lipid also have noteworthy effect on bioavailability of MTX. Similarly, the time for attaining peak plasma drug concentration in case of plain drug solution (1 h) was less as compared to MTX-EML formulations (4 h) suggesting possible lymphatic transport of MTX.

Trevaskis et al. (2008) focused in a mechanistic update of lipid-based delivery systems and their role in intestinal lymphatic drug transport. It was defined that the attendant delivery benefits associated with lymphatic drug transport include a reduction in first-pass metabolism and lymphatic exposure to drug concentrations orders of magnitude higher than that attained in systemic blood. Intestinal lymphatic transport can be estimated by estimating drug concentration in the mescentric duct and/or thoracic duct cannulation as reported previously. We used to cannulate mesenteric duct as preferred site than thoracic duct since thoracic duct drains lymph from other areas of the body and may be sometimes misleading. Among several animal models that have been proposed for lymphatic study protocols, we selected an anaesthetized rat model for *in vivo* studies. The performances of EML were evaluated after intraduodenal administration of the formulations as discussed above. The lipid digestion and its reassembling into lipoproteins like chylomicrons transport the lipophilic molecules through mesenteric lymph duct. The lymphatic drug concentration profile as shown in Fig. 6 reveals lymphatic absorption of the drug. This may be due to co-absorption of drug and carrier lipid into chylomicrons and subsequent transportation, and finally release of the MTX at albuminal site of the endothelial during reassembling of derivatized lipid(s). However, a minor part of drug may remain associated yet to be carried by chylomicrons to the liver (Paliwal et al., 2009). Recently, Dahan et al. (2008) also proposed two mechanism of absorption for phospholipidic prodrugs such as permeation through the gut wall and entering intact to the enterocyte and association with chylomicron in the enterocyte and reaching the systemic circulation via the lymphatic route. *In vivo* results indicated that EML based formulation improved many fold MTX concentration in the lymphatic region as weigh against to MTX plain solution (Fig. 6). The MTX-EML-D formulation has shown highest uptake of the lipid particles, which may be credited to small size of the particles and higher drug loading. Thus, it may be anticipated from the above results that lymphatic uptake and well-defined transcellular mechanism of lipid processing played vital role for the increased bioavailability of MTX and thus may be accounted for a delay in time to attain peak plasma concentration. The transport of intact EML and/or the stimulation of chylomicron formation may be other liable aspect, which is a common process of lipid uptake and digestion.

## 5. Conclusion

Lymphatic delivery through intestinal milieu could be achieved through successful engineering of drug carriers. Nano-sized chylomicron mimicking cargos such as EML showed promising potential for delivery of MTX. EML mediated delivery supported synergistic effect of both the route and/or mechanism that is paracellular and transcellular as the higher concentration of MTX was observed in comparison to plain drug solution. The instability of formulations in acidic medium suggested that formulations should also be protected from harnessing gastric environment of stomach before oral administration. A much higher bioavailability of MTX may be achieved successfully with lipid based carrier system like EML. The present work opens new possibility of successful utilization of EML as carrier for delivery of bioactive(s) through chylomicron mimicking pathway. This mechanism could be further utilized in order to improve oral controlled delivery of drug, vaccine and bioamcromolecules.

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