International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 14, Issue 3, 2022

Original Article

EZETIMIBE NANOSTRUCTURED LIPID CARRIERS (NLCs): A NEW TECHNIQUE TO OVERCOME THE LIMITATIONS OF ORAL ADMINISTRATION

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Received: 07 Jan 2022, Revised and Accepted: 09 Feb 2022

ABSTRACT

Objective: Ezetimibe (EMB) is a commonly used lipid-lowering medication that lowers cholesterol and triglycerides. Because of its lower water solubility and hepatic metabolism, it necessitates the formulation of drug delivery systems that are capable of improving solubility and avoiding hepatic effect.

Methods: Ezetimibe nanostructured lipid carriers (EMB-NLCs) were formulated and examined. They were formulated through emulsification with a high homogenization speed and ultrasonication (The method and evaluation parameters have been mentioned under method section in Formulation of EMB-NLCs paragraph).

Results: The formulated NLCs have exhibited particle size (P. S.) between 163.6±7.20 and 866.66±18.65 nm and the zeta potential (Z. P.) values have ranged between-24±1.25 and-35±0.25 mV. Besides, they exhibited higher EE% than 77 percent and the drug encapsulated in lipid matrix was in amorphous state. Pharmacokinetics of optimized formula (F1; composed of 2% w/w Gelucire® 43/01, 8% w/w Miglyol® 812 N, 0.5% w/w lecithin and 2% w/w Poloxmer® 188) have exhibited 2.63-and 2.33-fold increase in oral bioavailability in comparison with EMB suspension and marketing product (Ezetrol® 10 mg tablet), respectively.

Conclusion: These studies have demonstrated that, NLCs are superior for enhancing *in vivo* behavior and oral bioavailability of EMB.

Keywords: Ezetimibe (EMB), Nanostructured lipid carriers (NLCs), Bioavailability and Oral delivery

DOI: https://dx.doi.org/10.22159/ijap.2022v14i3.44072. Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

A large population is said to be affected by hypercholesterolemia (hyperlipidemia). A decrease in high density lipoprotein (45 mg/dl) and an increase in low density lipoprotein (185 mg/dl) as well as triglycerides (TG) in blood plasma (200 mg/dl) cause atherosclerosis [1]. Conversely, high cholesterol raises the risk of heart disease [2]. Hypercholesterolemia can cause excessive cholesterol deposition on the coronary artery walls [3].

EMB is a drug that inhibits cholesterol absorption and is used to treat primary hypercholesterolemia. In hyperlipidemic patients, EMB lowers high levels of cholesterol, non-high density lipoprotein, apolipoprotein B and low density lipoprotein [4]. EMB is classified as class II in accordance with the Biopharmaceutical Classification System (BCS). It is an azetidine derivative with poor aqueous solubility and excellent permeability that is used in BCS class II therapeutics (log P of 4.56). EMB undergoes an extensive first-pass hepatic process of metabolism, which is inversely related to systemic bioavailability once it enters the portal vein [5]. The alkaline environment of the intestine limits the absorption of EMB. As a result, under alkaline conditions, the drug release is slow and limited, which often manifests as low oral bioavailability. Furthermore, pre-systemic clearance, high lipophilicity and the P-gp efflux technique all have an impact on bioavailability. A better treatment requires improved dissolution and/or apparent solubility, especially for drugs like EMB that are poorly water-soluble [6].

Drug delivery systems based on lipids are promising drug carriers because they improve the solubility of drugs that are water insoluble and/or lipophilic, thereby increasing oral bioavailability [7]. The oldest generation of lipidic nanostructure is solid lipid nanoparticles (SLNs) which are an aqueous formulation of nanoparticles with a solidified lipid and settled with more than one surfactant layers. However, there have been some disadvantages to using SLNs, including a limitation in a loading capacity as well as drug expulsion during storage. Consequently, in nanostructured lipid carriers (NLCs), a new generation of lipidic nanoparticles, is required [8]. Unlike SLNs, NLC dispersions are made up of a combination of solid and liquid lipids that allow a larger payload while simultaneously preventing drug expulsion during storage [9]. NLC formulae also have the advantage of longer release of the drug, ease of production scaling and biocompatibility [10]. When NLCs reach the small intestine, they are digested internally, by lipases and co-lipases, into micelles which are organized for drug absorption. The lymphatic pathway or patches may aid the absorption of NLC-encapsulated drugs [11].

The current study aims to investigate the feasibility of encapsulating EMB into NLCs in order to increase the rate of dissolution and, ultimately, of oral bioavailability. Glyceryl monostearate (GMS), Gelucire® 43/01, and Precirol® ATO 5 were selected as solid lipids and Miglyol® 812 N was chosen as liquid lipid. Lecithin was used as lipophilic emulsifier while Poloxamer® 188, Tween® 60 and their combination (1:1) were chosen as hydrophilic surfactants. The physicochemical properties of NLCs were optimized, and the bioavailability study in rats was investigated.

MATERIALS AND METHODS

Materials

EMB was purchased from Chemipharm (Cairo, Egypt). Ezetrol® 10 mg tablet was bought from GNP/Schering (Cairo, Egypt). Glyceryl monostearate (GMS), Gelucire® 43/01(mono-, di-and triglyceride esters of fatty acids), Precirol® ATO 5(Glyceryl distearate/Glyceryl palmitostearate) and Miglyol® 812 N (Triglyceride ester of saturated coconut/palmkernel oil) were purchased from Gattefosse (Paris, France). Poloxmer® 188 (polyoxyethylene-polyoxypropylene block copolymer), Lecithin (Phosphatidylcholine 70%) and Tween® 60 (polyoxyethylen-60-sorbitanmonooleat) were brought from Merck Specialties (Mumbai, India). The chemicals used were of high analytical quality.

Methods

Formulation of EMB-NLCs

The different formulations of EMB-NLCs were formulated by using the emulsification method by using high-speed homogenization and

then ultra-sonication [12]. The aqueous and lipid phases were formulated separately: the lipid phase is formed out of solid lipid (GMS, Precirol® ATO 5 or Gelucire® 43/01; 2%), liquid lipid (Miglyol® 812 N; 8%) and lipophilic emulsifier (lecithin; 0.5%). However, the hydrophilic phase is composed of hydrophilic emulsifier (Poloxmer® 188, Tween® 60 or their combination (1:1); 2%) which have been dispersed in water. EMB (10 mg) was dispersed in Miglyol® 812 N and blended with lipid phase constituents. All constituents of the lipid phase were individually melted at Celsius degrees above solid lipid transition temperatures (GMS, 58-60 °C; Precirol® ATO 5, 53-55 °C and Gelucire® 43/01, 43-46 °C) for 10 min before being combined.

The hyrophilic phase then was drop by drop added to the melted lipid phase and mixed with a high velocity homogenizer (miccra–D–9 IKA, Germany) at 12,000 rpm for 15 min. The dispersion was then subjected to some additional processing by using a probe-type sonicator (250 HT Sonicleanpty, Australia) for 15 min. After that, the formulated emulsions were then cooled to room temperature. Table 1 shows various formulations made by varying the type of surfactants and solid lipids.

Table 1: EMB-NLCs different formulations (F1-F9)

EMB-NLCs different formulae*		
Formula No.	**Solid lipid (2%, w/w)**	**Hydrophilic surfactant (2%, w/w)**
F1	Gelucire® 43/01	Poloxmer® 188
F2	Gelucire® 43/01	Tween® 60
F3	Gelucire® 43/01	Poloxmer® 188-Tween® 60 (1:1)
F4	GMS	Poloxmer® 188
F5	GMS	Tween® 60
F6	GMS	Poloxmer® 188-Tween® 60 (1:1)
F7	Precirol® ATO 5	Poloxmer® 188
F8	Precirol® ATO 5	Tween® 60
F9	Precirol® ATO 5	Poloxmer® 188-Tween® 60 (1:1)

*Each formula contains 10 mg EMB, 0.5%, w/w lecithin and 8%, w/w Miglyol® 812 N

Characterization of the formulated EMB-NLCs different formulae

The different formulae of EMB-NLCs have been examined for particle size (P. S.), zeta potential (Z. P.), entrapment efficiency (EE %) and polydispersity index (PDI). The mean and standard deviation were calculated for each trial in triplicate.

Determination of EE %

Various formulae suspensions (1 ml) was subjected to centrifugation at 14,000 rpm for 1 h at 25 °C in a centrifuge (Sigma Laboratory centrifuge, Germany). The supernatant was then discarded, and the remaining residue was solubilized in methanol and measured with a UV spectrophotometer (UV-1800, Shimadzu, China) at 230 nm. The EE % of EMB in the different formulae of EMB-NLCs were examined by using the following equation (Eq. 1) [13]:

$$EE\ (\%) = (C_{total} - C_{free\ drug}/C_{total}) \times 100 \quad \text{..... (Eq. 1)}$$

Where C_{total} is the quantity of the EMB loaded while $C_{free\ drug}$ is the quantity of the free EMB in the supernatant.

Calculation of P. S., Z. P. and PDI

Particle size, zeta potential and PDI were calculated for each of the formulated EMB-NLCs formulation. In brief, one milliliter of each trial was dispersed in distilled water and then examined by using dynamic light scattering technique (Zetasizer, Malvern Instruments Ltd., Malvern, UK)[14].

Morphology of EMB-NLCs

The shape of the different formulae of EMB-NLCs was determined by transmission electron microscopy (TEM) (Model HT7700, Hitachi, Japan). One drop of each formula was applied to a collodion-coated copper grid and allowed to dry for 2 min. After 5 min, the samples were tainted with uranyl acetate stain and tested after 5 min by TEM [15].

Thermal analysis

Thermal study trials were done by differential scanning calorimeter (DSC) (TA-50 ESI, Shimadzu, Japan). EMB and Gelucire® 43/01 pure samples were subjected to a direct analysis and the optimized formulation (F1) was subjected to lyophilization using lyophilizer (Model SS3241, Stellar Series, Thailand) and mannitol was used as a cryoprotectant. In aluminum pans sealed with lids, one milligram of each of the different components and lyophilized F1 was analyzed alongside the standard reference aluminum. To obtain the endothermic peaks, thermograms were determined between 25 °C and 400 °C and at a rate of scan 10 °C/min at a nitrogen flow rate of 30 ml/min [16].

***In vitro* release experiments**

In vitro release trials were done for pure EMB, and optimized formula (F1) by using the reverse dialysis method in which dialysis membrane has a molecular weight of 12,000 Dalton. The membrane was soaked in distilled water for 1 h and then placed in the medium for a night. Five milligrams of EMB and the volume equivalent to the prepared F1 formula was examined in the USP dissolution apparatus II vessel (Copley scientific, UK) which comprises 500 ml of the dissolution medium (phosphate buffer, pH 6.8) at 37±0.5 °C and 75±2 rpm. Prior to the experiments, various dialysis membranes containing a small quantity of the dissolution medium were equilibrated and previously filled with the dissolution medium for 2 h. To preserve a sink condition, one of the dialysis bags was removed from the dissolution medium and was substituted by an equal quantity of a fresh medium at predetermined time intervals. At 244 nm, the EMB content in the dialysis bag was determined by spectrophotometer (Shimadzu, model UV-3254, Japan). All results were taken in triplicate and compared to a blank. A special program was used to find the best order or model of EMB release by determining the best kinetic models for the *in vitro* release data. The trials included the following models: Higuchi diffusion model [17], first-order release kinetic model, zero-order release kinetic model and Hixson–Crowell model. The actual model of release is the one with the highest value of the correlation coefficient (r) value [18].

***In vivo* studies**

The purpose of the present study is to compare the pharmacodynamic efficacy and bioavailability of EMB-NLCs optimized formula to EMB dispersion and commercial product (Ezetrol® 10 mg). Four months old male albino rats weighing about 200 to 250 g each was brought from the National Research Center's (NRC) animal house (Dokki, Egypt). Animal experiments were done in accordance with committee procedures and animal care procedures. Prior to the experiments, rats were maintained for one week in the animal house under constant environmental conditions (50±5% relative humidity; 25±0.5 °C) with a free access to water and food pellets.

Bioavailability study

Animals were assigned to one of three random groups (n=3); Group I were given F1; Group II were given EMB dispersed in 2% carboxymethylcellulose as a blank drug [11] while Group III were given a commercial product (Ezetrol® 10 mg). The animals received only one oral dose of EMB at a rate of 25 mg/kg. A heparinized capillary tube was used to collect blood from the orbital sinus of rats

at predetermined intervals. One-milliliter samples were placed in heparinized Eppendorf tubes and centrifuged at 12,000 rpm for ten minutes (Sigma Laboratory centrifuge, Germany). Plasma samples were placed and frozen at-20 °C till the time when they were analyzed by HPLC valid method.

Chromatography

Plasma samples of (100 µl) were placed in Eppendorf different tubes containing 100 µl of 10% perchloric acid and then vortexed at room temperature for 30 sec. The tubes were then extracted with 250 µl of diethyl ether and subjected to centrifugation at 4000 rpm for 15 min (Sigma Laboratory centrifuge, Germany). The supernatant was removed, dried under nitrogen at 40 °C, and then analyzed.

The stationary phase which is composed of C-18 reverse-phase column (250 x 4.6 mm, Phenomenex, USA) was employed for the chromatographic analysis. The mobile phase was a degassed filtered mixture of 25 mmol potassium dihydrogen orthophosphate and acetonitrile (50:50, v/v) which have been adjusted to pH 6.5, and the flow rate was 1.0 ml/min. At 244 nm, the eluent was determined by using a UV detector.

Statistical analysis

All results were calculated as a mean±SD and statistically analyzed by one-way ANOVA by using standard non-compartmental software (WinNonlinR®, Pharsight Corporation, USA). Any difference at $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Characterization of the formulated EMB-NLCs formulations

Encapsulation efficiency

As stated by the solubility trial performed by using the test tube technique, various components were chosen for the formulation of EMB-NLCs [19]. The choices were made on the basis of high degree of solubilization for EMB, confirming the EMB solubility in the developed formulation as critical for optimal drug loading [20]. EMB was most soluble in GMS (348±16 mg/gm), Precirol® ATO 5 (67±7.42 mg/gm), and Gelucire® 43/01 (9±2.14 mg/gm), that may be due to GMS's emulsifying characteristic [10], HLB of Precirol® ATO 5 (HLB =2) and Gelucire® 43/01 (HLB =1). Lipophilic lipids have a lower HLB value and more hydrophilic lipids have a higher HLB value. So, lipids with lower HLB values are better suited for hydrophobic EMB solubility than those with higher HLB values. EMB had the highest degree of solubility in Miglyol® 812 N (19.43±1.51 mg/ml) than any other liquid lipid tested.

EMB was also well soluble in Poloxmer® 188 (10.15±1.25 mg/ml) and Tween® 60 (34.29±2.25 mg/ml). The EE percent of EMB in the formulated nanostructured different formulae ranged from 77.1±1.87 percent to 97.7±1.68 percent, as shown in table 2.

In terms of the type of solid lipid, there was not any significant difference ($p>0.05$) in EE% among the examined different formulae. The relatively insignificant and large difference may be due to the high solubility of lipophilic EMB in Miglyol® 812 N, that resulted in defects in the crystal order which created spaces to entrap EMB and thus increased drug EE% [21].

When compared to the use of individual surfactants, the mixture of Poloxmer® 188 and Tween® 60 showed lower EE% (except F9). This pattern could be explained by the greater solubilizing effect of the mixture on EMB than any single surfactant use. The higher degree of solubilization aided the partitioning of EMB between the aqueous and oil phases. Because EMB is fairly well soluble in the used surfactants and the solubilizers, It was extracted from the oil droplets, reducing the quantity of EMB entrapped in the formulated NLCs [22]. The encapsulated EMB was calculated by dissolving NLCs after centrifugation in a mixture of solvent of ethanol and phosphate buffer pH 6.8 (1:1). The total amount of encapsulated and un-encapsulated EMB reached 100 percent of the use dose, according to the results.

Table 2: Characterization of the prepared EMB-NLCs formulations (F1-F9)*

Formula No.	EE% ±SD	Mean P. S. (nm)±SD	Z. P.(Mv) ±SD	PDI
F1	91.2±0.25	163.6±7.20	-35±0.25	0.306
F2	92.4±1.42	200.25±9.51	-32±0.98	0.445
F3	88.3±0.68	169.65±11.21	-32±0.68	0.272
F4	96.4±1.85	185.99±14.77	-30±14.23	0.287
F5	93.3±1.74	235.11±9.52	-29±1.58	0.462
F6	88.1±0.87	255.50±12.54	-30±2.32	0.582
F7	95.7±1.22	866.66±18.65	-29±0.58	0.743
F8	77.1±1.87	225.80±12.55	-24±1.25	0.63
F9	97.7±1.68	450.41±14.87	-25±1.32	0.703

*Results are represented as mean±SD, n = 3

Particle size

The particle size of NLCs as drug carriers is an important factor to consider. As shown in table 2, the mean P. S. for all prepared formulae was less than 500 nm, with the exception of F7 (866.66±18.65 nm), which used Precirol® ATO 5 and Poloxmer® 188 as lipid and surfactant, respectively. This finding is consistent with the previous study on thymoquinone NLCs; it could be due to the incompatibility of Precirol® ATO 5 and Poloxmer® 188, which has been verified via stability tests [23].

The solid lipid type had a significant impact on the mean P. S. of different formulations. NLCs made with Precirol® ATO 5 had the largest mean P. S., while smallest mean P. S. appeared with NLCs made from Gelucire® 43/01. This may be attributed to differences in lipid melting point, with Precirol® ATO 5 melting at 67–74 °C, GMS at 57–59 °C, and Gelucire® 43/01 at 42–44 °C. Since solid lipids have a high melting range, they have a greater melt viscosity that reduces the efficiency of the homogenization phase in reducing P. S. The mean viscosity of the melted lipid was calculated by the aid of Brookfield viscometer (LW, Brookfield, USA), and the viscosities of GMS, Gelucire® 43/01, and Precirol® ATO 5 were determined to be 34±3.7, 23±3.3, and 48±3.5 centipoise, respectively [24].

Additionally, the emulsifying characteristics of GMS and Gelucire® 43/01 helped in the development of NLCs with smaller particle sizes by facilitating emulsification. Since the PDI values were smaller than 0.3, the particle size distribution of F3 and F4 was very homogeneous. Other formulations seemed to be heterogeneous, with PDI more than 0.3, implying that they were unstable. A smaller PDI (0.2) confirmed a homogeneous vesicle, while a PDI larger than 0.3 reflects a high degree of particle size heterogeneity [25].

Zeta potential

Table 2 shows the Z. P. values of the various formulations. The Z. P. is an important tool for predicting the stability of the formed NLCs. Zeta potential with values greater than 30mV or smaller than-30mV are considered sufficient for NLC stabilization [26]. The Z. P. values ranged from-24±1.25 to-35±0.25 mV. Surfactants with steric hindrance characteristics, such as Tween® 60 and Poloxmer® 188, improved the stability of the formulated NLCs. As a result, the majority of the formulated NLCs are stable.

Morphology

TEM image of the optimized F1 is shown in fig. 1. The TEM image showed that the NLCs were oval, discrete or spherical and uniform

in shape indicating that the formulated NLCs had been uniformly dispersed. NLCs sizes of obtained by TEM were similar to zetasizer results.

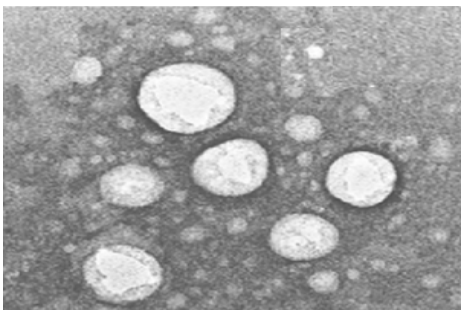

Fig. 1: TEM photograph of optimized EMB-NLCs formula (F1)

Thermal analysis

DSC is an excellent tool for investigating the crystallization of drug and its interactions with various components of NLCs by showing energy variations and temperature at phase transition.

DSC different thermograms of EMB, Gelucire® 43/01 and the lyophilized powder of formulated F1 are shown in fig. 2.

EMB's thermogram revealed the endothermic peak at about 163 °C, corresponding to its melting point and hence indicating its crystalline nature. The melting of Gelucire® 43/01 occurred at the peak at 44 °C. Lyophilized F1 indicated endothermic peak at 98.43 °C, which corresponds to the melting point of Gelucire® 43/01.

The shifting of peak of Gelucire® 43/01 from 44 °C to 98.43 °C is due to the nano size of NLCs and the use of surfactant and dispersion of lipid. The shift in peak, combined with the disappearance of the characteristic EMB endothermic peak at 163 °C, suggested that EMB entrapped in lipids phases was in an amorphous state.

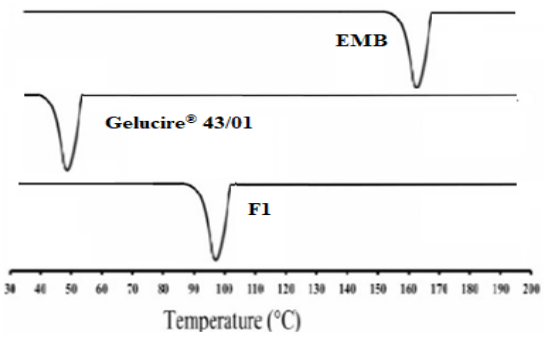

Fig. 2: DSC thermograms of EMB, Gelucire® 43/01 and formula (F1)

In vitro release studies

The *in vitro* experiment was done by using a reverse dialysis method in phosphate buffer (pH 6.8). The *in vitro* release of F1 compared to EMB suspension is showed in fig. 3. Formula F1 was selected for the *in vitro* release experiment based on the smallest value of P. S., highest value of Z. P. and appropriate EE%. It is clear that EMB released from formula F1 was 99.6±1.14% while EMB suspension released about 62.4±3.2% after 10 h. Class II drugs, in particular those whose absorption is limited by a rate-limiting step, have as their primary goal the improvement of dissolution. Formula F1 exhibited biphasic release stages. The first stage that lasted for 4 h was characterized by a rapid release pattern which could be attributed to EMB located around the particles and the steric stabilization of Poloxmer® 188 which surround surface of NLCs and could entrap some EMB molecules. The second stage was characterized by a slower release pattern that ended within 10 h as a result of the slow diffusion of EMB from the lipid [27]. Using kinetic fit, the EMB released from formula F1 in pH 6.8 was best fitted to the Higuchi diffusion model (r = 0.9845), which could be attributed to erosion of lipid matrix erosion.

Linear regression of formula F1 revealed that the time requested for 50% EMB release ($t_{1/2}$) was 2.8 h, and the release rate constant was approximately 30 h^{-1}.

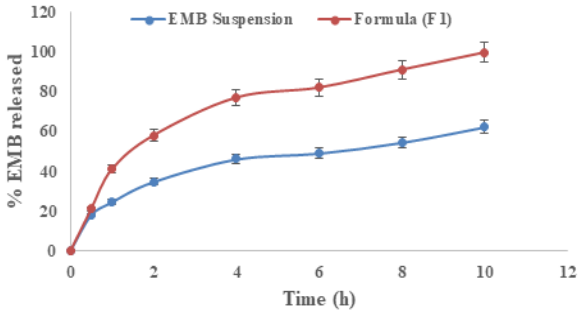

Fig. 3: *In vitro* release of EMB suspension and formula (F1)*, *Results are represented as mean±SD, n = 3

Bioavailability study

A pharmacokinetic study was conducted to investigate the possibility of using NLCs to increase EMB oral bioavailability. Pharmacokinetic parameters for formula F1, EMB suspension and commercial product (Ezetrol® 10 mg) were, then, calculated. Fig. 4 depicts the mean EMB plasma concentration–time profiles, and table 3 lists the calculated pharmacokinetic parameters. The pharmacokinetic parameters of formula F1 differed significantly ($p<0.05$) from those of EMB suspension and Ezetrol® 10 mg. EMB suspension and Ezetrol® 10 mg had T_{max} of 1.4 h and C_{max} of 9.5±1.7 µg/ml and 10.7±1.6 µg/ml, respectively. Formula F1, on the other hand, required 0.5 h longer to reach a maximum concentration. The C_{max} of formula F1 (25±2.4 µg/ml) was significantly ($p<0.05$) higher than that of EMB suspension and Ezetrol® 10 mg, which could be explained by the decrease in particle size from micron (EMB suspension and Ezetrol® 10 mg) to nanometer range (F1). This improved the surface area and thus the solubility extent of EMB and dissolution rate [28]. The relative bioavailability of formula F1 was 2.63-and 2.33-fold higher than that of EMB suspension and Ezetrol® 10 mg, respectively, indicating that incorporating EMB into NLCs significantly improves oral delivery of EMB.

Table 3: Pharmacokinetic parameters after single oral dose (25 mg/kg) of formula F1, EMB suspension and Ezetrol® 10 mg tablet to rats*

Pharmacokinetic parmeters	F1	EMB suspension	Ezetrol® 10 mg
C_{max} (µg/ml)	25±2.4	9.5±1.7	10.7±1.6
T_{max} (h)	1.9±0.15	1.4±0.2	1.4±0.25
Relative bioavailability		2.63	2.33

*Results are represented as mean±SD, n = 3

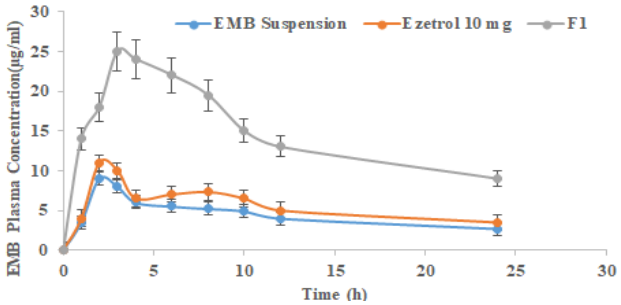

Fig. 4: Average plasma concentration-time curve after single oral dose of EMB-suspension, commercial product and formula (F1)*, *Results are represented as mean±SD, n = 3

CONCLUSION

EMB-NLCs have successfully formulated by using a variety of solid lipids and surfactants. Different formulae have been characterized and found to have P. S. ranging from 163.6±7.20 to 866.66±18.65 nm, Z. P. values ranging from-24±1.25 to-35±0.25 mV, and EE% greater than 77 percent. The DSC study has demonstrated the presence of amorphous state EMB with improved extent and rate of dissolution. The optimized formula (F1) has released more than the corresponding suspension. Formula F1 oral bioavailability has increased greater than 2.63-and 2.33-fold when compared to EMB suspension and Ezetrol® 10 mg tablet respectively. The improved plasma concentration of EMB-NLCs could be due to their nanosize and the presence of formulation excipients, which may aid lymphatic absorption. Finally, NLCs of poorly water soluble EMB has proved to be an effective method for increasing its pharmacological bioactivity and oral bioavailability.

ACKNOWLEDGMENT

The authors thank the entire Pharmacy staff at Mansoura University for their assistance in completing the bioavailability and pharmacokinetic studies.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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