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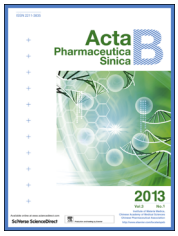

ORIGINAL ARTICLE

Tween 80 containing lipid nanoemulsions for delivery of indinavir to brain

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KEY WORDS

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Abstract Indinavir is a protease inhibitor used in the treatment of HIV infection. However, it has limited efficacy in eradicating the virus in the brain due to efflux by P-glycoprotein (P-gp) expressed at the blood–brain barrier (BBB). The objective of this work was to develop an o/w lipid nanoemulsion (LNE) of indinavir using Tween 80 as co-emulsifier to improve its brain specific delivery. LNEs were prepared with different compositions and were characterized for globule size, polydispersity index, zeta potential and *in vitro* drug release. Five formulations were then evaluated for drug content, entrapment efficiency and stability after which brain uptake studies were carried out using fluorescent labeled LNEs and pharmacokinetic (PK) and tissue distribution studies were conducted after intravenous administration in mice. Brain uptake of indinavir was shown to be improved for a 1% Tween 80 containing formulation (F5) compared to a formulation containing 0.3% cholesterol (F2). In PK studies, the brain level of indinavir subsequent to administration of F5 was significantly ($P<0.05$) higher than produced by administration of a drug solution (2.44-fold) or a control nanoemulsion (F1) (1.48-fold) or formulation F2 (1.6-fold). The increased brain specific accumulation of indinavir from F5 is probably due to enhanced low density lipoprotein-mediated endocytosis and P-gp inhibition by Tween 80 at the BBB. These results suggest Tween 80 containing LNEs could provide a simple but effective means of delivering indinavir to brain.

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1. Introduction

Infection with the human immunodeficiency virus (HIV) often results in progression to the acquired immune deficiency syndrome (AIDS). The HIV retrovirus is found in the brain soon after infection and leads to a variety of central nervous system adverse effects[1]. HIV in the periphery is significantly reduced by highly active antiretroviral therapy (HAART) comprised of multiple small molecule therapeutics. However, certain tissues including brain, macrophages and testis remain reservoirs for HIV infection even with HAART[1,2]. This is because the drugs used in HAART have only limited ability to cross the blood–brain barrier (BBB) into brain parenchyma.

Among the drugs used to treat HIV infection, indinavir is an anti-retroviral protease inhibitor used as a part of the HAART regimen in patients with AIDS. The sub-therapeutic concentration of indinavir in brain leads to failure of treatment and results in the development of drug-resistant viral strains in brain despite the presence of adequate plasma concentrations[3]. The reason for the sub-therapeutic concentration in brain is due to efflux by P-glycoprotein (P-gp) expressed at the BBB[4–7]. Increasing the brain concentration of indinavir by improving its permeation of the BBB is therefore a key to reducing the viral burden in brain during antiretroviral therapy.

Different strategies can be used to improve levels of indinavir in brain, including co-administration of P-gp inhibitors[8] and use of colloidal carriers[9]. Among the various colloidal carriers, lipid nanoemulsions (LNEs) have been used as drug carriers for poorly aqueous soluble drugs such as indinavir to improve their oral bioavailability[10], sustained release[11] and targeting[12,13]. The LNEs of indinavir coupled with holo-transferrin[14] or pegylation[15] or by incorporation of lipoaminoacids as ligands[16] have been reported to provide brain specific/targeted delivery of indinavir. Furthermore, administration of a suspension of a lipid-associated indinavir complex reduced the HIV viral RNA load in macaques[17].

Tween 80 (Polysorbate 80, polyoxyethylene sorbitan monooleate) is a hydrophilic nonionic surfactant widely used in emulsification and dispersion of substances in medicinal and food products[18,19]. Not only it is useful as an emulsifying agent in emulsions[20] and multiple emulsions[21,22], and as a co-emulsifying agent for reducing the globule size in lipid emulsions[23], but also has the advantage in relation to indinavir of being an inhibitor of intestinal P-gp. Thus it has been used to increase the permeability of numerous drugs in rats *in vivo*[24], in Caco-2 cells *in vitro*[25], in tissues such as rat intestinal membrane *ex vivo*[26] and in the inverted rat intestinal sac[27]. In addition, it has been used in conjunction with nanoparticles to improve brain specific delivery of several drugs including tacrine[28], doxorubicin[29], the hexapeptide dalargin[30], loperamide[31] and tubocurarine[32], by increasing LDL-mediated endocytosis.

The objective of this work was to develop indinavir LNEs using lecithin as emulsifier and Tween 80 as co-emulsifier. The effect of Tween 80 containing LNEs on brain delivery of indinavir was investigated in mice by determining plasma and tissue levels of drug after intravenous administration. The therapeutic availability and targeting potential were calculated to evaluate the efficacy of Tween 80 containing formulations in comparison to drug solution and control LNEs.

2. Materials and methods

2.1. Materials

Indinavir was generously provided by Matrix Laboratories (Hyderabad, India). Egg phosphatidyl choline and EPC-80 were gifts from Lipoid (Germany). Refined soyabean oil was obtained from Fluka (Mumbai, India). Tween 80, cholesterol, glycerol, methanol and acetonitrile were products of Merck (Mumbai, India). DiD oil (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate), α-tocopherol, triethylamine and phosphoric acid were purchased from Sigma (Mumbai, India). Sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride and oleic acid were from S.D. Fine Chemicals (Mumbai, India). Centrisart tubes and dialysis membranes were products of Sartorius (Germany) and HiMedia (Mumbai, India), respectively. All other chemicals were of reagent grade and used as received.

2.2. Preparation of LNEs

LNEs were prepared with the compositions shown in Table 1 using a hot homogenization and ultrasonication process. The formula consisted of 10% *w/v* soybean oil as the oil core, 1.2% *w/v* EPC-80 as a phospholipid emulsifier, oleic acid (0.3% *w/v*) as a negative charge inducer, α-tocopherol (0.25% *w/v*) as antioxidant and glycerol (2.25% *w/v*) to maintain isotonicity for intravenous

Table 1 Composition of lipid nanoemulsions of indinavir.

Formulation ingredient (% *w/v*)	No cholesterol	Cholesterol (0.3%)		Tween 80			
	F1	F2	*F2	F3	F4	F5	*F5
Indinavir	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Soya bean oil	10	10	10	10	10	10	10
Egg lecithin	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Cholesterol	0	0.3	0.3	0.3	0.3	0.3	0.3
α-Tocopherol acetate	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Oleic acid	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Tween 80	0	0	0	0.2	0.6	1.0	1.0
Glycerol	2.25	2.25	2.25	2.25	2.25	2.25	2.25
Did oil (Dil C_{18})	–	–	0.1	–	–	–	0.1
Doubly distilled water (mL)	10	10	10	10	10	10	10

*Fluorescent dye (DiD oil) containing LNEs.

administration. Oil soluble substances were added to soya bean oil (oil phase) and water soluble substances to the aqueous phase. Both phases were then heated to 70 °C after which the aqueous phase was added to the oil phase with stirring and the mixture homogenized for about 3 min at 15,000 rpm (Homogenizer Diax 900, Heidolph, Germany). Finally, the mixture was sonicated (750 W, Vibra Sonics, Sonics and Materials Inc., USA) for about 20 min at 50% amplitude. LNEs were prepared using 0.2%, 0.6% and 1% *w/v* Tween 80 keeping the other ingredients constant.

2.3. Determination of globule size (GS) and polydispersity index (PDI)

LNEs diluted 1:100 with doubly distilled water were put in a cuvette and placed inside the sample holder of a Malvern Nano ZS90 zeta sizer (Malvern, UK) for measurement of GS and PDI. The observations for GS determination were recorded at a light scattering angle of 90° and a temperature of 25 °C. The photon correlation spectroscopic principle was used to determine the hydrodynamic diameter of globules *via* Brownian motion.

2.4. Determination of zeta potential (ZP)

LNEs diluted 1:100 with doubly distilled water were placed in a disposable cuvette and inserted into the sample holder of the Malvern Nano ZS90 zeta sizer. The ZP value was measured by the built-in software which uses the Helmholtz–Smoluchowski equation describing the electrophoretic mobility of globules.

2.5. In vitro drug release

Dialysis through cellulose membranes (DM 60, molecular weight cut-off 12000, Himedia, Mumbai, India) was used to study *in vitro* drug release based on a reported method[8] with slight modification. A 100 mL mixture of 30% ethanol and phosphate buffer pH 7.4 in a 250 mL beaker was used as dialysis medium. The amount of drug in samples collected over 12 h was determined by UV absorption at 215 nm using an ELICO SL 159 UV–vis spectrophotometer (Hyderabad, India). Cumulative percentage drug release *vs*. time was plotted to evaluate the release pattern of drug from a drug solution and the different formulations.

2.6. Determination of drug content and entrapment efficiency (EE)

Indinavir in LNEs and aqueous media was determined by reversed phase HPLC[8] using an LC-10AT VP solvent delivery system (Shimadzu, Kyoto, Japan), an SPD-10 A VP variable wavelength detector (Shimadzu) set at 215 nm, a Lichrospher® C18 analytical column (250 nm × 4.6 mm, 5 µm) (Merck, Mumbai, India) at ambient temperature and an injector fitted with a 20 µL loop. The mobile phase consisted of 68% phosphoric acid solution adjusted to pH 5.5 with triethylamine and 32% acetonitrile delivered at a flow rate of 1 mL/min. Sample preparation and assay validation is described in Section 2.9.2. Drug content of indinavir loaded LNEs (0.5 mL) was determined by first diluting samples with 10 mL chloroform/methanol mixture (1:1) and making a final dilution in mobile phase. EE was determined by measuring the concentration of free drug in the aqueous medium after ultrafiltration[33] through the filter membrane (molecular weight cut off 20 k Da) of Centrisart tubes at the base of the sample receiver chamber. About 2 mL of a formulation was placed in the donor chamber and centrifuged at 6500 rpm for 15 min (C24, Remi instruments Ltd., Mumbai, India).

2.7. Stability studies

Stability of optimized LNEs was evaluated for the effects of centrifugal, thermal and dilution stress and on storage at 4 and 25 °C for 6 months. Centrifugal stress involved taking 1 mL aliquots of LNEs in 1.5 mL Eppendorf tubes and centrifuging (Heraeus Biofuge, Germany) at 13,000 rpm for 10 min. The creaming volume percentage for each LNE was calculated as previously described[34] and the formulations compared. Thermal stress involved subjecting LNEs to autoclaving at 121 °C for 15 min and then determining any changes in GS, PDI and ZP. Dilution stress involved diluting LNEs with doubly-distilled water in ratios of 1:50, 1:100, 1:200, 1:500, 1:1000 and 1:5000 and evaluating GS and ZP. Stability on storage was investigated by taking samples (1 mL) into 2 mL Eppendorf tubes after 1 day and after 1, 2, 3, 4 and 6 months and measuring GS, PDI and ZP.

2.8. Brain uptake studies by fluorescence microscopy

Male Swiss-albino mice (Mahaveera Enterprises, Hyderabad, India) weighing 24–26 g were used in the study. The animals were kept in cages and provided with water and standard diet (NIN, Hyderabad) *ad libitum*. After animals were acclimatized to the environmental conditions, they received single 5 mg/kg doses of formulations *F2 or *F5 containing a fluorescent dye (DiD oil) by tail vein injection. After 0.25 h, animals were anaesthetized, decapitated and the whole brains removed and frozen at −80 °C. Frozen brains were immediately treated with tissue freezing medium (Leica) and 5 µm microsections prepared using a cryomicrotome (Cryostat, Leica CM 3050 S). The microsections were mounted on glass slides and covered with aluminum foil to prevent the loss of fluorescence due to light. The microsections were photographed using a fluorescent microscope (Nikon E 800, Nikon, Japan) with excitation and emission wavelengths of 520 and 540 nm, respectively.

2.9. Pharmacokinetic (PK) and tissue distribution studies

These studies were performed using protocols, procedures and methods reported earlier[14] and briefly described below.

2.9.1. Animals and sample collection

The protocol for the animal experiments was reviewed and approved by Animal Ethical Committee of our institution. A total of 72 male Swiss-albino mice (weight 24–26 g) were randomly divided into four groups ($n = 18$ per group) and kept in cages provided with water and standard diet (NIN, Hyderabad) *ad libitum*. Before the day of an experiment, mice were fasted overnight with water provided *ad libitum*. On the day of an experiment, groups of mice were anaesthetized with ether and given a single intravenous (tail vein) injection of either indinavir or an indinavir formulation (F1, F2 or F3; 5 mg/kg indinavir) dissolved in a saline-propylene glycol-ethanol vehicle (5:4:1, *v/v/v*; 1 mg/mL).. The animals were kept unrestrained during the entire study. At 0.25, 0.5, 1, 2, 4 and 6 h after the dose, animals were anesthetized with ether and a blood sample withdrawn from the retro-orbital sinus vein into a heparinized polypropylene tube. Animals were then decapitated and tissue

Table 2 Effect of concentration of Tween 80 on globule size, PDI and zeta potentials of formulations ($n=3$).

Formulation	Variable ingredient (% *w/v*)	Size (nm)	PDI	Zp (mV)
F1	No cholesterol	329.5 ± 3.08	0.079 ± 0.05	−35.8 ± 6.04
F2	Tween 80 (0)	237.0 ± 5.08	0.245 ± 0.01	−31.3 ± 1.80
F3	Tween 80 (0.2)	226.0 ± 2.00	0.145 ± 0.03	−37.3 ± 1.20
F4	Tween 80 (0.6)	208.5 ± 3.50	0.107 ± 0.09	−38.5 ± 3.50
F5	Tween 80 (1)	196.0 ± 3.54	0.068 ± 0.03	−40.1 ± 4.02

samples (whole brain, lungs, heart, liver, kidney and spleen) were collected. Plasma was separated by centrifuging blood at 13,000 rpm for 10 min and tissue samples were washed with normal saline. All samples were frozen at −80 °C until drug analysis.

2.9.2. *Determination of drug in biological samples*

The reversed-phase HPLC method described in Section 2.6 was slightly modified and used for the determination of indinavir concentration in biological samples. All solutions were prepared in water:acetonitrile (50:50 *v/v*). Stock solutions (1 mg/mL) of indinavir were prepared and diluted to give standard solutions of concentration 0.1, 0.25, 0.5, 1, 2.5 and 5 µg/mL. A stock solution (1 mg/mL) of verapamil hydrochloride as internal standard (IS) was diluted to give a working 5 µg/mL IS solution. To 500 µL tissue homogenate (either sample or control obtained from drug free animals) prepared in ice cold saline [0.2 g tissue per mL prepared using a tissue homogenizer (Remi Instruments Ltd., Mumbai, India) at 6000 rpm], 100 µL perchloric acid (60%) was added and, after vortex-mixing for 5 min, the resulting suspension was centrifuged at 13,000 rpm for 10 min (Biofuge, Heraeus, Germany). To 300 µL plasma or tissue homogenate supernatant, 100 µL standard indinavir solution, 100 µL IS working solution, 1 mL 4 M KOH solution and 5 mL diethyl ether were added and the resulting mixture vortexed for 15 min and then centrifuged at 6500 rpm for 10 min. The organic layer was separated and evaporated to dryness under vacuum (GL 66, Toshniwal, Mumbai, India) after which the residue was reconstituted in 100 µL 100 µM phosphoric acid and 20 µL of the resulting solution was injected to HPLC. Linear regression of calibration curves gave with the following equations and correlation coefficients: Plasma $Y=0.263X+0.001$, $R^2=0.998$; brain $Y=0.172X+0.001$, $R^2=0.997$; lung $Y=0.208X+0.026$, $R^2=0.992$; heart $Y=0.220X+0.017$, $R^2=0.990$; liver $Y=0.281X+0.003$, $R^2=0.996$; kidney $Y=0.258X+0.009$, $R^2=0.996$; and spleen $Y=0.204X+0.008$, $R^2=0.996$.

2.9.3. *Pharmacokinetic parameters and statistical analysis*

Pharmacokinetic parameters (C_{max}, T_{max}, AUC_{0-6h}, $t_{1/2}$, MRT) and therapeutic availability (TA) were calculated using Kinetica software (version 5.0) and are expressed as mean ± standard deviation (SD). Comparison between groups was done using the paired students *t*-test with $P<0.05$ considered statistically significant.

2.10. *Determination of targeting potential*

Tissue-to-serum ratios of drug after administration in different formulations and at different times after dosing were calculated to determine the targeting potential of the formulation to brain. A ratio greater than 1 indicates good brain targeting of the formulation.

3. Results

3.1. *Characterization of LNEs*

GS, PDI and ZP of LNEs are shown in Table 2. As the Tween 80 concentration increased, there was a slight reduction in GS with no appreciable increase in ZP values. In terms of the *in vitro* drug release profiles over 12 h, there was no significant difference in cumulative drug release from LNEs F2–F5 with all formulations giving better release than F1 and drug solution (Fig. 1). Overall, formulation F5 had lower GS, relatively better release and higher ZP than the other formulations and was used in further studies.

3.2. *Total drug content and EE*

The total drug content of formulations F1, F2 and F5 was 10.01 ± 0.09, 9.85 ± 0.14 and 9.80 ± 0.11 mg/mL respectively with corresponding EE values of 99.0 ± 0.15, 98.8 ± 0.03, and 98.9 ± 0.2, respectively.

3.3. *Stability studies*

Centrifugal stress is a rapid way to check the stability of LNEs. The percentage creaming values of F1, F2 and F5 were 96.0 ± 1.0, 97.8 ± 0.3 and 99.0 ± 0.5 and there was no appreciable change in GS, PDI and ZP of the selected formulations upon autoclaving (thermal stress) (Fig. 2) or dilution (Table 3). In terms of stability on storage, GS increased by 10%–25% but ZP remained fairly constant over the 6 months. On this basis, the LNEs were physically stable at 4 °C and room temperature for 6 months.

3.4. *Brain uptake studies by fluorescence microscopy*

Fluorescent images taken 0.25 h after injection of fluorescent dye (DiD oil, Dil C_{18}) incorporated LNEs *via* the tail vein indicate that formulation F5 gives more intense fluorescence of brain tissue than F2 (Fig. 3), indicating greater brain uptake of this emulsion.

3.5. *PK and tissue distribution studies*

Plasma levels of drug after adminstration of drug solution were higher than those after formulations F1, F2 and F5 at 0.25 h (Fig. 4) but were not significantly different at other time points. In tissue distribution studies, the brain level of indinavir (Fig. 5) from F5 was higher than that produced by drug solution. F1 and F2

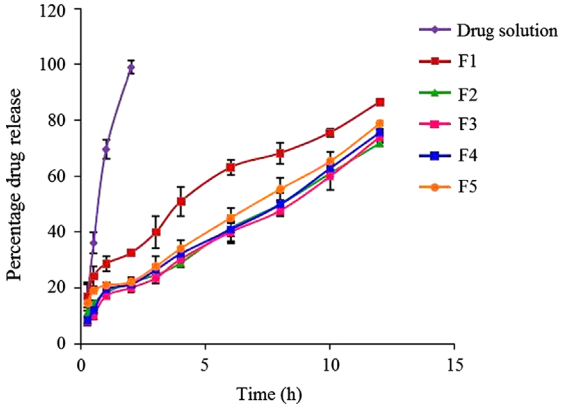

Figure 1 Cumulative percentage indinavir release from drug solution and formulations F1, F2, F3, F4 and F5 ($n=3$) as determined by equilibrium dialysis.

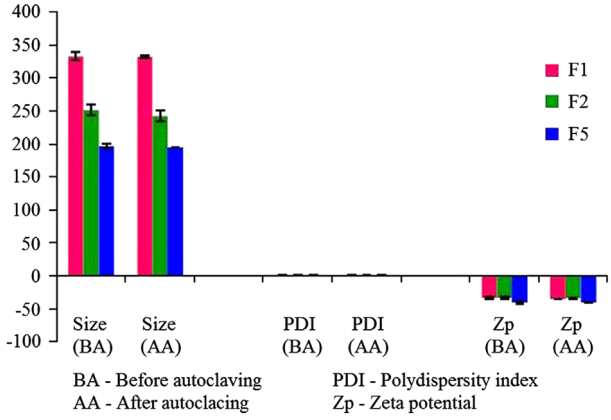

Figure 2 Effect of autoclaving on GS, PDI and ZP of formulations F1, F2 and F5 ($n=3$).

produced greater accumulation of drug in brain tissue at all time points but there was no significant difference between levels produced by drug solution, F1 and F2 at different time points.

The AUC_{0-6h} and therapeutic availability (TA) of indinavir are presented in Table 4 (other parameters are not shown). The AUC_{0-6h} of indinavir for drug solution[14] was higher in plasma than for formulations F1, F2[14] or F5 whereas the indinavir level in brain tissue for F5 was higher than for drug solution or formulations F1 and F2. The TA of drug for F5 in brain tissue was 2.44-fold than for drug solution, 1.48-fold that for F1 and 1.6-fold that for F2. In other tissues (lung, liver, kidney and spleen), TA was <1 except in heart where TA was >1 for F5 and drug solution but not for F1 or F2.

The brain-to-plasma ratio for F5 was higher (1–3-fold) than for all other formulations at all-time points (Fig. 6). Although the ratio was <1, F5 delivered indinavir to brain better than the other formulations.

4. Discussion

4.1. *Preparation and characterization of LNEs*

The content of EPC-80 of 1.2% *w/v* used in the preparation of LNEs is reportedly the minimum required for preparation of lipid emulsions[35]. Phospholipids are weak emulsifiers[36] such that the incorporation of cholesterol into the formulation is required to enhance rigidity at the interface, produce a more stable formulation[37] and retard drug release. In this study, the incorporation of cholesterol resulted in a decrease in GS but no appreciable change in ZP (results not shown). Incorporation of different concentrations of Tween 80 also resulted in a decrease in GS but little change in ZP possibly due to the non-ionic nature of Tween 80.

4.2. *In vitro drug release*

There was no difference in cumulative release of drug from formulations F2–F5 which was slower than from drug solution and F1. LNEs prepared with Tween 80 are smaller in GS and therefore have greater

Table 3 Effect of dilution on globule size, PDI and zeta potentials of optimized formulations ($n=3$).

Formulation	Dilution factor	Size (nm)	PDI	Zp (mV)
F1	1:50	340.8±3.8	0.08±0.04	−31.07±0.54
	1:100	351.0±2.5	0.10±0.01	−29.81±1.35
	1:200	358.7±5.9	0.21±0.06	−27.50±0.56
	1:500	365.7±7.4	0.26±0.10	−27.93±2.60
	1:1000	366.1±12.3	0.25±0.04	−27.43±5.39
	1:5000	379.1±13.5	0.29±0.05	−26.23±2.42
F2	1:50	256.3±5.2	0.07±0.20	−33.70±2.91
	1:100	263.5±1.6	0.17±0.20	−31.30±0.43
	1:200	265.5±8.9	0.25±0.41	−30.53±2.62
	1:500	262.4±5.2	0.29±0.12	−29.46±2.41
	1:1000	269.2±3.7	0.30±0.04	−28.50±1.53
	1:5000	272.5±1.7	0.29±0.10	−27.10±3.34
F5	1:50	200.1±3.2	0.05±0.04	−34.00±3.51
	1:100	202.4±5.0	0.10±0.11	−33.80±2.26
	1:200	216.0±5.4	0.15±0.05	−30.10±2.21
	1:500	233.8±4.5	0.19±0.05	−29.00±3.52
	1:1000	245.8±5.6	0.25±0.06	−28.20±3.84
	1:5000	257.5±3.0	0.15±0.13	−26.40±0.19

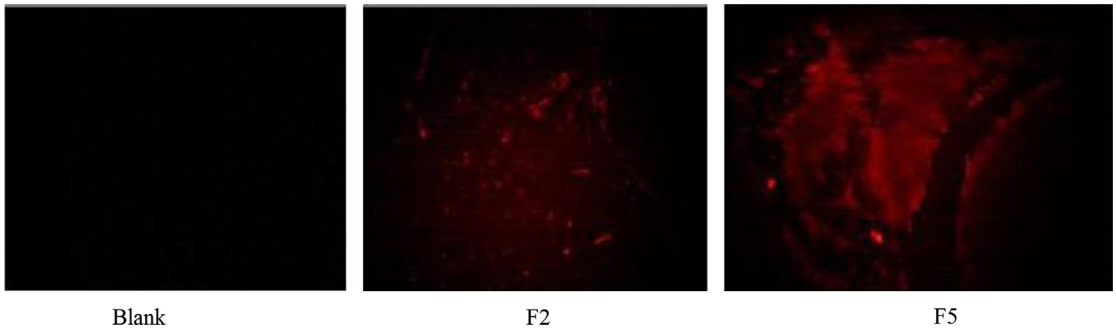

Figure 3 Fluorescent images (400 ×) of mice brain 0.25 h after intravenous injection of DiD oil incorporated formulations F2 and F5 in comparison to blank brain tissue.

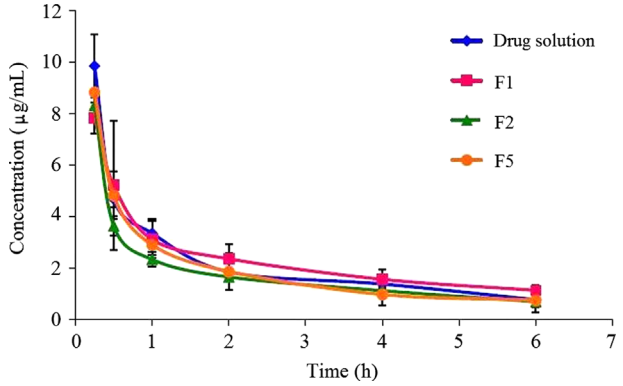

Figure 4 Plasma profiles of indinavir from drug solution and formulations F1, F2 and F5 after intravenous administration in mice ($n=3$).

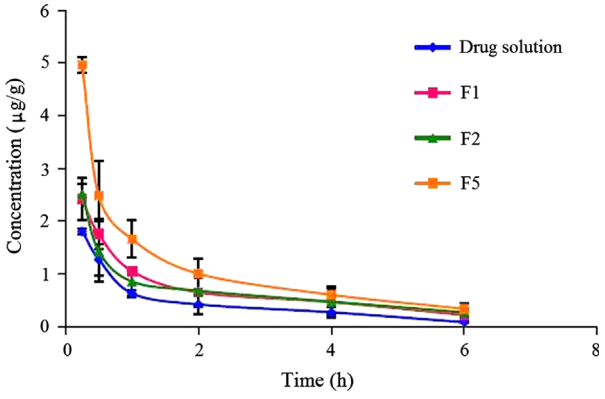

Figure 5 Brain profiles of indinavir after intravenous adminstration of indinavir solution and formulations F1, F2 and F5 in mice ($n=3$).

surface area for drug release. Furthermore, the hydrophilicity at the surface of globules is also increased. However, these two properties did not greatly affect the cumulative release of drug at 12 h. The decrease in GS produced by 1% Tween 80 concentration was optimal and this formulation was selected for further study.

4.3. *Stability studies*

It has been reported that emulsions with higher creaming values have better stability to centrifugal stress[34]. In this study, all the optimized formulations showed higher creaming values indicating the stability of the LNEs (data not shown). In terms of thermal stress to which LNEs for intravenous use would be subjected to ensure sterility, there were no considerable changes in GS or ZP values on autoclaving indicating the stability of LNEs F1, F2 and F5. Such stability of phospholipid emulsions after sterilization has also been reported elsewhere[38]. In relation to dilution stress, dilution of an emulsion disturbs the rigidity of the surfactant layers at the interface leading to changes in ZP[39]. In this study, there were no significant differences in GS and ZP between the tested formulations, indicating all the emulsions were stable.

In terms of physical stability of LNEs during storage, there were no appreciable changes in GS, ZP or PDI, indicating that the LNEs were stable on storage at 4 °C and room temperature for up to 6 months.

4.4. *Fluorescent dye studies*

Formulation F5 (Fig. 3) showed more intense fluorescence in brain than formulation F2 possibly due to a cooperative effect between cholesterol and Tween 80 at the interface of the emulsion globules leading to preferential adsorption of apolipoprotein E (Apo-E) and/or apolipoprotein B (Apo-B) onto the globule surface. Presumably these modified globules mimic LDL particles and are transported into brain by LDL receptor mediated endocytosis[9].

4.5. *PK and tissue distribution*

In the PK study, the higher drug levels in plasma after administration of formulation F5 compared to F2 may be due to the greater hydrophilicity of globules caused by the presence of Tween 80. Formulation F2 produced lower levels of drug in plasma than F1 and drug solution which could be due to the cholesterol in F2 causing preferential adsorption of Apo-E onto the surface of globules allowing them to mimic LDL particles and undergo rapid uptake by organs of the reticulo-endothelial system (RES)[40] leading to lower drug levels in plasma.

In brain tissue, formulations F1 and F2 produced higher indinavir levels ($P<0.05$) than drug solution. There are several possible reasons for this increased uptake as proposed by Kreuter[41] for entry of nanoparticles into brain. Solid lipid nanoparticles (SLNs) and LNEs differ only in their physical state (SLNs contain solid lipid; LNEs contain liquid lipid) and possess similar surface properties for the same emulsifier. However, LNEs may have the advantage of superior flexibility allowing them to squeeze through the endothelial lining. Just like SLNs, LNEs can enter the brain by absorption of LNE globules onto brain capillary walls leading to increased retention of LNE globules in brain capillaries and

Table 4 AUC_{0-6h} and therapeutic availability (TA) of optimized LNE formulations.

Sample	Parameter	Formulation			
		Dug solution[a]	F1	F2[a]	F5
Plasma	AUC_{0-6h} (μg h/mL)	13.09 ± 0.57	14.04 ± 2.91	10.69 ± 0.55	11.74 ± 1.85
	TA	1.00	1.07[b]	0.82[b]/0.76[c]	0.89[b]/0.83[c]/1.09[d]
Brain	AUC_{0-6h} (μg h/mL)	2.65 ± 0.57	4.36 ± 1.23[‡]	4.04 ± 1.35[‡]	6.48 ± 1.11[‡,†,¶]
	TA	1.00	1.64[b]	1.52[b]/0.92[c]	2.44[b]/1.48[c]/1.6[d]
Lungs	AUC_{0-6h} (μg h/mL)	6.82 ± 0.59	3.85 ± 0.64	4.36 ± 0.35	4.76 ± 1.30
	TA	1.00	0.56[b]	0.64[b]/1.13[c]	0.69[b]/1.24[c]/1.09[d]
Heart	AUC_{0-6h} (μg h/mL)	12.57 ± 1.53	7.7 ± 1.10	8.88 ± 0.42	10.04 ± 3.88
	TA	1.00	0.61[b]	0.71[b]/1.15[c]	0.8[b]/1.30[c]/1.13[d]
Liver	AUC_{0-6h} (μg h/mL)	6.49 ± 1.66	4.63 ± 0.09	4.97 ± 1.70	5.2 ± 2.07
	TA	1.00	0.71[b]	0.76[b]/1.07[c]	0.8[b]/1.12[c]/1.04[d]
Kidney	AUC_{0-6h} (μg h/mL)	9.15 ± 0.48	8.48 ± 2.24	6.75 ± 1.04	5.76 ± 1.42
	TA	1.00	0.93[b]	0.74[b]/0.79[c]	0.63[b]/0.67[c]/0.85[d]
Spleen	AUC_{0-6h} (μg h/mL)	6.6 ± 2.07	7.69 ± 0.97	6.94 ± 0.74	6.12 ± 1.55
	TA	1.00	1.17[b]	1.05[b]/0.90[c]	0.93[b]/0.8[c]/0.88[d]

[a]Data of drug solution and F2 are from reference 30.
[b]Indicates TA in comparison to drug solution.
[c]Indicates TA in comparison to F1.
[d]Indicates TA in comparison to F2.
[‡]Statistically significant in comparison to drug solution ($P<0.05$).
[†]Statistically significant in comparison to control emulsion ($P<0.05$).
[¶]Statistically significant in comparison to cholesterol emulsion ($P<0.05$).

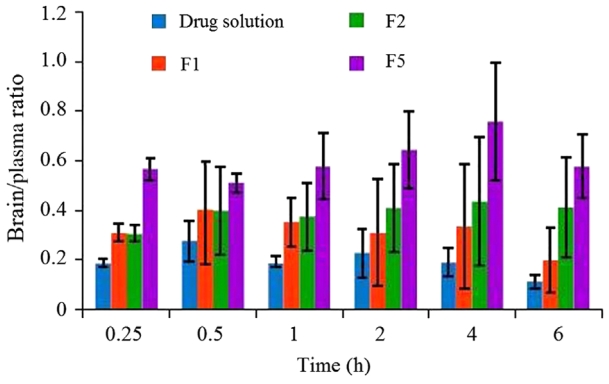

Figure 6 Brain-to-plasma ratios of indinavir after intravenous administration of drug solution and formulations F1, F2 and F5 in mice ($n=3$).

creating a higher concentration gradient across endothelial cells[42]. Endocytosis and transcytosis may also be possible mechanisms for entry of LNEs into brain because of their lipophilic nature.

The indinavir concentration in brain was increased significantly ($P<0.05$) for LNEs containing Tween 80 in comparison with drug solution, F1 and F2. Kreuter[43] proposed a mechanism for entry of Tween 80 containing nanoparticles into brain. The reason for the increased uptake of LNEs containing Tween 80 across the BBB is most likely endocytosis *via* the LDL receptor by endothelial cells lining brain capillaries. This endocytosis is mediated by the adsorption of Apo-B and/or Apo-E onto globules from the blood. The LNEs can then mimic the lipoprotein and the drug may either be released within these endothelial cells followed by passive diffusion into the brain or be transported into the brain by transcytosis. Furthermore, Tween 80 has the ability to inhibit intestinal P-gp and has been used to increase the permeability of numerous drugs in models of the intestinal wall. Inhibition of P-gp at the BBB may be another mechanism by which Tween 80 containing LNEs improve brain levels of indinavir.

In other tissues such as lung, liver, heart and kidney, the TA values of drug solution were greater than that for F1, F2 and F5 but in spleen the TA value was slightly greater than 1 for F1 and F2 formulations. In heart the TA for F5 was >1 compared to F1 and F2 possibly due to high levels of lipoprotein lipase present in heart which may induce lipolysis of LNE globules and result in the higher levels of indinavir observed. The observation that the TAs of F5, F2 and F1 in kidney were <1 may suggest LNEs of indinavir will produce less nephrolithiasis and crystalluria, common side effects of indinavir.

The brain-to-plasma ratio of F5 was much higher (1–3-fold) than for all other formulations indicating preferential accumulation of indinavir in brain for Tween 80 containing LNEs (Fig. 6). F2 exhibited higher brain:plasma ratios than drug solution which could be due to LDL mediated endocytosis. However, Tween 80 as co-emulsifier in F5 improved the drug level of indinavir in brain in comparison to drug solution and formulations F1 and F2. Together with the results of our previous studies[14,15], it can be concluded that transferrin, PEG 2000, PEG 5000, stearyl amine and Tween 80 can be used to prepare LNEs with improved brain specific delivery.

5. Conclusions

Stable lipid nanoemulsions containing Tween 80 were prepared, characterized and evaluated for improving the brain specific

delivery of indinavir. Fluorescent microscopy and pharmacokinetic studies in mice clearly demonstrated improved uptake of LNEs by brain tissue in comparison to control formulations. In fact, Tween 80 containing LNEs increased the brain specific delivery of indinavir and may prove to be useful in reducing the viral load in brain in chronic HIV infection.

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