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Research Article

Development of Indinavir Submicron Lipid Emulsions Loaded with Lipoamino Acids—*In Vivo* Pharmacokinetics and Brain-Specific Delivery

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Abstract. The aim of our present work was to develop indinavir O/W submicron lipid emulsions (SLEs) loaded with lipoamino acids for specific delivery to brain. Tetradecyl aspartic acid (A) and decyl glutamic acid (G) loaded stable SLEs of indinavir having a mean size range of 210–220 nm and average zeta potential of −23.54±1.2 mV were developed using homogenization and ultrasonication. The cumulative % drug release from different SLEs varied in between 26% and 85%. The formulations, SLE, SLE-A3, and SLE-G3 were stable to the centrifugal stress, dilution stress, and storage at RT. The total drug content and entrapment efficiency were determined by HPLC method. During pharmacokinetic studies in male Wistar rats there was no significant difference in the serum levels of indinavir for SLE, SLE-A3 and SLE-G3 formulations at all time points. In tissue distribution studies, the therapeutic availability (TA) of indinavir in brain and kidneys for SLE-A3 were 4.27- and 2.66-fold whereas for SLE-G3 were 2.94 and 2.12 times, respectively, higher than that of indinavir solution. But when compared with that of SLE, in brain tissue the levels of indinavir from SLE-G3 and SLE-A3 varied in between 2.5- and 3.38-fold. While in case of the kidney, it was between 1.23- and 1.54-fold only. However, the TA is not significantly different in tissues like the heart, liver, and spleen. Thus, brain-specific delivery of indinavir was improved by including tetradecyl aspartic acid and decyl glutamic acid in submicron lipid emulsions.

KEY WORDS: brain targeting of drugs; indinavir; lipoamino acids; submicron lipid emulsion; tissue distribution studies.

INTRODUCTION

Indinavir, an HIV protease inhibitor used in treatment of AIDS, possesses favorable antiviral, immunological, and clinical characteristics (1). The anti-HIV activity of indinavir was found to be concentration dependent (2), sub-optimal concentrations leading to therapeutic failure as well as the emergence of drug-resistant viral strains in tissues particularly the central nervous system (CNS), despite of adequate plasma concentrations (3). The reason for sub-therapeutic concentration of indinavir in brain is due to efflux by P-glycoprotein (Pgp) expressed in blood–brain barrier (BBB) (4). The maintenance of adequate therapeutic concentration of drugs in CNS is the key to the success of antiretroviral therapy. Thus, permeation of adequate amount of indinavir across BBB is essential.

L-amino acid transporter 1 (LAT 1) or system L is a major route for transportation of branched or aromatic amino acids into living cells. This transport system is also essential for the penetration of amino acids through the BBB and the placental barrier (5). The transport of large neutral amino acids across the BBB is mediated by a large neutral amino acid transporter (6). A remarkable characteristic of system L is its broad substrate selectivity, which enables the transporter to accept amino acid-related compounds. Lipidic α-amino acids (LAAs) are the class of compounds combining structural features of amino acids with those of fatty acids. Lipoamino acids are substrates for System L as they have α-amino and carboxylic acid groups attached to the same carbon atom plus a hydrophobic side group (7). LAAs combine the physico-chemical properties of both lipids and amino acids due to their amphipathic structure; LAAs linkage to drugs, apart from enhancing their lipophilicity, can also facilitate their interaction with cell membranes and penetration across absorption and biological barriers (8). Conjugation of drug molecules to lipoamino acids (LAA) has shown to increase biological uptake and intracellular concentration of drugs (8–10). The LAAs are also used to develop lipid core peptide systems useful for delivery of genes, drugs, and vaccines (11). The mechanism of uptake of lipoamino acid-loaded lipid emulsions (SLEs) is by carrier-mediated endocytosis, which may involve the binding of lipoamino acids to the receptor and internalization of the attached carrier by endocytosis mechanism.

Many novel drug delivery systems (12) like liposomes, submicron lipid emulsions, and nanoparticles were proposed to deliver the pharmacological agents to desired site at therapeutically optimal dosage regimen. This site specific or targeted delivery using specific ligands combined with delivery at an optimal rate would not only improve the efficacy of drug but would also reduce the possible unwanted side effects of drug thus improving the therapeutic index (13).

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Many parenteral lipid emulsions are currently available in market for nutritional (Lipofundin®, Intralipid®, Elolipid®, Neutralipid®, *etc.*) and therapeutic (Diagemuls®, Diprivan®, Liple®, *etc.*) applications. Submicron lipid emulsions are potential drug carriers for lipophilic and amphiphilic drugs with many favorable properties such as biocompatible, biodegradable, stable and easy to prepare and handle (14). The basic structure is a neutral lipid core (*i.e.*, triglyceride) stabilized by a monolayer of amphiphilic lipid (phospholipids). Lipid emulsion systems can be scaled up industrially. Lipid emulsions have been studied as parenteral drug carriers for sustained release and organ targeting (14). By using the lipid emulsions, direct contact of the drug with the body fluids and tissues can also be avoided.

Indinavir is a known substrate for Pgp and prone for efflux transport at BBB and resulting in low concentrations in brain. For improving drug levels in brain, indinavir being a lipophilic drug (log P=3.2) can be formulated as parenteral SLEs. The aim of this work is to prepare, characterize and evaluate submicron oil in water (O/W) lipid emulsions containing lipoamino acids for brain-specific delivery of indinavir using an animal model. In this work, we have used two lipoamino acids with varying lipid chain length, tetradecyl aspartic acid (A14) and decyl glutamic acid (G10). The fatty acid chains were linked to the amino acids by ester linkage and possessed amphiphilic properities (15). Different concentrations were used to prepare SLEs and optimized formulations were developed based on size, zeta potential, and *in vitro* drug release. Furthermore, the pharmacokinetics and tissue distribution studies were conducted in male Wistar rats; the therapeutic availability and tissue to serum ratios were calculated to assess the brain-specific delivery.

EXPERIMENTAL

Materials

Indinavir was a gift from Matrix Laboratories, Hyderabad, India. Egg Lecithin (Lipoid E80) was also a kind gift from Lipoid, Ludwigshafen, Germany. Tetradecyl aspartic acid and decyl glutamic acid were gifts from Dr. A. Muralidhar Rao, NATCO Pharma Ltd, Hyderabad. Acetonitrile, chloroform, methanol, cholesterol, and glycerol (98%) were purchased from Merck Ltd, Mumbai, India. Oleic acid was purchased from Loba chemicals Pvt Ltd, Mumbai, India. Alpha-tocopherol acetate, dialysis membranes were purchased from HiMedia, Mumbai, India. Phosphoric acid (assay 86%), triethylamine, soy bean oil were purchased from Sigma chemicals Ltd, Mumbai. Diethyl ether, potassium hydroxide, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, and perchloric acid were purchased from SD fine chemicals, Mumbai.

Methods

Preparation and Characterization of Indinavir SLE Formulations Containing Lipoamino Acids

Preparation of Submicron Lipid Emulsions. The SLEs were prepared with different compositions (Table I). In brief indinavir, egg lecithin, cholesterol, oleic acid, and α-tocopherol acetate were added to soyabean oil (oil phase) and heated to 70° C on a water bath. Glycerol was dissolved in sufficient amount of double-distilled water (aqueous phase) heated to 70°C. The aqueous phase was added to oil phase at the same temperature, homogenized (Shear homogenizer DIX 900, Heidolph, Kelheim, Germany) for 3 min to get coarse oil in water emulsion (10 ml). The homogenized emulsion was sonicated (750W, Vibra Cell, Sonics and Materials Inc. CT, USA) using 12 T probe for about 20 min.

In case of tetradecyl aspartic acid and decyl glutamic acid containing emulsions, a stock solution of each of LAA was prepared (250 μg/ml) in a solvent system consisting of chloroform/methanol (1:1). Appropriate dilutions of the LAA solutions (equivalent for 1, 10, and 100 μg) were prepared with same solvent mixture and transferred initially to a glass tube (used for preparation of emulsion) and allowed to evaporate in vacuum oven (Toshniwal, Mumbai, India) maintained at 37°C. Then all the ingredients needed for SLE were added and the above procedure was used for preparation of LAA-SLE formulations. The control SLE was prepared without any lipoamino acid.

Determination of Particle Size and Zeta Potential. The SLEs were characterized for globule size (hydrodynamic diameter), polydispersity index and zeta potential. The emulsions were initially diluted (100 times) with double-distilled water and kept in the cuvette to which a dip cell was attached. The cuvette was placed inside the instrument (Malvern Nano ZS90, Malvern, UK) for measurement of size and zeta potential (Zp). During the measurement, average particle count rate was maintained between 50 and 500 kcps (16). The principle of photon correlation spectroscopy determined the hydrodynamic diameter of the globules via Brownian motion. The observations for globule size were

Table I. Compositions of the Prepared Indinavir SLEs

Ingredients	SLE control	SLE-A1	SLE-A2	SLE-A3	SLE-G1	SLE-G2	SLE-G3
Indinavir (mg)	10	10	10	10	10	10	10
Soybean oil (mg)	1,000	1,000	1,000	1,000	1,000	1,000	1,000
Egg lecithin (mg)	120	120	120	120	120	120	120
Glycerol (mg)	225	225	225	225	225	225	225
α-Tocopherol acetate (mg)	20	20	20	20	20	20	20
Oleic acid (mg)	25	25	25	25	25	25	25
Cholesterol (mg)	30	30	30	30	30	30	30
A-14(tetradecyl aspartic acid) (μg)	–	1	10	100	–	–	–
G-10(decyl glutamic acid) (μg)	–	–	–	–	1	10	100
Double-distilled water up to (ml)	10	10	10	10	10	10	10

recorded at 90° light scattering angle and temperature maintained at 25°C. The Zp was measured based on the electrophoretic mobility of globules using in-built software, which used the Helmholtz–Smoluchowski equation.

Determination of the Effects of Centrifugal Stress, Autoclaving, Desorption Stress, and Storage on Stability of SLEs

Effect of Centrifugal Stress on Stability. Formulations were filled in 2-ml eppendorf tubes, and the emulsions were subjected to centrifugation (Heraeus Biofuge) at 1,529×*g* (15,000 m/s^2) for 10 min as reported (17). To avoid modifications induced by heating, the temperature was maintained below 30°C. The creaming volume percentage for each emulsion was calculated by using the following formula (17) and compared.

$$C = 100\frac{V_t - V_s}{V_t}$$

Where C is creaming volume percentage, V_t=total volume of sample and V_s is volume of the lower phase layer.

Effect of autoclaving on physical stability. The SLEs were subjected to the effect of thermal stress on the stability of emulsions by autoclaving the emulsions at 15 lbs/in.^{2}, 121°C for 15 min. The physical state of emulsions was observed and the size of globules and zeta potentials were measured.

Effect of Dilution (Desorption Stress) on Stability. The optimized SLEs (SLE, SLE-A3, and SLE-G3) were diluted with double-distilled water 50–5,000 times (1:50, 1:100, 1:200, 1:500, 1:1,000, and 1: 5,000), and the effect of dilution on globule size and zeta potential was studied by using zeta sizer.

Stability of SLEs during storage. The optimized formulations (SLE, SLE-A3, and SLE-G3) were filled in eppendorf tubes and were stored at 25°C temperature for 2 months and analyzed for globule size, PDI and zeta potential.

Determination of Total Drug Content and Entrapment Efficiency of SLEs

Total Drug Content. About 0.1 ml of formulation was diluted to 1 ml with chloroform/methanol (1:1) solution. Final dilution was made with mobile phase phosphoric acid (50 mM, pH adjusted to 5.5 with triethylamine)/acetonitrile—(55:45) and indinavir content was estimated by high-performance liquid chromatography (HPLC; Shimadzu, Japan) method (18) at 215 nm using a C18 column (Phenomenex, Luna 5 μm, 250× 4.6 mm) with a flow rate of 1 ml/min at ambient temperature.

Entrapment Efficiency. Entrapment efficiency was determined by measuring the content of free drug (unentrapped) in aqueous medium. The aqueous medium was separated by ultrafiltration (19) using centrisort tubes (Sartorius, USA), which consisted of a filter membrane (molecular weight cut-off, 20,000 Da) at the base of the sample recovery chamber. About 1 ml of the formulation was placed in the donor chamber and centrifuged at 6,500 rpm for 15 min in Biofuge (Heraeus, Germany). The SLEs along with encapsulated drug remained in the donor chamber and aqueous phase moved into the receiver chamber through filter membrane. The amount of indinavir in the aqueous phase was estimated by HPLC method.

In Vitro Drug Release from SLE Formulations

Drug release was studied by dialysis bag method. Initially, the dialysis tubing was soaked in sterile water or phosphate buffer pH 7.4 for overnight. Emulsions (1 ml) were placed in dialysis bag made up of membrane having molecular weight cut-off 12,000–14,000 (HiMedia, Mumbai) and then suspended in 100 ml of dialysis medium consisting of phosphate buffer pH 7.4/ethanol (70:30) in a 250-ml beaker, which is stirred on a magnetic stirrer at 100 rpm maintained at 37°C. One-milliliter sample was collected at different time intervals, *i.e.*, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, and 12 h and was replaced with equal volume of fresh medium at each time interval. A standard graph was plotted with known concentrations of indinavir in phosphate buffer pH 7.4 by measuring the absorbance at 215 nm (18) on a UV-Visible spectrophotometer (ELICO SL 159, India).The amount of indinavir in collected samples was estimated by using the calibration curve $y = 0.0293x + 0.0165 (R^2 = 0.999)$. The cumulative percentage release of the drug *versus* time was plotted to know the release pattern of the drug from different formulations.

Determination of Bioavailability of Indinavir SLEs—In Vivo Animal Study

Study Protocol. Healthy male Wistar rats (280–300 g) were used for the pharmacokinetic and tissue distribution studies. Male Wistar Rats were purchased from Mahaveera enterprises, Narepally, Ranga Reddy Dist, Andhra Pradesh, India. The studies were conducted with prior approval of our institutional animal ethics committee (IAEC) file no. IAEC/03/UCPSc/KU/2008. The animals were given a standard diet approved by NIN (National Institution of Nutrition, Hyderabad, India) and had free access to water. The rats were fasted overnight, divided into four groups of 18 each. They were randomly administered with each formulation containing indinavir (5 mg/kg body weight) was given by intravenous bolus injection via tail vein. At predetermined intervals 0.25, 0.5, 1.0, 2.0, 4.0, and 6.0 h, rats (n=3) were anaesthetized with ether. Using heparinized capillary tubes, the blood samples were collected from retro-orbital sinus and serum samples were separated by centrifugation (Biofuge, Heraeus, Germany). At the same time points above, the rats (n=3) were decapitated and organs such as the brain, heart, liver, spleen, and kidney were removed, washed with ice cold saline, and kept in individual vials. The serum and tissue samples were stored under frozen conditions (−80°C) until analysis.

Estimation of Indinavir in Serum and Tissue Samples

Preparation of Calibration Curves, Extraction, and HPLC Analysis. A simple and validated reversed-phase HPLC

method (18) was modified and used for the determination of indinavir concentrations in biological samples from rats. The indinavir and verapamil hydrochloride (internal standard) stock solutions (1 mg/ml) were prepared with water-ACN (50:50 *v/v*). From this stock, 0.1, 0.25, 0.5, 1, 2.5, and 5 μg/mL standard concentrations of indinavir were prepared. Verapamil hydrochloride (internal standard) stock solution (1 mg/ml) was further diluted to produce the concentration of 5 μg/ml. To 500 μl of the tissue homogenates (obtained from tissues without any drug treatment to animal), 100 μl of perchloric acid (70%) was added and, after vortex-mixing for 5 min, the resulting suspension was centrifuged at 13,000 rpm for 10 min. To 300 μl of serum or tissue homogenate supernatants, prepared as described above, 100 μl of above prepared standard indinavir solutions, 100 μl of internal standard solution (5 μg/mL Verapamil hydrochloride aqueous solution), 1 ml of 4 M KOH solution, and 3 ml of diethyl ether were added and the resulting mixture was vortexed for 15 min and then centrifuged at 6,500 rpm for 10 min. The organic layer was separated and evaporated to dryness in a vacuum dryer (Toshniwal, Mumbai, India). Finally, the dried sample was re-dissolved in 100 μl of mobile phase and 20 μl of the resulting solution was injected to HPLC. The peak area ratios thus obtained from different concentrations of the drug were plotted against the concentration of the drug. The slope of the plot determined by least square regression analysis was used to calculate indinavir concentration in the unknown tissue samples.

For determination of indinavir concentration from biological samples (serum, brain, heart, liver, kidney, and spleen) in rats, the tissue samples were weighed and homogenized with ice cold saline (0.2 g of tissue per milliliter of saline) using tissue homogenizer at 6,000 rpm (Remi Motors, Mumbai, India). For drug analysis, 500 μl of the tissue homogenates or serum samples were used. The above described procedure was used for extraction. The concentration of indinavir in tissues was calculated by using standard calibration equation.

Calculation of Pharmacokinetic Parameters and Targeting Index

The pharmacokinetic parameters, mean c_{max}, t_{max}, AUC $_{(0–6\ h)}$, and therapeutic availability (TA) of indinavir from various SLEs following administration in rats (n=3) were calculated for different tissues by using Kinetica software; all data were expressed as mean ± SD. Two sample comparisons were performed by using paired student's t test. P value below 0.05 was considered to be statistically significant. The therapeutic availability is the ratio of AUC (test) to that AUC (control). Targeting index is the ratio of concentration of drug in tissue to that of serum at particular time point after administration of a formulation. If the ratio is more than one, indicates the drug targeting to that tissue.

RESULTS AND DISCUSSION

Preparation and Characterization of SLEs

The SLEs with varying amount of lipoamino acids were prepared (Table I). The amount of oil phase in the emulsions was fixed at 10% and that of emulsifying agent was at 1.2% to produce stable emulsions (20) was used as the basis for the development of SLEs in this study. All the prepared SLEs were characterized for the globule size, poly dispersity index, zeta potential, and *in vitro* drug release. The average globule sizes varied in the range of 210–220 nm. There was no significant change in zeta potential and ranged in between −22 to −25 mV with different concentrations of LAAs when compared with the control SLE without LAA (Table II). The LAAs are amphoteric in nature and do not contribute for big changes in zeta potentials of oil globules at these low concentrations. The polydispersity indices were below 0.5 for prepared SLEs. The sizes of the globules were uniform among the series of prepared SLEs.

In Vitro Drug Release

The *in vitro* drug release was carried out by dialysis method for a period of 12 h. The cumulative percentage drug release from SLE, SLE-A1, SLE-A2, and SLE-A3 were 85%, 41%, 34%, and 27%, respectively (Fig. 1a). In case of SLE-G1, SLE-G2, and SLE-G3 the cumulative percentage drug release was 35%, 31%, and 26%, respectively (Fig. 1b). As the concentrations of A and G were increased, retardation in drug release was noticed. The retardation in drug release could be due to the orientation of LAA molecules at the interface resulting in higher rigidity. The other reason could be due to the charged

Table II. Globule Sizes, Polydispersity Indices, and Zeta Potentials of the SLEs (n=3) Before and After Sterilization and Creaming Volume Percentages of Different Formulations—Effect of Centrifugation

	Before sterilization			After sterilization (filtration)			Creaming volume (%)
Formulation code	Size (nm)	(PDI)	Z.P (mV)	Size (nm)	(PDI)	Z.P (mV)	–
SLE (control)	212.3±7.73	0.080±0.03	−23.16±0.92	222.0±2.16[a]	0.11±0.03[a]	−23.1±0.66[a]	97.83±0.28
SLE-A1	216.2±3.93	0.409±0.07	−22.26±0.66	188.4±5.14	0.37±0.02	−22.6±0.61	96.16±1.25
SLE-A2	210.2±4.05	0.195±0.05	−23.83±0.45	187.3±1.74	0.24±0.04	−22.8±0.90	95.33±0.57
SLE-A3	215.5±2.46	0.135±0.10	−24.16±0.35	180.7±7.36	0.20±0.11	−23.8±1.64	94.66±1.52
SLE-G1	213.0±5.60	0.425±0.06	−22.30±0.65	178.5±4.35	0.39±0.06	−23.3±0.20	94.66±1.52
SLE-G2	214.2±4.40	0.193±0.02	−23.56±0.77	186.5±6.40	0.25±0.05	−23.9±1.66	94.33±1.15
SLE-G3	214.0±4.67	0.376±0.04	−25.53±0.68	194.8±4.16	0.36±0.06	−25.6±1.04	94.60±1.52

[a] Indicates after steam sterilization (autoclaving)

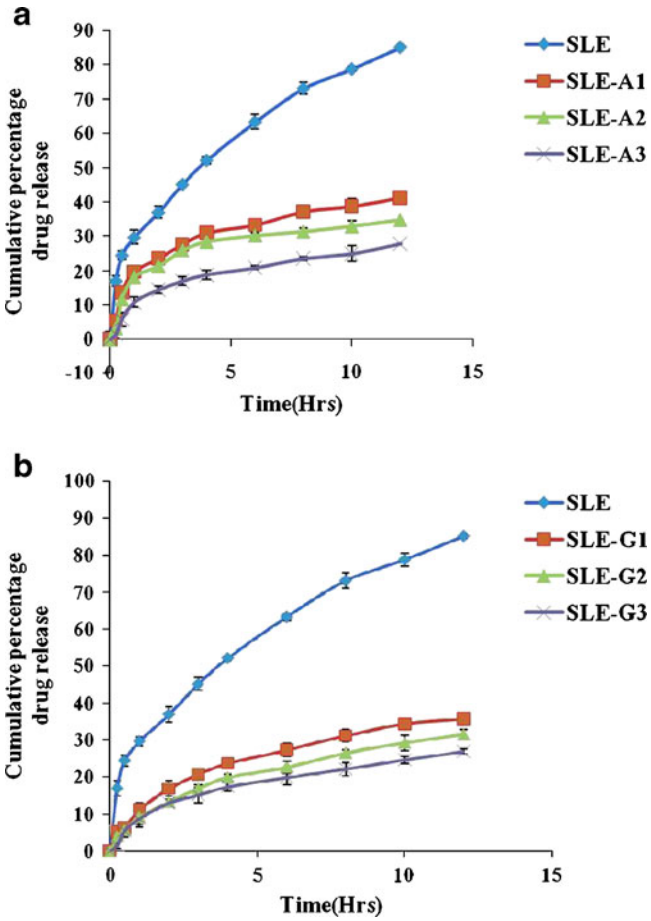

Fig. 1. **a** Cumulative percentage drug release from SLEs loaded with different concentrations of tetradecyl aspartic acid. **b** Cumulative percentage drug release from SLEs loaded with different concentrations of decyl glutamic acid

interactions between the drug molecules and amino acid moieties. It was known that the amino acids such as aspartic acid and glutamic acid were positively charged when pH of solution was 4.6 or 5.6, but negatively charged at higher pH values (21) and the drug has no charge when present in oil core but when entered the aqueous environment, due to opposite charges of attraction; there might be retarded release of the drug. Based on the size, PDI, zeta potential and *in vitro* drug release studies SLE-A3 and SLE-G3 were selected for further studies.

Stability Studies of SLEs

Effect of Centrifugal Stress

The centrifugal stress on the emulsions results in the creaming, coalescence and separation of oil phase. The extent of creaming volume indicates the status of stability. Stable emulsions have very high percentage creaming volumes. This is a rapid method to find out the instability in emulsions. In general submicron lipid emulsions were thermodynamically stable. This study indicated that all the SLEs were stable to the centrifugal stress. It was found that the SLE control was having slightly higher percentage creaming value than SLE-A3 and SLE-G3 as shown in Table II. It was known that following increase in amino acid concentration at interface leads to destabilization of emulsions (22). However, the role of lipoamino acid is not yet known on the stability of emulsions, as lipid portion of these molecules is expected to dissolve in the oil phase.

Effect of Thermal Stress

It was known that on steam sterilization of the emulsions, the phospholipids rapidly relocate from the aqueous phase to the oil phase. The reason being, phospholipids concentrate in the oil/water meso phase, forming a cubic liquid crystalline phase, the bulk of which was converted to a lamellar phase on cooling, and that this organization of interfacial material accounts for the enhanced stability of phospholipids emulsions after sterilization (23). When control SLE was subjected to autoclaving, there was decrease in the size and increase in zeta potentials (Table II), whereas SLEs containing lipoamino acids separated into two phases. It is known that O/W emulsions having the reduced negative charges were found to be unstable due to autoclaving (24). The instability is due to consequential effect of coalescence of oil globules during thermal stress. In subsequent studies, the emulsions prepared were passed through Millipore filters, having 0.2 μm pore for the purpose of sterilization.

Effect of Dilution Stress

The control and optimized formulations, SLE, SLE-A3, and SLE-G3 were subjected to dilution (50–5,000 times) stress. The dilution of an emulsion disturbs the rigidity of the surfactant layers at the interface leading to instability of system. The extent of stability of an emulsion can be rapidly checked by measuring the changes in zeta potential due to dilution. Stable emulsions withstand higher dilution effect and do not undergo changes in zeta potential due to dilution (25). There was no significant difference found in size and zeta potentials of all the tested

Table III. Effect of Dilution on Globule Size, Polydispersity Index and Zeta Potentials of the Optimized Formulations

Formulation code	Dilution factor	Size (nm)	PDI	Zp (mV)
SLE	1:50	226.3±3.3	0.245±0.12	−22.1±1.2
	1:100	228.5±2.5	0.285±0.05	−23.6±3.4
	1:200	230.9±3.6	0.316±0.25	−25.5±2.6
	1:500	234.2±4.3	0.279±0.89	−24.8±4.2
	1:1,000	237.5±1.8	0.325±0.45	−23.7±3.8
	1:5,000	240.4±5.2	0.365±0.01	−22.9±4.0
SLE-A3	1:50	217.6±5.2	0.247±0.21	−21.6±3.2
	1:100	221.5±3.8	0.228±0.12	−22.5±5.6
	1:200	224.1±4.5	0.336±0.09	−24.6±6.0
	1:500	229.6±2.6	0.375±0.16	−25.2±4.5
	1:1,000	232.4±3.5	0.392±0.25	−24.7±1.2
	1:5,000	236.5±2.4	0.369±0..13	−25.6±2.3
SLE-G3	1:50	214.7±4.5	0.228±0.25	−23.6±2.5
	1:100	218.6±2.3	0.235±0.6	−24.2±2.7
	1:200	224.7±5.6	0.242±0.2	−23.8±3.2
	1:500	227.4±2.9	0.289±0.12	−24.6±2.2
	1:1,000	230.4±3.4	0.295±0.18	−27.8±4.4
	1:5,000	232.6±5.2	0.303±0.24	−26.4±3.5

Table IV. Effect of Storage on Globule Size, PDI, and Zeta Potential of the Optimized Formulations

	Size			Zeta potential			Polydispersity index (PDI)		
Day	SLE	SLE-A3	SLE-G3	SLE	SLE-A3	SLE-G3	SLE	SLE-A3	SLE-G3
1	216.1±2.1	217.6±1.2	214.1±4.3	−24.4±0.1	−24.16±0.4	−25.53±0.2	0.247±0.06	0.217±0.5	0.228±0.05
15	220.3±3.1	224.3±2.1	220.9±0.6	−23.6±2.9	−25.2±1.6	−26.82±1.6	0.207±0.1	0.212±0.2	0.292±0.2
30	232.3±0.6	225.2±3.1	218.7±0.7	−26.2±0.2	−24.8±1.0	−24.32±1.2	0.217±0.2	0.276±0.1	0.350±0.15
45	228.8±1.6	236.4±2.6	223.4±1.5	−25.9±0.3	−28.5±0.9	−28.1±0.3	0.279±0.6	0.218±0.08	0.278±0.06
60	262.2±0.5	228.2±2.1	220.2±4.0	−27.2±0.7	−23.8±0.5	−25.64±0.1	0.245±0.09	0.213±0.04	0.342±0.26

formulations during the dilution studies indicated that all the emulsions were stable (Table III).

Effect of Storage on Stability

Stability of parenteral emulsion was mainly studied in terms of maintaining of its physical integrity, namely the dispersed phase particle size and size distribution. The optimized formulations were tested for stability under storage at room temperature for 8 weeks and their size and zeta potentials were measured. There was no significant difference found in sizes and zeta potentials of the submicron emulsion formulations up to 8 weeks, and indicated that the optimized formulations were stable at room temperature (Table IV).

Entrapment Efficiency and Drug Content

The entrapment efficiency and total drug content of the optimized SLES were determined by HPLC method (18). Entrapment efficiencies for SLE, SLE-A3 and SLE-G3 were 99%, 95%, and 93%, respectively, whereas the total drug content was found to be 9.68, 9.58, and 9.54 mg for SLE, SLE-A3, and SLE-G3, respectively.

Pharmacokinetics and Tissue Distribution

Preparation of Calibration Curve and Estimation of Drug Concentration. The *in vivo* pharmacokinetic and tissue distribution studies were performed in male Wistar rats and the plasma and tissue concentrations of indinavir at different time points following IV administration of indinavir solution, SLE control, SLE-A3 and SLE-G3 were determined by using HPLC method. The amount of indinavir in serum and other tissue homogenates *viz*., brain, heart, liver, kidney, and spleen at different time intervals were calculated by using the individual calibration curves $y=1.074x-0.0245$ ($R^2=0.993$), $y=0.439x+0.0453$ ($R^2=0.998$), $y=0.289x-0.0472$ ($R^2=0.989$), $y=0.2348x-0.0171$ ($R^2=0.988$), $y=0.2373x-0.0185$ ($R^2=0.995$), and $y=0.2056x-0.034$ ($R^2=0.990$) respectively. The concentration *vs*. time profiles of different formulations in serum, brain, kidney, liver, heart and spleen are plotted as shown in Figs. 2, 3, 4, 5, 6, and 7. There was no significant difference in the serum levels of indinavir from SLE-A3 and SLE-G3 (Fig. 2) and the control formulations at all time points. The brain levels (Fig. 3) of indinavir from SLE-A3 and SLE-G3 were significantly higher than control formulations at all time points. This resulted in higher availability of indinavir from SLE-A3 and SLE-G3.

In this study, the indinavir drug solution and SLE were used as controls to study the effect of incorporating the LAAs in SLEs. The mean c_{max}, t_{max}, $AUC_{(0-6\ h)}$, and TA of indinavir from various SLEs following administration in rats ($n=3$) were calculated for different tissues (Table V). The c_{max} values of SLE, SLE-A3, and SLE-G3 were higher than that of the drug solution in all the tissues. The c_{max} values of SLE-A3 and SLE-G3 in comparison to control SLE was significant in brain and spleen but insignificant in other tissues. However, there were some changes observed in case of t_{max} values for all

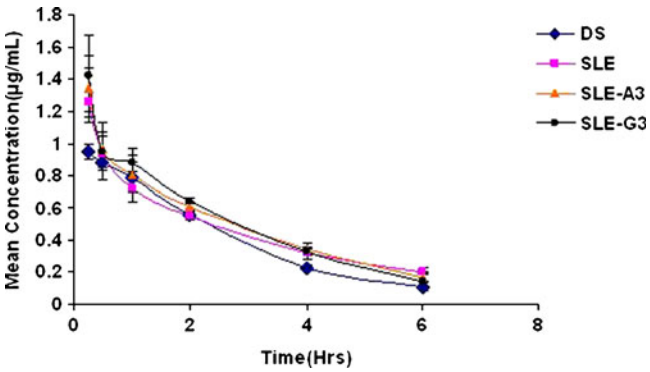

Fig. 2. Concentration *versus* time profile of indinavir in serum from different formulations upon i.v. administration. *DS* drug solution, *SLE-3* control emulsion, *SLE-A3* tetradecyl aspartic acid-loaded SLE, *SLE-G3* decyl glutamic acid-loaded SLE

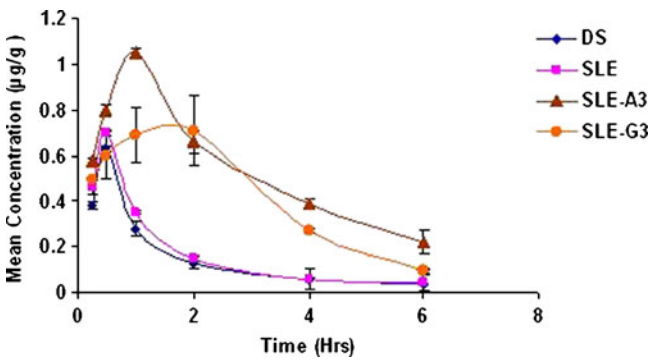

Fig. 3. Concentration *versus* time profile of indinavir in brain from different formulations upon i.v. administration. *DS* drug solution, *SLE-3* control emulsion, *SLE-A3* teradecyl aspartic acid-loaded SLE, *SLE-G3* decyl glutamic acid-loaded SLE

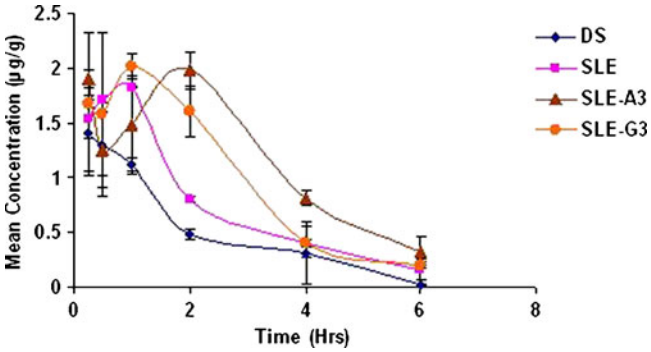

Fig. 4. Concentration *versus* time profile of indinavir in kidney from different formulations upon i.v. administration. *DS* drug solution, *SLE-3* control emulsion, *SLE-A3* tetradecyl aspartic acid-loaded SLE, *SLE-G3* decyl glutamic acid-loaded SLE

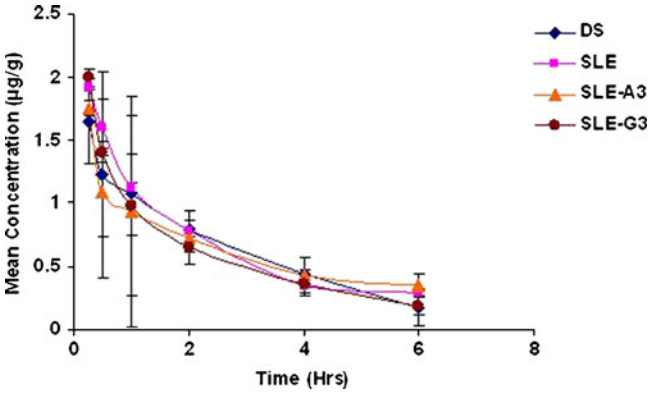

Fig. 6. Concentration *versus* time profile of indinavir in heart from different formulations upon i.v. administration. *DS* drug solution, *SLE-3* control emulsion, *SLE-A3* tetradecyl aspartic acid-loaded SLE, *SLE-G3* decyl glutamic acid-loaded SLE

control and test formulations. The difference in AUC is significant ($P<0.05$) for SLE, SLE-A3, and SLE-G3 formulations for all tissues in comparison to drug solution, however, insignificant in heart and spleen in case of SLE-G3 formulation. The difference in $AUC_{(0–6\ h)}$ value is significant in brain and kidney tissues for SLE-A3 and SLE-G3 in comparison to control SLE. Here, the design of submicron lipid emulsion of indinavir clearly improved the brain-specific delivery, when compared with drug solution.

The TA is a parameter to assess drug-targeting potential of drug carrier system (26). It shows the extent of localization of a drug in the tissue. The SLE-A3 and SLE-G3 systems were found to have greater therapeutic availability than drug solution and SLE (control formulations) indicating the localization of indinavir in brain tissue. Furthermore, using of LAAs of glutamic acid and aspartic acid as specific ligands significantly improved by 2–3-fold, respectively, in the brain localization of indinavir when compared with control SLE. In kidney, the indinavir levels due to SLE-A3 and SLE-G3 were significantly higher than control formulation at all time points after 1 h. However, in between 0 and 1 h the levels were fluctuating. The TA values of SLE, SLE-A3, and SLE-G3 were significant ($P<0.05$) in comparison to drug solution. In heart, there is no significant difference in the indinavir levels in case of SLE—A3 and SLE—G3 in comparison to drug solution and SLE. In general, in case of liver and spleen the indinavir levels from SLE were higher than that of drug solution, SLE-A3 and SLE-G3 at almost all the time points, where as the serum drug levels were low in case of drug solution in comparison to SLE-A3 and SLE-G3.

Tissue to serum drug concentration ratio (T/S) is commonly employed as an index of targeting (27). If the ratio is more than one, it indicates the drug targeting to that tissue. The ratios of brain to serum concentrations of indinavir at different time points following IV administration of indinavir solution, SLE, SLE-A3, and SLE-G3 in rats are shown in Fig. 8. From this histogram, it was found that for SLE-A3 formulations, tissue to serum drug concentration ratio (T/S) for brain tissue was consistently greater than one after 1 h interval indicating the preferential localization of indinavir in brain for SLEs prepared with LAAs. However, for SLE-G3 the ratio was slightly less than one at 1st-, 4th-, and 6th-hour intervals. These results indicate that the system L present in brain can be utilized for improving the brain-specific delivery of drug and confirms the previous findings of others (15).

Based on the above results, it is concluded that the indinavir levels due to emulsions containing lipoamino acids are significantly higher than control formulations at all time points in brain and also in case of kidney. However, the

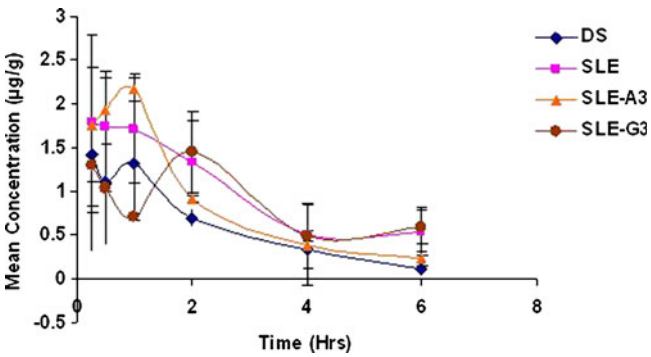

Fig. 5. Concentration *versus* time profile of indinavir in liver from different formulations upon i.v. administration. *DS* drug solution, *SLE-3* control emulsion, *SLE-A3* tetradecyl aspartic acid-loaded SLE, *SLE-G3* decyl glutamic acid-loaded SLE

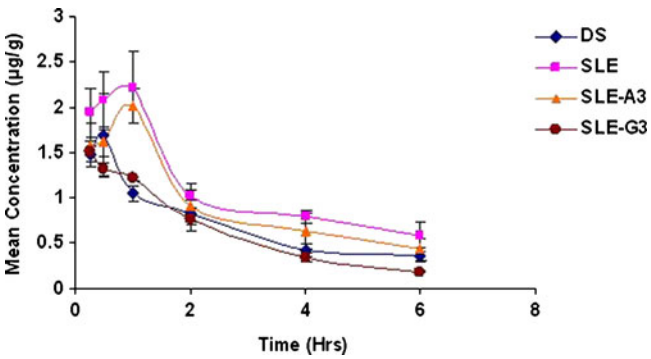

Fig. 7. Concentration *versus* time profile of indinavir in spleen from different formulations upon i.v. administration. *DS* drug solution, *SLE-3* control emulsion, *SLE-A3* tetradecyl aspartic acid-loaded SLE, *SLE-G3* decyl glutamic acid-loaded SLE

Table V. Mean c_{max}, t_{max}, $AUC_{(0–6\ h)}$, and Therapeutic Availability (TA) of Indinavir from Drug Solution, SLE (Control), SLE-A3, and SLE-G3 Formulations

Organ	Pharmacokinetic parameters	Formulation code			
		Drug solution	SLE	SLE-A3	SLE-G3
Serum	c_{max} (μg/mL)	0.95±0.04	1.26±0.06[a]	1.34±0.21[a]	1.42±0.25[a]
	t_{max} (h)	0.33±0.14	0.25	0.25	0.25
	$AUC_{(0–6\ h)}$ (μg h/mL)	2.80±0.11	3.56±0.05[a]	3.43±0.02[a]	3.45±0.49[a]
	$t_{1/2}$ (h)	1.79±0.24	2.66±0.18	2.0±0.26	1.90±0.21
	TA	1.00	1.27[a]	1.22[a, c]/0.96[d]	1.23[a, c]/0.96[d]
Brain	c_{max} (μg/mL)	0.63±0.015	0.69±0.01[a]	1.05±0.01[a, b]	0.78±0.04[a, b]
	t_{max} (h)	0.50	0.50	1.00	1.66±0.57
	$AUC_{(0–6\ h)}$ (μg h/mL)	0.92±0.10	1.16±0.43[a]	3.93±0.26[a, b]	2.7±0.21[a, b]
	$t_{1/2}$ (h)	1.58±0.43	2.4±2.21	2.39±0.36	1.5±0.22
	TA	1.00	1.26[a]	4.27[a, c]/3.38[d]	2.94[a, c]/2.5[d]
Kidney	c_{max} (μg/mL)	1.44±0.31	1.83±0.01[a]	2.02±0.10[a, b]	2.20±0.21[a, b]
	t_{max} (h)	0.41±0.14	1.00	1.41±1.01	0.58±0.38
	$AUC_{(0–6\ h)}$ (μg h/mL)	2.81±0.65	4.85±0.43[a]	7.49±0.91[a, b]	5.98±0.58[a, b]
	$t_{1/2}$ (h)	0.89±0.19	1.75±0.70	1.55±0.4	1.40±0.01
	TA	1.00	1.72[a]	2.66[a, c]/1.54[d]	2.12[a, c]/1.23[d]
Liver	c_{max} (μg/mL)	1.68±0.65	2.27±0.45[a]	2.16±0.14[a]	1.68±0.25[a]
	t_{max} (h)	0.5±0.43	0.33±0.14	1.00	1.41±1.01
	$AUC_{(0–6\ h)}$ (μg h/mL)	3.66±0.54	7.30±2.33[a]	5.26±1.23[a]	6.33±2.15[a]
	$t_{1/2}$ (h)	1.34±0.12	2.49±0.32	1.54±0.18	2.62±0.90
	TA	1.00	1.99[a]	1.43[a, c]/0.72[d]	1.72[a, c]/0.86[d]
Heart	c_{max} (μg/mL)	1.69±0.30	1.92±0.011[a]	1.82±0.14[a]	1.97±0.04[a, b]
	t_{max} (h)	0.58±0.38	0.25	0.5±0.43	0.25
	$AUC_{(0–6\ h)}$ (μg h/mL)	4.29±0.88	4.81±0.04[a]	6.58±1.46[a]	4.18±0.90[b]
	$t_{1/2}$ (h)	1.81±0.64	2.28±0.20	5.10±3.06	2.19±0.93
	TA	1.00	1.12[a]	1.53[a, c]/1.36[d]	0.97[c]/0.86[d]
Spleen	c_{max} (μg/mL)	1.78±0.32	2.33±0.27[a]	2.01±0.20[a]	1.51±0.11
	t_{max} (h)	0.41±0.14	0.58±0.38	1.00	0.25
	$AUC_{(0–6\ h)}$ (μg h/mL)	5.37±0.12	12.52±5.10[a]	7.87±1.95[a]	4.15±0.15
	$t_{1/2}$ (h)	2.80±0.34	6.51±4.52	3.74±0.59	1.86±0.12
	TA	1.00	2.33[a]	1.46[a, c]/0.62[d]	0.77[c]/0.33[d]

By Student's paired t test, $P<0.05$ is considered as significant
[a] In comparison to drug solution
[b] In comparison to control SLE
[c] TA in comparison to drug solution
[d] TA in comparison with control SLE

extent of difference in indinavir levels in brain tissue is 2.5–3.3-fold, but in case of kidney, it is around 1.2–1.5-fold. The slightly higher levels in kidney are possibly achieved due to the presence of LAT system (5) apart from in brain tissue.

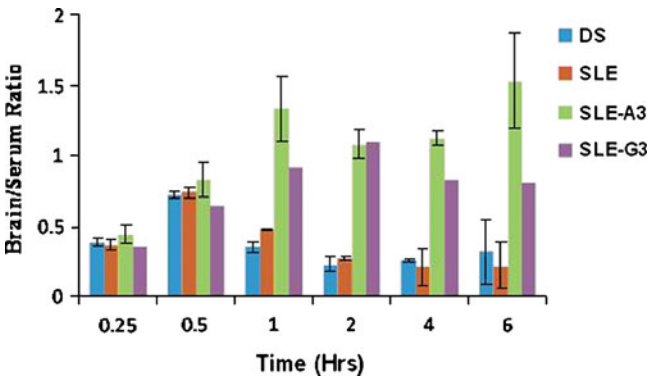

Fig. 8. Brain to serum ratio of indinavir levels of different formulations upon i.v. administration. *DS* drug solution, *SLE-3* control emulsion, *SLE-A3* tetradecyl aspartic acid-loaded SLE, *SLE-G3* decyl glutamic acid-loaded SLE

In conclusion, using a commercially viable manufacturing process stable submicron emulsions of indinavir containing lipoamino acids as specific ligands were prepared, characterized and the *in vitro* and *in vivo* behavior was studied in Wistar rats. The tissue distribution studies clearly indicated that the lipoamino acid containing SLE-A3 and SLE-G3 emulsions improved the brain-specific delivery of indinavir in comparison to drug solution and control SLE.

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