RESEARCH ARTICLE

RES and brain targeting stavudine-loaded solid lipid nanoparticles for AIDS therapy

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Cells of the reticuloendothelial system (RES, e.g., macrophages) play an important role in the immunopathogenesis of Acquired Immunodeficiency Syndrome (AIDS). The objective of the present study was to investigate the possibility of specifically targeting antiviral drugs Stavudine to RES (like Liver, Spleen etc.) and brain using solid lipid nanoparticles (SLNs) as colloidal drug carriers. Various lipids like stearic acid, cetyl palmitate, glyceryl behenate and phospholipid in combination have been used and effect of lipid on properties of SLNs has been investigated. The SLNs of Stavudine were prepared by double emulsion solvent evaporation method. The diameter of SLNs was determined and found in range of 175 ± 6.027 to 393 ± 2.309 nm depending on the type of lipid used. Percentage drug entrapment was found to be influenced by the concentration and type of lipid used, which was found in the range of 18.1 to 65.2%. The drug release behavior was studied by dialysis bag method and the release pattern of drug was found to follow Higuchi model. Results of stability evaluation showed a relatively long-term stability after storage at 4°C for 1 month. Stavudine-loaded SLNs were successfully prepared, optimized and effectively targeted to RES and brain. SLNs of Stavudine have been shown to be taken up by brain 11 fold greater as compared to pure Stavudine. This finding is more important since Human Immunodeficiency Syndrome (HIV) infect the central nervous system.

Key words: *Drug targeting, reticulo endothelial system, solid lipid nanoparticles, stavudine (d4T)*

INTRODUCTION

Human immunodeficiency virus (HIV) is a retrovirus, which causes irreversible destruction of the immune system, leading to the occurrence of AIDS.[1] The HIV is best known for targeting the T cells of the immune system. In the body, various monocytes derived macrophage cells, dendritic cells of brain, lungs, spleen, liver, kidney, lymph nodes and bone marrow serves as active HIV sanctuaries.[2-4] The ability of anti-AIDS drugs to access cells of the reticulo endothelial system (RES) is therefore essential for the success of AIDS therapy.[5] Though antiretroviral drugs are able to combat replication of the HIV by inhibiting reverse transcriptase and thereby viral DNA synthesis, but a major limitation associated with the clinical use of these agents is their dose limiting toxicity.[6] For anti-HIV drugs to be effective, adequate distribution to specific sites in the body must be achieved, and effective drug concentrations must be maintained at those sites for the required period of time.[7]

Currently available anti-HIV drugs bear some significant drawbacks such as relatively short half-life, low bioavailability, poor permeability and undesirable side effects. Stavudine (d4T) is one of the important drugs, belonging to the class of reverse transcriptase inhibitors approved by the FDA for the treatment of AIDS. Stavudine has greater bioavailability (88-99%). However, long-term administration of stavudine over a period of 6 months results in life threatening adverse effects such as a dose limiting peripheral neuropathy, anemia, hypersensitivity, insomnia, and malaise. Lactic acidosis, hepatitis or liver failure has also been reported. Efforts have been made to design drug delivery systems for anti-HIV agents to obtain an effective controlled delivery of the drug. An attempt has been made to deliver the antiretroviral via SLNs by implementing the principles of novel drug delivery.

SLNs are colloidal drug carrier systems, which are very much similar to nanoemulsions, but differing in lipid

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nature.[8-10] The diameter of SLNs ranges from 10-900 nm. They are prepared from biocompatible lipids and possess excellent biodegradability.[11] In addition to its low toxicity, SLNs are cost effective and have less regulatory problems compared to polymeric nanoparticles. SLNs have been shown to accumulate in the RES, which is very important for AIDS therapy since HIV targets the RES.[12] For drug delivery into the brain, an increase in osmotic pressure can be aggressive and may bring other substances into brain, although tight junction may be opened by high osmotic pressure to efficiently increase drug permeability across blood-brain barrier.[13] On the other hand, carrier-mediated systems like SLNs, which may alter body drug distribution without severe intervention in the structure of tight junction, would be an excellent technique for brain-targeting delivery.[14] An SLNs drug delivery system may be ideal in the case of stavudine as it may alleviate drug toxicity by delivering the drug directly to the macrophages and brain in a passive manner.[15]

In the present study, the feasibility of the strategy to target drugs to the RES and brain was tested in rats. In addition to that various lipids have been used to prepare Stavudine-loaded SLNs and effect of lipids on various parameters like particle size, percentage drug entrapment, have been investigated. Attempt has been made to reduce the dose and side effects by targeting the drug to specific site.

MATERIALS AND METHODS

Materials

Stavudine was received as a gift sample from Smilax Laboratories Limited, Hyderabad, INDIA and Cipla Ltd, Vikhroli, India. Cetyl palmitate and Glyceryl behenate were gifted by Phoenix Chemical, Inc. NJ, USA. Stearic acid was purchased from S.D. Fine-Chem Ltd., Mumbai, INDIA. Dipalmitoylphosphatidylcholine (DPPC) and Dimyristoylphosphatidylglycerol (DMPG) were received as a gift from Lipoid, Germany. All other ingredients used were of analytical grade.

Animals

Albino Wistar rats were used after obtaining the due permission for conducting *in vivo* studies, from institutional ethics committee (KLE University's College of Pharmacy, Belgaum, India). The protocol according to form B was approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and Institutional Animal Ethics Committee (IAEC) (Registration number 221/CPCSEA).

Preparation of stavudine-loaded solid lipid nanoparticles

SLNs were fabricated by using one of the promising techniques - Double Emulsion Solvent Evaporation method. The method involves preparation of an organic phase consisting of lipids and span 60 dissolved in 20 mL dichloromethane. While 100 mg of Stavudine dissolved in 2 mL of distilled water was used as aqueous phase. Drug solution was added into organic phase containing lipid and w/o emulsion was prepared by homogenizing the solution at 1000 rpm for 3 min. Then w/o/w were prepared by adding w/o emulsion to 1% aqueous PVA solution and stirred at 4000 rpm using rotor stator homogenizer for 120 min. After evaporation of organic phase under stirring, the SLNs were isolated by centrifugation at 16,000 rpm for 60 min. The SLNs were washed three times, resuspended in deionized water, and freeze dried to obtain lyophilized particles, which were stored in freezer.[16,17]

Mainly three lipids (cetyl palmitate, stearic acid, and glyceryl behenate) were used in different concentration [Table 1]. Phospholipids have been used in combination with C4, G4, and S4 formulation, which were optimized formulations of each lipid.

Determination of particle size and zeta potential

Particle size of SLNs was measured using Nanotrac, Japan. A10% solution of SLNs was made with deionised water before measurement. The zeta potential of SLNs was determined using a Zeta meter + 3 M, USA. The system was maintained at 25°C. Each sample was analyzed in triplicate for both particle size and zeta potential.[16]

Drug entrapment efficiency

The entrapment efficiencies of prepared systems were determined by measuring the concentration of free drug in the dispersion medium. The supernatant of the aqueous dispersion obtained after centrifugation for 1 h at 16000 rpm at 0°C was separated and then filtered through 0.45-µm millpore (Millipore Filter). The filtrate was diluted using phosphate buffer pH 6.8 and measured spectrophotometrically (Shimadzu, UV 1700, Japan). The

Table 1: Formula for stavudine-loaded solid lipid nanoparticles

Formulation	Lipid used	Drug:lipid
C 1	Cetyl palmitate	1 : 1
C 2		1 : 2
C 3		1 : 3
C 4		1 : 4
G 1	Glyceryl behenate	1 : 1
G 2		1 : 2
G 3		1 : 3
G 4		1 : 4
S 1	Stearic acid	1 : 1
S 2		1 : 2
S 3		1 : 3
S 4		1 : 4
CP 4	Cetyl palmitate + DPPC + DMPG	1 : 4*
GP 4	Glyceryl behenate + DPPC + DMPG	1 : 4*
SP 4	Stearic acid + DPPC + DMPG	1 : 4*

*Lipids used in the ratio of × : DPPC: DMPG was 15 : 3 : 2. (× = Cetyl palmitate or Glyceryl behenate or Stearic acid); DPPC: Dipalmitoylphosphatidylcholine; DMPG: Dimyristoylphosphatidylglycerol

amount of free drug was detected in the filtrate and the amount of incorporated drug was determined as the result of the initial drug minus the free drug. The entrapment efficiency was calculated using the following Eq. 1.[16,18]

$$\text{Percent drug entrapment efficieny} = (W_{\text{initial drug}} - W_{\text{free drug}})/(W_{\text{initial drug}}) * 100 \quad (1)$$

Where, "$W_{\text{initial drug}}$" is the weight of initial drug used and the "$W_{\text{free drug}}$" is the weight of free drug detected in the supernatant after centrifugation of the aqueous dispersion.

Scanning electron microscopy

Optimized formulation, CP4 was viewed using a conventional scanning electron microscope (JSM-5900LV, JEOL, Japan) at an accelerating voltage of 20 kV. One drop of the SLNs suspension was placed on a graphite surface. After oven-drying, the sample was coated with gold using an Ion Sputter.

IR studies

The IR spectrum of pure Stavudine, freeze-dried SLNs of C4, S4, G4, and CP4 was performed using FTIR, Perkin Elmer (Spectrum-100), Japan. In the present study, potassium bromide pellet method was employed. The samples prepared after grinding the drug and freeze dried SLNs separately in micronized IR grade potassium bromide were compressed to form a disc using dies, which were scanned over a wave number range of 4000 cm^{-1} to 500 cm^{-1}.

Differential scanning calorimetry analysis

DSC was performed with an EXSTAR 6000 thermo gravimetric analyzer (EIKO, Japan). Stavudine, the physical mixture of stavudine and lipid used for CP4 (optimized formulation) and CP4 SLNs obtained by lyophilisation were placed in conventional aluminium pans and a scan speed of 5°C/min was employed.

In vitro release

Optimized formulation of SLNs (CP4, SP4, and GP4) in each lipid and pure drug were subjected to *in vitro* release studies, which were carried out using dialysis bag and modified apparatus. The dissolution medium used was freshly prepared phosphate buffer of pH 6.8. Dialysis bag, previously soaked overnight in the dissolution medium, was tied to both the end after filling the pure drug or SLNs in it. This dialysis bag was suspended in 200 ml of dissolution medium maintained at 37 ± 5°C. The dissolution medium was stirred at low speed using magnetic stirrer. Aliquots, each of 5 ml volume, were withdrawn at various intervals of time over a period of 72 h and each time fresh buffer were replaced by an equal volume of receptor medium. The aliquots were suitably diluted with receptor medium and analyzed by UV-Vis spectrophotometer at 265 nm. The quantity of drug equivalent to 30 mg of Stavudine was taken for the dissolution study.[19,20] All the experiments were performed in triplicate and the average values were taken.

In vivo study

The *in vivo* studies were performed to compare the targeting efficiency of drug-loaded nanoparticles with that of pure drug. The animals were kept under standard conditions, with free access to food and water. The optimized formulation, CP4, and pure Stavudine solution were given orally. For each preparation and each sampling time point, six rats, three males and three females (Wistar Albino rats), of body weight between 180 and 220 g were treated with a single dose of 4 mg Stavudine per kg body weight. After dosing the animals were kept in individual cages until they were sacrificed.

At 2 h and 6 h, animals were dissected and each tested organ (heart, liver, spleen, lung, kidney, brain, GI track, muscle, bone marrow, lymph node) was removed. Every organ sample was accurately weighed and homogenized. Homogenized tissue (0.5 g) was mixed with 0.1 mL Didanosine (50 mcg/mL), internal standard solution and 0.9 ml of phosphate buffer pH 6.8. The sample was stirred for 30 sec, centrifuged for 5 min at 3000 rpm and loaded into cartridge (The cartridge was previously conditioned with 2 ml of methanol and 2 mL of water). The cartridge was washed with 1 mL of water that was collected, filtered through 0.45 µm arcodisk and injected into the HPLC column attached to the UV detector.[21-23] The samples were separated on a Shimpack CLC-ODS C_{18} column (5 mm, 150 µm × 4.6 mm, ID) maintained at 30°C. A flow rate of 1.0 mL/min was maintained.

Stability study

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of variety of environmental condition such as temperature, humidity, and light. The best formulation CP4 was tested for its stability. CP4 was divided into 3 sets; 4 ± 1°C in refrigerator, 30± 2°C at 60% ± 5% RH in humidity control oven and 40° ± 2°C at 75 % ± 5% RH in humidity control oven.[24]

After 3 months particle size, percent drug entrapment, and *in vitro* drug release of formulations were determined by the method discussed previously. (In case of percentage drug entrapment efficiency one batch of frieze dried CP4 were dispersed in 200 mL de-ionized water and free drug were measured by method discuss previously. The value obtained is added to previous value of free drug to get total unentrapped drug after stability studies).

RESULTS AND DISCUSSION

Particle size and zeta potential

Particle size of SLNs of Stavudine was found in the range of 175 -393 nm, which is dependent on type of lipid used [Table 2].

It was found that particle size of SLNs is dependent on the melting point of the lipid used. Particle size of formulation

decreased with decrease in melting point of the respective lipids. The melting point of Glyceryl behenate, Stearic acid, and Cetyl Palmitate were 83°C, 69°C, and 54°C, respectively, and range of the particle size of respective SLNs were found in the range of 295-393 nm (Glyceryl behenate), 227-277 nm (Stearic acid), and 197-219 nm (Cetyl Palmitate).

Surface charge of the S1-S4 ranged from -4.35 to -5.13, which indicates low stability of the formulation. While in case of Cetyl palmitate and Glyceryl behenate, surface charge was in the range of -26.45 to -28.86, which indicates moderate stability. Formulations CP4 and GP4 showed maximum surface charge, i.e., -52.25 and 53.14, respectively, which indicates maximum stability. Formulation SP4 also showed considerable increased in surface charge compare to S4, which might be due to the negative charge of the DMPG used in combination with Stearic acid [Table 2].

% Drug entrapment efficiency

The % drug entrapment of stavudine SLNs were depicted in Table 2. Results indicate that as the lipid concentration increased from 100-400 mg, the encapsulation efficiency also increased irrespective of type of lipid used. The nature of lipids played a major role in drug entrapment efficiency. Drug entrapment efficiency was found to be higher in case of Cetyl palmitate made SLNs. This is because of the complex nature of Cetyl palmitate as compared to Stearic acid and Glyceryl behenate made SLNs. High encapsulation efficiency in CP4, GP4, and SP4 as compared to C4, G4, and S4, respectively, indicates that encapsulation efficiency can be significantly increased by using combination of lipids [Figure 1].

Table 2: Particle size, Zeta potential, and % drug entrapment of all formulations

Formulation	Particle size (nm)	Zeta potential	% Drug Entrapment
C 1	208 ± 2.645	-26.45 ± 0.01	21.40
C 2	219 ± 5.507	-27.52 ± 0.04	38.90
C 3	207 ± 10.214	-26.58 ± 0.01	51.40
C 4	197 ± 1.732	-26.76 ± 0.02	62.20
G 1	393 ± 2.309	-28.45 ± 0.026	18.9
G 2	357 ± 16.623	-27.95 ± 0.03	18.1
G 3	317 ± 7.00	-28.86 ± 0.02	32.8
G 4	295 ± 4.041	-27.76 ± 0.01	43.8
S 1	277 ± 6.658	-4.58 ± 0.01	18.38
S 2	250 ± 8.577	-4.95 ± 0.01	37.68
S 3	245 ± 10.598	-5.13 ± 0.01	47.45
S 4	227 ± 2.645	-4.35 ± 0.01	51.2
CP 4	175 ± 6.027	-52.25 ± 0.02	65.2
GP 4	276 ± 14.754	-53.14 ± 0.04	51.08
SP 4	210 ± 9.415	-22.25 ± 0.02	57.56

Scanning electron microscopy

The SEM of CP4 formulation reveals the smooth and spherical shape of SLNs.

IR studies

IR studies indicate that there was no interaction between Stavudine and various lipids used. Characteristic peak of OH stretching, C = O stretching, C-H bending were observed at 1114 cm^{-1}, 1688 cm^{-1}, 1458 cm^{-1}, respectively, in IR spectrum of pure drug. The same peaks were also reported in all stavudine-loaded SLNs prepared using different lipids [Figure 2]. There was no change or major shifting of characteristic peaks of stavudine in drug-loaded SLNs suggested that there was no significant drug lipids interaction, which indicates the stable nature of drug in all formulations.

Differential scanning calorimetry analysis

Any possible drug polymer interaction can be studied by thermal analysis. DSC studies were performed on pure drug, physical mixture of drug and lipid used in CP4, and drug-loaded SLNs (CP4). Stavudine exhibits a sharp endothermic peak at 169.40°C, which corresponds to its melting point. The peak of the drug appear in the thermogram of physical mixture indicating no drug lipids interaction but did not appear in drug loaded SLNs [Figure 3]. It may indicate that the drug was uniformly dispersed at the molecular level in the SLNs.

In vitro release

Different formulations showed different degree of drug release [Figure 4]. It was observed that the drug release

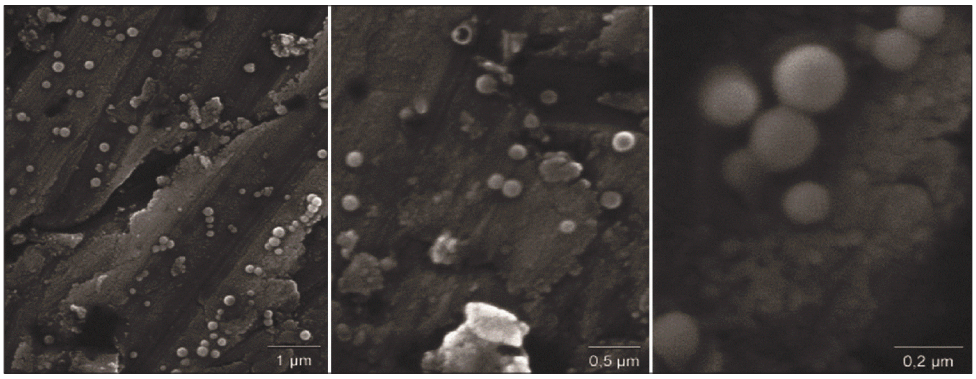

Figure 1: Scanning Electron Microscopy of formulation CP4

from the formulations slightly increases as the particle size of the formulation decreases and all the four formulations showed a biphasic release with initial burst effect up to 72 h.

Although the significance of burst release in controlled delivery systems has not been entirely considered, no successful theories have described the phenomenon yet. The mechanism for the burst release may be attributed to the drug adsorbed on SLNs or due to leakage of drug from SLNs. Despite the fast release of drug in a burst stage is used strategically in certain drug administration, the negative effects caused the burst may be pharmacologically dangerous and non-viable economically. Therefore, a thorough understanding of the burst effect in controlled release systems is undoubtedly necessary. Calculated regression co-efficient values for different formulations were tabulated in Table 3. Based on the high regression values (r), the best–fit model for formulation C4, S4, G4, CP4 was found to be Higuchi Model and '*n* value >0.5' value in Peppa's model indicating non-fickian diffusion release.

In vivo organ distribution study

Particulate substances or drug carriers with an average size below 7 µm are normally taken up by the macrophages of the RES, particularly by the Kupffer-cells of the liver.[25,26] This was also apparent in the current study for stavudine-loaded SLNs. Since stavudine is a hydrophilic in nature, it cannot pass Blood Brain barrier (BBB) but SLNs of stavudine can pass BBB because of its lipophilicity.

Results clearly indicate effectively uptake of stavudine-loaded SLNs by RES and brain (as depicted in Tables 4a, and 4b and Figures 5a and 5b). In case of SLNs, the total amount of stavudine in the organs of the RES (liver, spleen, lungs, bone marrow, and lymph nodes) amounted to around 60% of the dose after 2 h, while in case of pure drug it accounted for only 19% [Table 4a and Figure 5a]. In the brain, almost 11 times higher concentrations were found compare to pure drug indicating accessibility of SLNs into brain through BBB. Concentration of drug in lymph node was found almost 4 times greater in SLNs compared to pure drug. This may be because of direct absorption of SLNs to lymph system via M cells of Payer's Patches. In contrast to this, drug concentrations in muscle and GIT were found less in case of SLNs compare to pure drug.

After 6 h, concentration of drug was almost negligible in animals treated with pure drug compared to SLNs. In SLNs treated animals concentration of drug was found 30% in RES even after 6 h indicating sustained release of stavudine form SLNs [Table 4b and Figure 5b].

The results of the present *in vivo* study show, as anticipated, that the antiviral drug Stavudine reaches higher drug concentrations in the organs of the RES after binding to the SLNs. This enhancement is probably due to the uptake of drug bound to nanoparticles by macrophages that phagocytize the colloidal drug carrier. Most notably higher brain levels of stavudine were observed, indicative of a facilitation of antiviral drug passage across the blood–brain barrier. This would be an important benefit during AIDS therapy with such preparations, since HIV infects the central nervous system to a significant degree, causing neurological pathology.

Stability study

In vitro drug release for the formulation C4, G4, C4, and CP4 after 90 days of stability testing at different storage conditions are shown in Figure 6. By comparing this data with previous data it was observed that there is overall decrease in % drug entrapment and increased in particle size at 25° ± 2°C / 60 ± 5% RH [Table 5] and on comparing with the previous data of cumulative % drug release data of CP4, it was observed that there is overall increase in the drug release [Figure 6].

These results may be attributed to bulk erosion of lipid to some extent during storage. It was also observed that there were no any significant changes in the particle size, percentage drug entrapment efficiency, and *in vitro* drug release of the selected formulation CP4 when stored at 4 ± 1°C temperature.

CONCLUSION

Stavudine-loaded SLNs were successfully prepared, optimized, and effectively targeted to RES and brain.

Table 3: Model fitting for release profile of formulations

Formulation	Mathematical models						Best fit model
	First order	Zero order	Higuchi model	Korsemeyer Peppa's model		Hixon-Crowell model	
				R^2	n		
C4	0.910	0.973	0.994	0.993	0.524	0.972	Higuchi
S4	0.966	0.924	0.985	0.986	0.502	0.064	Higuchi
G4	0.963	0.926	0.986	0.985	0.512	0.966	Higuchi
CP4	0.986	0.908	0.996	0.992	0.536	0.981	Higuchi

Cetyl palmitate was found to be best lipid as compared to Stearic acid and Glyceryl behenate and combination of lipid would be more beneficial for preparation of SLNs of Stavudine. The study demonstrate that hydrophilic drug like Stavudine can be targeted to brain and RES. The importance of macrophages and central nervous system in the progression of AIDS is well established. For this reason, the need to target drugs specifically to macrophages and brain was stressed. The SLNs of Stavudine have been shown to be taken up by brain 11 fold greater as compared to pure Stavudine. This finding is more important, since Human Immunodeficiency Syndrome (HIV) infect the central nervous system. Results of stability study indicate best condition for storage of SLNs is at 4 ± 1°C. This novel strategy reported here may provide a mechanism for improving therapy and reducing the dose of stavudine and might be attractive to the development of other HIV or brain targeting of other hydrophilic drugs.

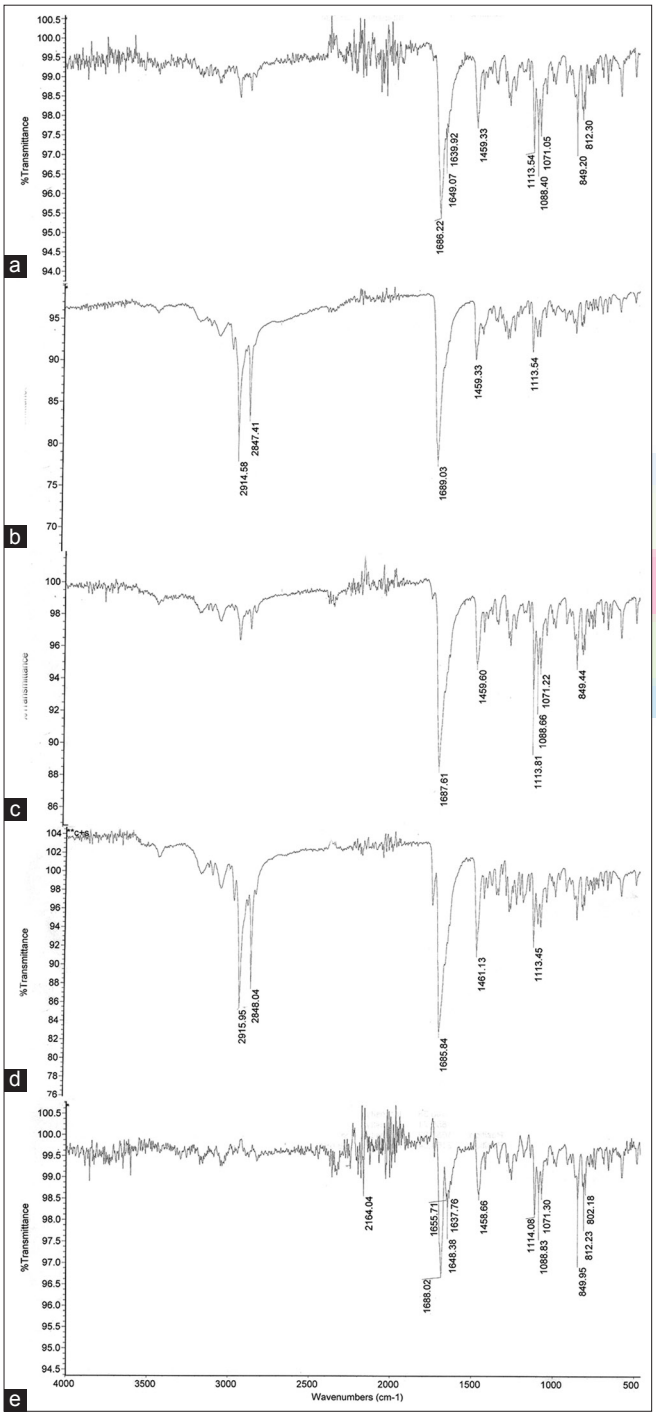

Figure 2: IR spectra of (a) Pure Stavudine (b) C4 (c) G4 (d) S4 and (e) CP4

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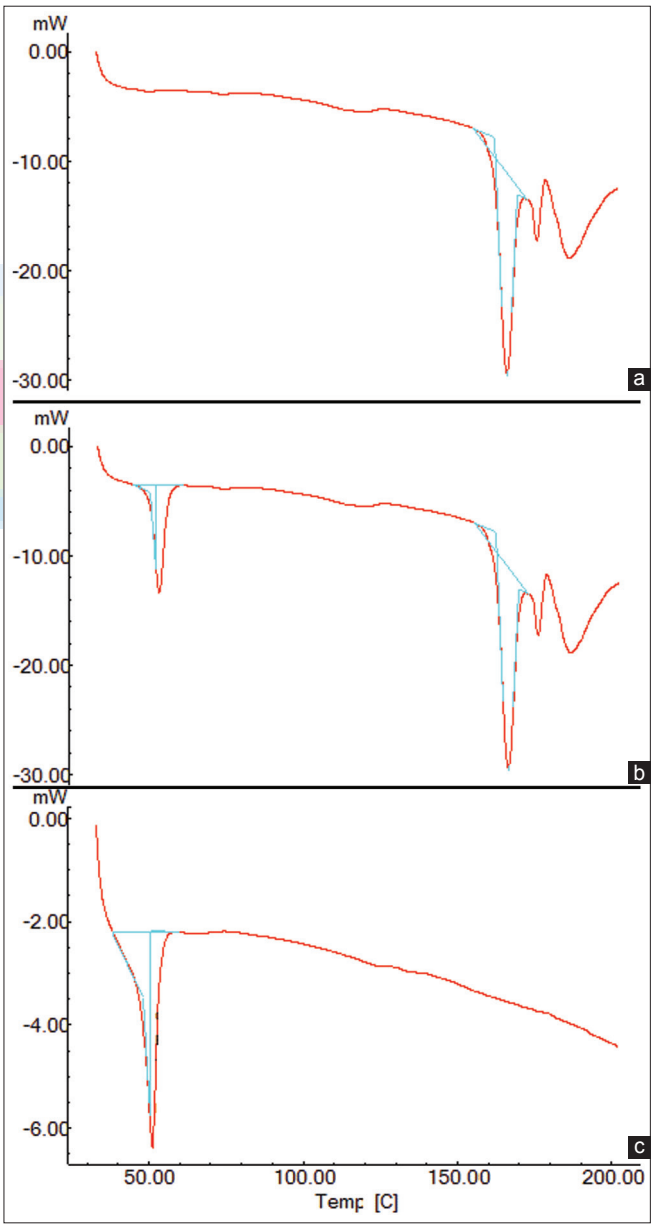

Figure 3: Differential Scanning Calorimetry of (a) Pure Stavudine (b) Physical mixture of drug + lipids used in CP 4 (c) Formulation CP4

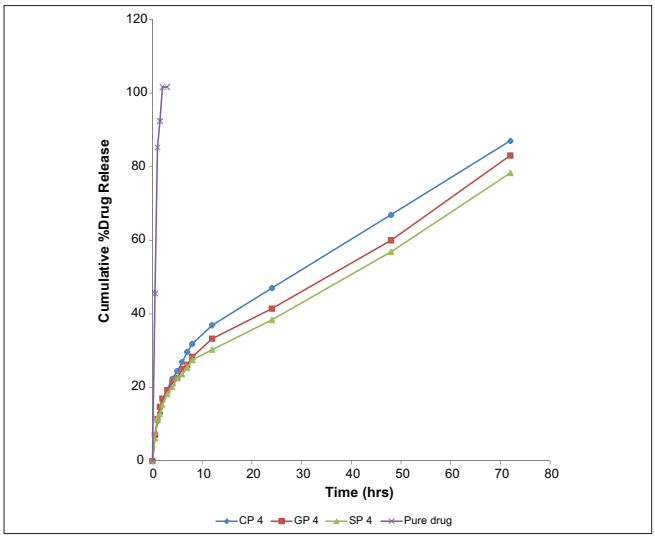

Figure 4: *In vitro Drug* release profile for the formulations CP4, GP4, SP4 and Pure drug

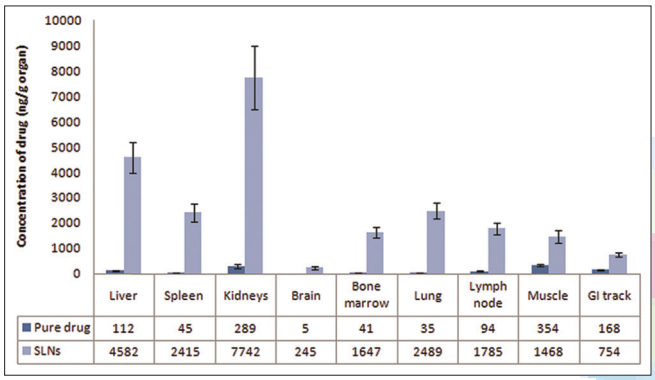

Figure 5b: Comparative *in vivo* tissue distribution studies of Stavudine SLNs (CP-4) with pure drug after 6 h

of Satvudine; to Phoenix Chemical, Inc. NJ, USA, for providing Cetyl palmitate and Glyceryl behenate; to Lipoid, Germany,

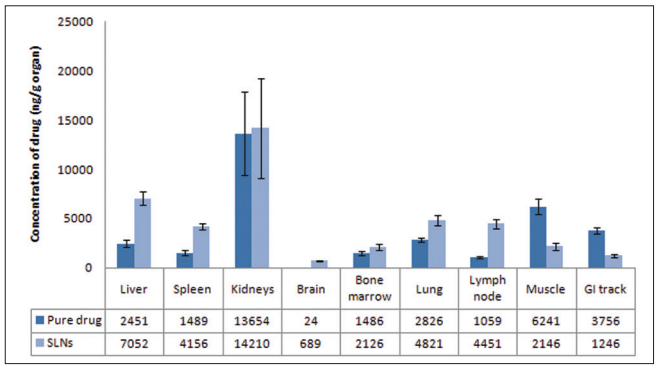

Figure 5a: Comparative *in vivo* tissue distribution studies of Stavudine SLNs (CP-4) with pure drug after 2h

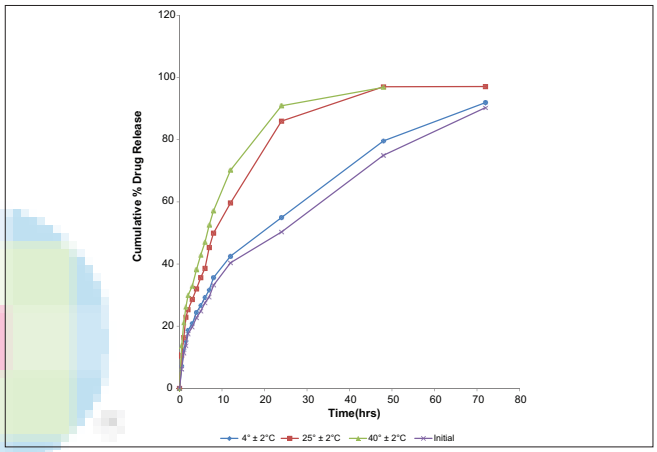

Figure 6: *In vitro* drug release profile of optimized formulation CP 4 for stability studies at different condition after 90 days

Table 4a: Concentration of Stavudine in different organs after orally given SLNs (ng stavudine g^{-1} organ) (*n* = 6)

Time	After 2 h	After 6 h
Organ	Mean (ng) ± S. D.	Mean(ng) ± S. D.
Liver	7052 ± 2.27	4582 ± 2.59
Spleen	4156 ± 1.52	2415 ± 1.32
Kidneys	14210 ± 2.09	7742 ± 0.185
Brain	689 ± 1.85	245 ± 0.59
Bone marrow	2126 ± 1.43	1647 ± 1.21
Lung	4821 ± 1.58	2489 ± 1.59
Lymph node	4451 ± 2.24	1785 ± 1.04
Muscle	2146 ± 2.07	1468 ± 2.35
GI track	1246 ± 1.68	754 ± 0.86

Table 4b: Concentration of Stavudine in different organs after orally given pure Stavudine (ng Stavudine g^{-1}organ) (*n* = 6)

Time	After 2 h	After 6 h
Organ	Mean (ng) ± S. D.	Mean(ng) ± S. D.
Liver	2451 ± 1.73	112 ± 0.34
Spleen	1489 ± 1.06	45 ± 0.08
Kidneys	13654 ± 1.89	289 ± 0.85
Brain	24 ± 0.95	05 ± 0.00
Bone marrow	1486 ± 2.06	41 ± 0.17
Lung	2826 ± 1.36	35 ± 0.11
Lymph node	1059 ± 1.18	94 ± 0.05
Muscle	6241 ± 2.18	354 ± 0.41
GI track	3756 ± 1.48	168 ± 0.24

Table 5: Entrapment efficiency and particle size after 90 days storage of selected formulation CP4

Time	Evaluation parameters			
	Initial	4 ± 1°C	25° ± 2°C/60 ± 5 % RH	40° ± 2°C/75 % + 5% RH
Paricle size (nm)	176 ± 6.027	177 ± 7.289	245 ± 6.452	316 ± 7.627
% Drug Entrapment	65.2	63.7	39.5	35.4

for providing Dipalmitoylphosphatidylcholine (DPPC) and Dimyristoylphosphatidylglycerol (DMPG).

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