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Formulation And Evaluation Of Antiretroviral Drug Loaded Unsaturated Phospholipid Based Stealth Liposome

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ABSTRACT

Purpose: The present work aims to develop the didanosine loaded stealth liposomes prepared by reverse phase method using Lipoid-S-100 base and DSPE-m-PEG-2000 as stealth forming agent.

Methods: In the process of the development of liposomes, amount of Lipoid-s-100 and cholesterol plays key role and changes the physicochemical properties of liposomes. Then current study focuses on development of different didanosine loaded liposomes screening batches by altering the amount of Lipoid-s-100 and cholesterol using reverse phase technique. The liposomes were evaluated for particle size, zeta potential, percentage of encapsulation and in vitro drug release.

Results: The particle size was found as 287.4 nm, PDI 0.345 and zeta potential was found as – 27.8mV respectively. The assay was found around 100% and the percentage encapsulation was found 89.42±0.9%. The in-vitro release studies of stealth liposomes presented 100 % of drug release up to 28hrs.

Conclusion: The present studies indicated that didanosine liposomes suitable tool for antiretroviral therapy.

Keywords: Phospholipid, Stealth coating, Parenteral Delivery, Small Unilamellar Vesicles

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INTRODUCTION

Didanosine is a synthetic purine nucleoside analogue active against HIV-1. Didanosine is indicated in combination with other antiretroviral agents for the treatment of HIV-1 infection. Didanosine is available as Videx, a pediatric powder for oral solution. Didanosine belongs to BCS-III, which has high solubility and low permeability. It has 27.3 mg/ml aqueous solubility at room temperature. On oral administration, the didanosine having degradation in presence of acid medium at pH 1 to 3 (stomach pH 1 to 3) [1]. To overcome this side effect, we are planning to develop didanosine liposomes for parenteral delivery.Liposomes are heterogeneous bilayer vesicular systems, made up of phospholipid with or without cholesterol. Liposomal technology is the approved novel tool to overcome the associated disadvantages with conventional therapy for various applications. Liposomes are versatile dosage forms to justify the various categories of active pharmaceutical substances and can be administered by various routes such as oral, nasal, parenteral, dermal, transdermal, pulmonary and ocular delivery etc. Liposomes offer desired advantages by non-toxic, biocompatible, biodegradable and bio-absorbable respectively. They have ability to improve the therapeutic properties of drugs by encapsulating in to their suitable portion of the liposomes with lower dose leads to reduced toxicity. Liposomes has ability to release the drug by prolong circulating in the systemic circulation by avoid the adsorption of opsonin proteins molecules (improved *in vivo* stability) due to stearic stability property leads to improve the patient compliance by prolong systemic circulation[2-4].

There are few literature reports on stealth liposomes for antiretroviral therapy. Biswajit et al Investigated acyclovir loaded stealth liposomes by parenteral delivery. The stealth liposomes improved mean residence time of didanosine and repeat administration was reduced by their sustained delivery, leads to decrease the dose related toxicity [5]. Sudhakar B et al reported on ritonavir loaded stealth liposomes using DSPC and PEG-20000. They reported that solubility of ritonavir and encapsulation was improved. In their study, the stealth liposomes followed dose independent pharmacokinetic whereas the classic liposome and drug followed dose-dependent pharmacokinetics [6].

Lakshmi Narashimhan et al., reported on saquinavir stealth liposomes. The anti-viral activity of saquinavir stealth liposomes shown superior properties compared to conventional liposomes, by using cytotoxicity studies on Jurkat T-cells [7]. Madhavi et al reported on didanosine loaded stealth liposomes using synthetic DSPC as base and PVA as stealth forming agent. They reported that liposome was found 43-91% of % EE. The *in vitro* drug release was found 86% for 12 hr respectively [8].

In the present study, didanosine loaded stealth liposomes was studied using Lipoid-s-100, is an unsaturated phospholipid, DSPE-m-PEG-2000 was chosen as stealth forming agent by reverse phase technique method. Basically, the saturation and unsaturation state of phospholipid and their composition directly influence the physicochemical properties of liposomes. Hence in the current study, we have selected Lipoid-s-100 is unsaturated to determine their impact on physichochemical properties of liposomes. Didanosine, selected as a model drug, having high solubility 27.3 mg/mg/ml and low permeable rate. The permeability can be expected to be more in the form of liposomes. Didanosine is susceptible to acid hydrolysis and is degraded rapidly at low gastric pH (pH<3). These problems can be overcome by loading the selected drug into stealth liposomes and delivered by parenteral route.

Materials

Didanosine procured from Sigma-Aldrich Pvt Ltd., Lipoid-s-100 and DSPE-m-PEG2000 from Lipoid Germany. Cholesterol and stearic acid were purchased from loba cheme pvt. Ltd., mumbai. Ehanol from Merck pvt. Ltd,

sodium hydroxide and potassium dihydrogen phosphate from Merck pvt. Ltd., Mumbai. All materials used were of analytical grade.

Preparation of liposome by Reverse Phase Method

In the present study, amount of Lipoid-S-100 and cholesterol use to change their concentrations for each batch whereas amount of drug was 50 mg, DSPE-m-PEG-2000 (3 mg/ml) and final liposomal dispersion medium was 10 mL and it kept constant (Table1).

Formulation development process has an impact on their liposomes morphology (unilamelar, multilamellar and multivesicular), encapsulation efficiency and drug release properties. Reverse phase method is suitable for development of hydrophilic drug loaded liposomes which is encapsulated in internal aqueous core. Didanosine, being as a hydrophilic agent, we have adopted reverse phase method for the development of didanosine liposomes. Required amount of Lipoid-S-100, cholesterol and DSPE-m-PEG-2000 were dissolved in requisite volume of ethanol in glass beaker with slight heating (≥50°C) on a hot plate and filtered through 0.2 µm filter. The didanosine was dissolved in 10 mL of sterilized purified water by magnetic stirrer (Heidolph) with 600 to 800 rpm. Then the aqueous drug solution added to the organic phase under stirring, leads to formation milky suspension which indicates the formation of the liposomes. The system was remaining under stirring up to 1 hour to facilitate ethanol removing. The whole formulation development was carried out in aseptic area under laminar air flow. Finally, pH was adjusted to around pH 6.0 using 0.1 N sodium hydroxide solutions. The liposomal dispersion volume was made up to 10 ml with the sterile pH purified water. The prepared liposomes taken into 10 ml clear type-1 of glass vials then nitrogen purging and stoppered by serum coated chlorobutyl stoppers (West Pharmaceutical Services Pvt Ltd) with flip off seals and stored at 2-8°C[9].

Table 1: Composition of Didanosine Liposome

Formulation code	Lipoid-S-100 (mg)	Cholesterol (mg)	mPEG-DSPE-2000 (mg)	Stearic acid	Didanosine	Distilled water
L1	50	20	30	10	50	10
L2	50	30			50	10
L3	75	20			50	10
L4	75	30			50	10
L5	100	20			50	10
L6	100	30			50	10

Note: All the excipients and drug was taken in milligrams whereas distilled water in milli litter respectively.

Characterization of liposome

Percentage of assay: The liposomes dispersion equivalent to 5.0 mg was taken and lysed with methanol and subsequent dilutions were made with distilled water and given concentration of drug by UV Spectroscopy at 250 nm [10].

Percent entrapment efficiency (%): The percent entrapment efficiency of didanosine loaded liposomes was carried out by using ultra centrifugation (Eppendorf). 2 ml of liposomes dispersion was subjected for ultra-centrifugation with 20000 rpm at 4°C for 2 hr. The supernatant was collected from pellet and pellet was lysised with methanol and concentration of PPS in supernatant and pellets was estimated by HPLC [10].

$$\%\ \text{Entrapment Efficacy (EE)} = \frac{(C_d - C)}{C_d} \times 100$$

Where C is unentrapped drug concentration and C_d is total drug concentration .

Size distribution and Zeta potential (ζ) measurement: The disposable capillary cell was used for measurement of zeta potential. Didanosine loaded liposomes directly into a disposable capillary cell and put inside the sample holder of the instrument (Malvern Nano ZS90, Malvern, UK) for zeta potential measurement. The zeta potential was measured at 90° light scattering angle and at 25°C. The ζ was measured based on the mobility of vesicles [11 -13].

pH: The Didanosine loaded liposomes was checked for pH at room temperature using thermo scientific model: Orion Star A211 pH Meter.

***In-vitro* release study:** *In vitro drug* release study was done by dialysis process in a beaker filled with 500 ml of PBS pH 6.8 kept under agitations of 100 rpm using teflon coated bead at 37±2ºC temperature. The liposomal suspension equivalent to 10 mg of didanosine was filled into the cellulose dialyzing membrane (dialysis membrane 60 from HIMedia pharmaceuticals, Mumbai, India whose molecular cut-off is 600D) and immersed in the receptor medium overnight before experiment and was tied with thread at both ends. 5ml aliquots were taken from dialysis medium and were displaced with an equal volume of fresh buffer to adjust constant dialysis volume at predetermined time intervals (1, 2, 4, 6, 8, 10, 12 and 24, hrs). The samples were analyzed by UV spectroscopy at 250 nm [11-13].

Drug release kinetics: To know the kinetics and mechanism of drug release, the data of *in vitro* drug release study of optimized liposomes fitted with various kinetic equations like Zero order (cumulative % released vs. time), First order (log % drug remaining vs. time), Higuchi's model (cumulative % drug released vs. square root of time) and Peppas (log % drug released vs. log time) and corresponding K and r values were calculated [14].

Visualization by scanning electron microscopy: The didanosine liposomes surface morphology was elaborated by scanning electron microscopy using HITACHI S3400 SEM instrument. The sample was examined at magnification of ~20x to ~20,000x.[15].

Statistical analysis: All the physicochemical parameters were measured as mean±s.d. Statistical analysis was carried out using Prism 8.4.2. Software trial version (Graph pad Inc. USA) Paired t-test was used for comparison of the stealth liposomes physicochemical parameters. All the statistical tests were performed at a significance level $p<0.05$.

RESULTS AND DISCUSSION

The present work aims to develop the didianosine stealth liposomes for intravenous delivery (IV). Various methods have been reported in the literature for preparation of stealth liposomes. Initially, we have tried methods feasible in our laboratory like film hydration method; it however resulted in extensive aggregation of vesicles

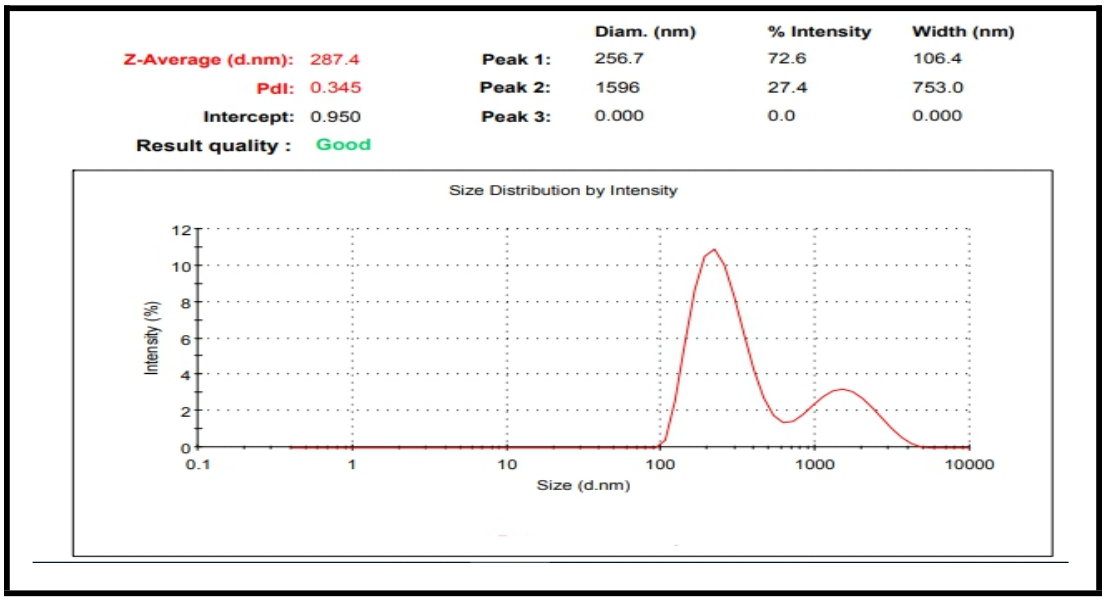
when observed microscopically. Subsequently reverse phase method was utilized as satisfactory and reproducible results were obtained. The percentage drug content of didanosine stealth liposomes formulation batches varied between 90.0 to 101.2 % as per the values given in the table 2 and values are found within the range of 90% to 110% of USP standards. The percent drug content of didanosine stealth liposomes indicates that there was no drug loss during the preparation of liposomes. The didanosine stealth liposomes % EE was given after separating entrapped and unentrapped drug by ultra-centrifugation. It is varied from 53 % to 89% for all the formulations. The highest %EE of 89% was observed for L3 formulation. The results are tabulated in table 2. The %EE was influenced by particle size, amount of the Lipoid-S-100 and cholesterol. As didanosine is a BCS Class-III drug, lower amount of cholesterol allows maximum internal aqueous volume of liposomes bilayered vesicle which leads to more encapsulation of didanosine whereas the higher concentration of cholesterol restrict the entry of internal core volume of vesicles and leads to decreased % EE of didanosine. Cholesterol is used as stabilizer for liposomes, when Lipoid-S-100 forming self-assembled bilayer, cholesterol fills the gaps between the liposomal bilayered gaps and restricts the movement of internal and external aqueous medium. So, the amount of Lipoid-S-100 and cholesterol is crucial for liposomes development. The didanosine liposomes mean vesicle size was found in the range of 287 nm to 320 nm and it was hardly affected by the selected excipients. The polydispersity index (PDI) of liposomes was found in the range of 0.171-0.345 which indicates the uniform distribution of particles. The vesicle size and PDI of all the liposomal batches were shown in table 2.

Table 2: Physico-chemical characterization of didanosine liposome

Batch Code	Assay (%)	vesicle size (nm)	PDI	Zeta potential (mV)	%EE	pH
L1	100.1±1.02	305±1.07	0.186±0.08	-23.1±1.6	59.4±1.93	6.09±0.9
L2	99.99±1.9	286±1.04	0.224±0.15	-21.8±1.1	53.12±2.12	6.01±1.2
L3	100.10±1.2	287 ±1.26	0.345±0.02	-27.8±1.2	89.42±0.9	6.08±0.66
L4	99.91±0.3	230±0.12	0.252±0.09	-26.4±0.9	83.2±2.65	6.02±0.81
L5	99.73±0.50	296±0.09	0.171±0.18	-21.3±1.6	78.21±1.8	6.06±1.21
L6	99.95±0.67	320±0.17	0.312±0.23	-24.4±1.1	65.34±1.9	6.04±1.42

Each value is mean ± s.d. (n=3)

Zeta potential is the primary property of colloidal dispersion systems which reflects their physical stability. Zeta potential values of prepared vesicular systems ranged from -21.3 to -27.8mV. This may be due to the surface charge giving nature of stearic acid. This result might have come because of the incorporation of the negatively charged stearic acid in the formulation. Vesicular systems were reported to have greater stability comparative to other colloidal dosage forms. Zeta potential vales in the range of +30mV to -30mV are required for the stability and to inhibit aggregation of vesicular systems. The results of zeta potential specified that didanosine stealth liposomes have adequate charge to prevent aggregation of liposomal vesicles due to electric repulsion. The liposomal zeta potential results are shown in the table 2. The pH of didanosine liposomes were found to be around pH 6.0., indicated that liposomes are suitable for parenteral delivery by intravenous route respectively. Figure1 showed the particle size and zeta potential respectively



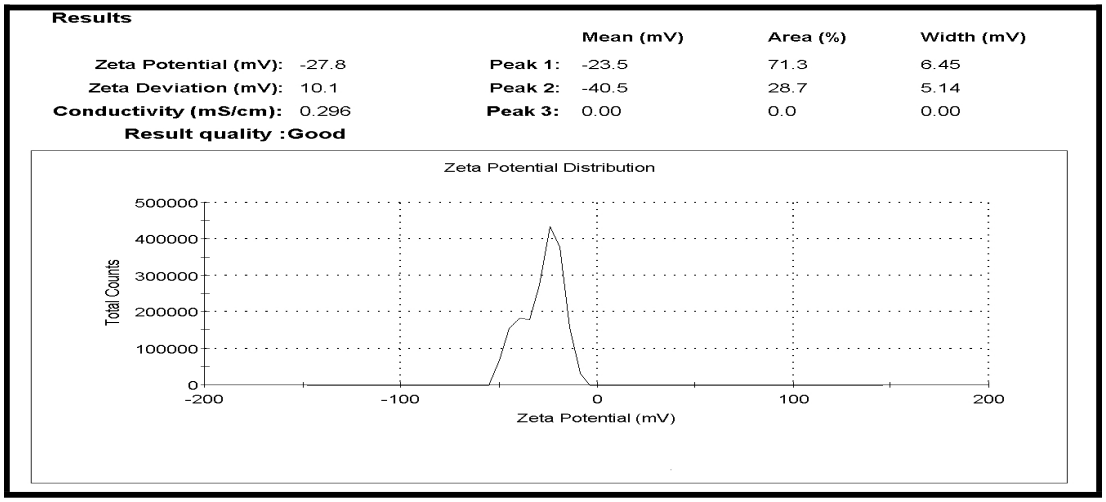


Figure 1: Particle size and zeta potential of didanosine loaded stealth liposome

All the liposomal formulations showed the drug release up to 100% with various initial burst releases and their release of drug duration was appearing in the range of 24 to 28 hrs respectively. The drug release of didanosine from liposomes impacted by various physicochemical properties i.e. size and % of EE respectively. The drug release percentage and the release duration are proportional to %EE and their particle size of liposomes respectively. The particle size of all the liposomes were found in the nanometers in range, the lower particle size of liposomes has more surface area which can accelerate the drug release during dissolution by dialysis process whereas the larger the particle size of liposomes provides the lower surface area which delays drug release rates. The ratio between didanosine liposomes dose volume and dialysis medium was maintained at 1:250 ratios. This step is necessary to reserve a driving force for drug transport to the outside and to keep sink conditions.On other side, the drug release from liposomes is influenced by the synergetic effect of Lipoid-S-100 and cholesterol respectively. At lower amount of cholesterol, liposomes showed extended drug release time than the higher amount of cholesterol of the liposomes. At lower amount of cholesterol, the liposomes are bigger in size and lead to more % of EE and release the drug over period of time. The drug release pattern in figure 2. Indicates that didanosine liposomes shown biphasic release profile due to unentrapped drug was dumped immediately by burst release tendency of liposomes. The initial burst release also varied with respective % EE of the liposomes dispersions.

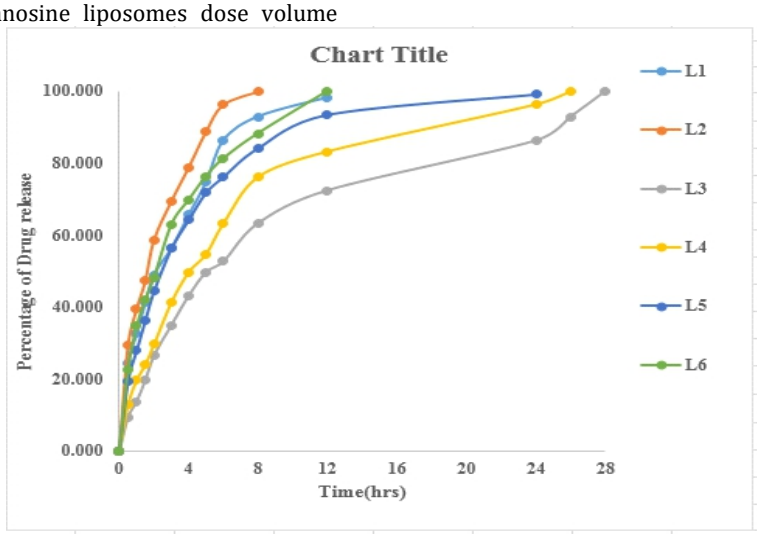


Figure 2: *In vitro* percentage drug release of didanosine liposomes by dialysis process

The data obtained from the *in-vitro* release of optimized formulation was fitted to various kinetic equations like zero order, first order, Higuchi, Hixon Crowel and Peppas to give the drug release mechanism and the results are shown in table 3. All the liposomal formulation followed first order release kinetics in compared with their zero-order release kinetics as specified by their correlation value 'r' except L3 batch. L3 batch drug release pattern follows zero order kinetics as clear by its higher 'r' value (0.9938) than first order (0.9870). All the prepared liposomes release the drug through diffusion mechanism and erosion which was confirmed by higher 'r' value of Higuchi, Hixon Crowel and the type of diffusion was fickian as given by 'n' value of Peppas plot. As per theory drugs encapsulated in the vesicular systems are released possibly by the following mechanisms: a) passive diffusion b) vesicle erosion.

Table 3: Drug release kinetics of the screening liposomal batches

Batch Code	Zero order	First order	Higuchi	Hixon Crowel	Peppas

	K_0	r	K_1	r	n	r	r	n
L1	2.27	0.9529	0.029	0.9682	0.9816	0.8851	0.9791	0.393
L2	2.43	0.9364	0.035	0.9762	0.9850	0.7404	0.9860	0.344
L3	2.37	0.9938	0.038	0.9870	0.9879	0.8647	0.9828	0.496
L4	1.83	0.9276	0.019	0.9336	0.9459	0.6769	0.9586	0.370
L5	2.04	0.9516	0.026	0.9641	0.9792	0.8499	0.9714	0.386
L6	2.04	0.9462	0.028	0.9581	0.9744	0.8259	0.9737	0.347

Scanning electron microscopy (SEM) confirmed the formation of stealth vesicles.The scanning of the grids showed the presence of spherical vesicles. SEM micrographs of the sample replica were taken to visualize the vesicles. The stealth liposomes morphology has given in the figure 3.

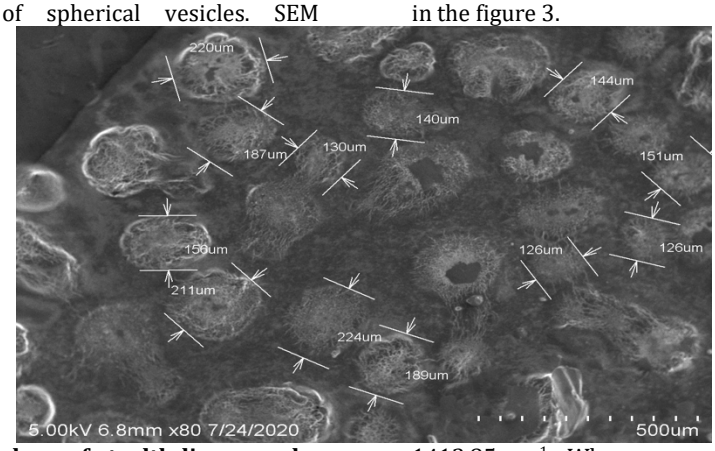
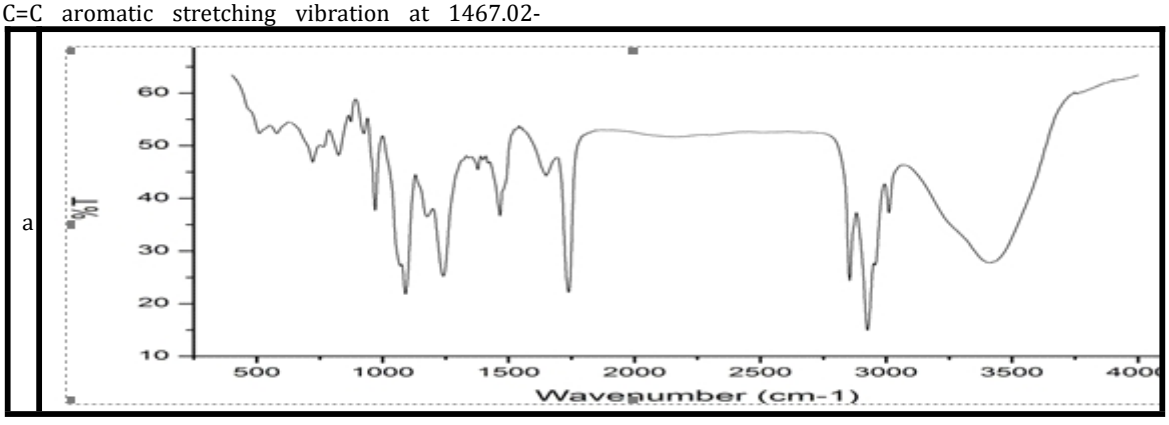


Figure 3: Surface morphology of stealth liposome by SEM

Drug and other excipients along with the optimized formulations were subjected to FTIR studies and the results obtained were shown in figure 4. The FTIR spectrum of didanosine pure drug exhibited characteristic absorption at 3400 cm^{-1} representing the presence of –CONH. Whereas a characteristic absorption band at 1820-1660 cm^{-1} is due to the presence of -C=O, and absorption band at 1300-1000 cm^{-1}. Cholesterol FTIR spectrum present C-O alcoholic stretching vibration at 1022.59 cm^{-1}, O-H stretching vibration at 3417.9 cm^{-1}, C-H (aliphatic) stretching vibration at 2932 cm^{-1} and C=C stretching vibration at 1465cm^{-1}. The FTIR spectrum of stearic acid shows C-H aliphatic stretching vibration at 2955 cm^{-1} and C=O (acid) stretching vibration at 1703cm^{-1}. Lipoid-S-100 FTIR spectrum , the band at 1735.93 cm-1 is attributed to C=O stretching vibration due to the ester group and 1238.30 cm-1 due to the Phosphate group. The bands appearing at 3300-3400 cm^{-1} are due to O-H stretching vibration and 3000-2800cm^{-1} are due to the stretching vibration of the bonds in –CH and $–CH_2$ groups and FTIR spectrum of liposomes suspension present N–H stretching vibration at 3344.66 $cm^{-1,}$ aliphatic C–H stretching vibration at 2928 cm^{-1}-2849.88 cm^{-1} and aromatic C=O stretching vibration at 1744.23 and C=C aromatic stretching vibration at 1467.02-1413.85 cm^{-1}. When comparing FTIR spectra of the pure drug didanosine, excipients and liposomes suspension, it is clear that there was no interaction of didanosine with excipients used in the formulations. In the liposomes suspension, the drug in combination with excipients did not produce major shift in principal peaks of didanosine, indicating no interaction due to presence of excipients.Madhavi N et al developed didanosine loaded liposomes using PVA as stealth converting agent and DSPC as synthetic lipid. They reported that 43-91% of encapsulation and in vitro drug release of liposomes showed 86% for 12 hrs whereas from our study, the particle size was found 287 nm to 320 nm, PDI 0.171-0.345 and encapsulation percentage efficiency was appear in the range of 53% to 89% respectively. The *in vitro* drug release found around 100% in the range of 8hrs to 28 hrs . The didanosine loaded liposomes results indicated that physicochemical properties of phospholipids (Lipoid-S-100) influenced the liposomes behavior. Especially from % of EE of didanosine from liposomes indicated that Lipoid-S-100 based liposomes has higher encapsulation (86%). This result in compatible with fact that the unsaturated fatty acyl chains of Lipoid S100 give high membrane flexibility, which would permit more didanosine to be entrapped into Lipoid S-100 liposomes [16].



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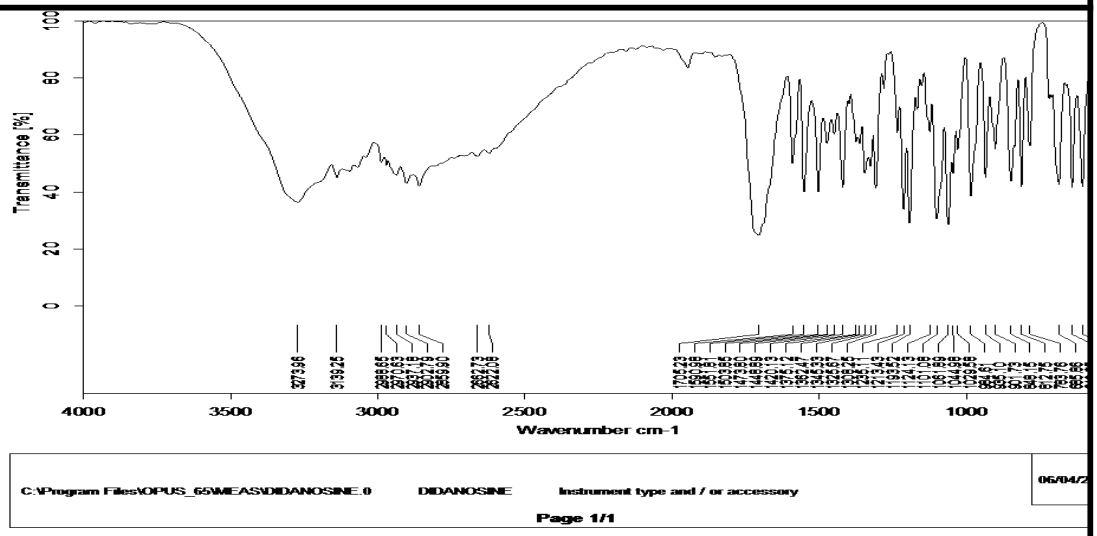
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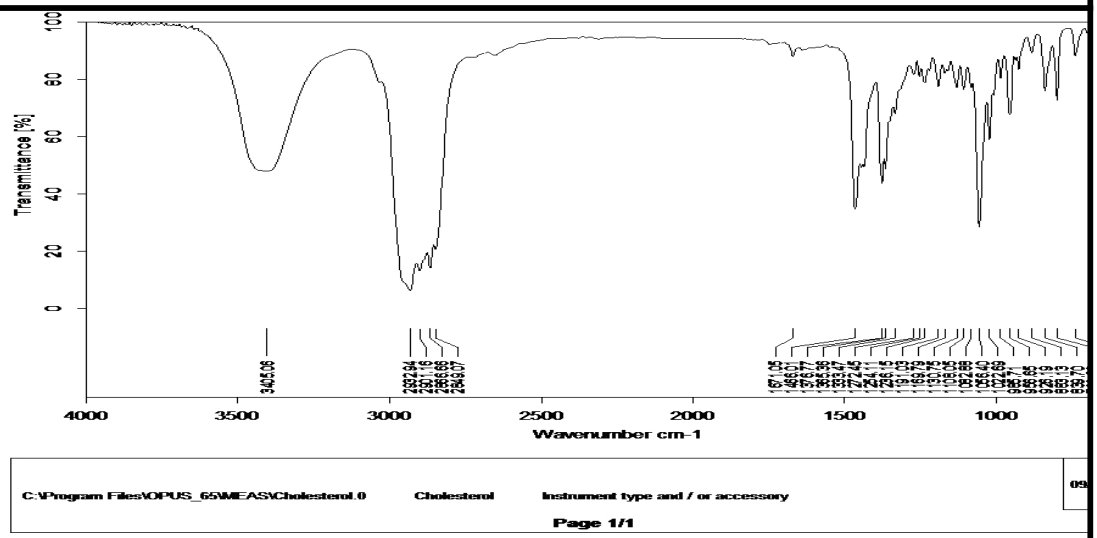
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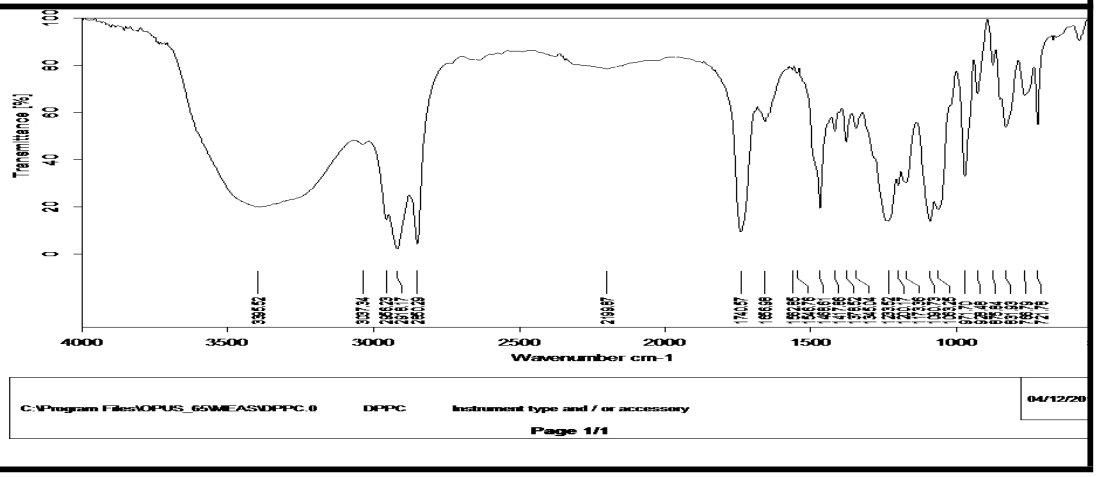
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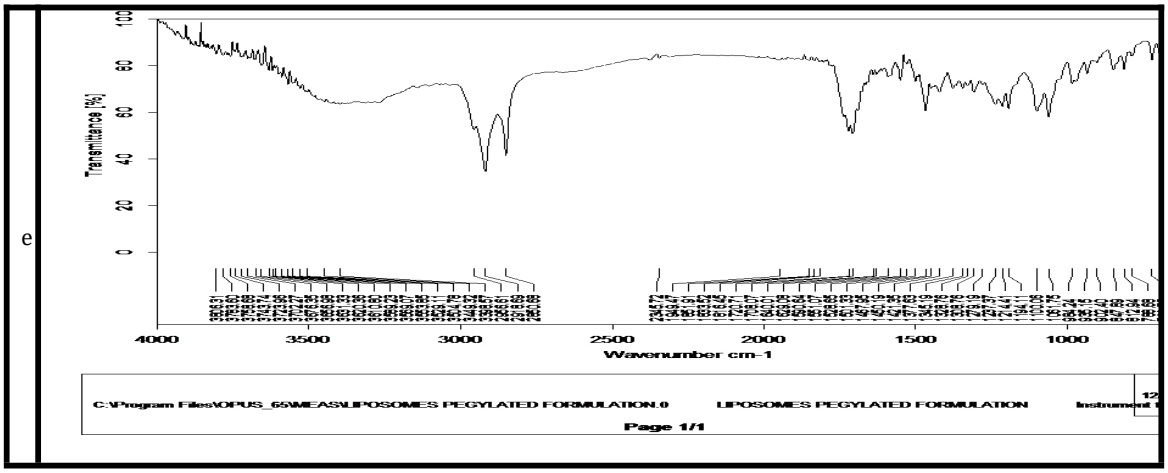

Figure 4: a) Lipoid-S-100 b)Didanosine C) Cholesterol D) DSPE-m-PEG-2000 , f) stealth liposomes

CONCLUSION

Didanosine loaded stealth liposomes were successfully developed by reverse phase method. All the physicochemical profile of prepared liposomes were found with satisfactory results. Stealth liposomes *in vitro* drug release was found around 100% within the range of 8 to 28 hrs. The stealth conversion of dspe-m-peg-2000 extends release up to 28 hrs which indicates the repeat administration can be ultimately declined. The reverse phase method successfully improved the encapsulated 86% respectively. Hence the didanosine stealth liposomes proved as useful tool in antiretroviral therapy to reduce the disadvantages and significantly improve the drug efficiency for AIDS treatment.

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