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Development and characterization of emulsomes for sustained and targeted delivery of an antiviral agent to liver

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Abstract

In this study we developed emulsomes, a novel lipoidal vesicular system with an internal solid fat core surrounded by a phospholipid bilayer. Plain emulsomal formulations composed of solid lipid (trilaurin or tristearin), cholesterol and soya phosphatidylcholine and stearylamine containing cationic emulsomes loaded with an antiviral drug (zidovudine) were prepared by a simple cast film method followed by sonication to produce emulsomes of nanometric size range. All different types of formulations were optimized for lipid ratios and characterized in-vitro for shape, morphology, size and in-vitro drug release profile. Emulsomal formulations displayed a sufficiently slow drug release profile (12–15% after 24 h). In-vivo organ distribution studies in rats demonstrated better uptake of emulsomal formulations by the liver cells. Further, a significantly higher ($P < 0.05$) liver concentration of drug was estimated over a period of 24 h for cationic emulsomes than for plain neutral emulsomes. We concluded that cationic emulsomes could fuse with the endosomal membrane due to charge–charge interaction and were released in the cytoplasm before lysosomal degradation and could sustain drug release over a prolonged period. The proposed cationic emulsome-based system showed excellent potential for intracellular hepatic targeting and the strategy could play a vital role in the effective treatment of life-threatening viral infections, such as hepatitis, HIV and Epstein-Barr virus infection.

Introduction

Intracellular liver infections caused by pathogenic viruses (hepatitis), parasites (malaria), bacteria (leishmania) and protozoa (amoebiasis), which are major cause of morbidity and mortality, are difficult to eradicate with conventional drug therapy because it fails to achieve the desired therapeutic concentration in the cytosol of the cells where the virus or infectious agent dwell and proliferate. Crossing the permeability barrier imposed by the cellular plasma membrane is the major problem in delivering the bioactive into the cytoplasmic compartment of a living cell. Administration of higher doses to attain the therapeutic drug concentration in the cytosol frequently results in dose-related toxic side effects. Moreover, development of multi-drug resistance (MDR) is another main cause of failure of therapeutic regimens in intracellular infections. MDR is often associated with the overexpression of a cell membrane glycoprotein (P-glycoprotein, a membrane spanning ATPase), which causes efflux of the drug from the cytoplasm and results in an ineffective drug concentration inside the cellular compartment (Liang 1987).

Several approaches for the targeted delivery of drugs to liver cells for the treatment of various microbial and viral diseases have been explored (Meijer et al 1992; Erion et al 2005). Colloidal carriers, particularly liposomes, have been widely investigated for the purpose (Kende et al 1985; Lambros et al 2002; Shi et al 2004). These are taken up by endocytosis, with later entry into the cytoplasmic matrix through early or late endosomal or lysosomal membranes (Friend et al 1996). However, the majority of such molecules are degraded in lysosomes without perturbing the homoeostasis of the recipient cell, thus curtailing the intracellular (cytosolic) concentration of the drug. This necessitates the development of systems for effective cytoplasmic delivery. Recently, cationic and pH-sensitive liposomes have been developed for the cytoplasmic delivery of bioactives. These systems can directly fuse with plasma membrane and transfer the contained bioactive across the cellular membrane into the

cytosol. They can also attain cytosolic delivery via destabilization of endosomes either by fusion of the cationic vesicle with the negatively charged endosomal membrane or by pH-dependent avoidance of interaction of early endosome with late endosome (prevents lysosomal degradation) (Wrobel & Collins 1995; Venugopalan et al 2002). However, this leads to a significantly localized higher drug concentration (dose dumping) in the cytosol (>optimum therapeutic concentration) due to destabilization of the carrier, which can trigger the cellular P-glycoprotein efflux system and cause rapid elimination of drug from the cytosol of the cells.

In this study, we developed a model antiviral drug (zidovudine)-loaded novel lipid-based system – emulsome, which is a lipoidal vesicular system with an internal solid fat core surrounded by phospholipid bilayers (Amselem et al 1994; Heiati et al 1996; Lowell et al 1997; Kretschmar et al 2001). Although the therapeutic potency of zidovudine in AIDS, hepatitis and other related viral infections is well recognized clinically, the drug is rapidly eliminated from the body with an elimination half-life (t½) of 0.9–1.2 h (Blum et al 1988; Cload 1989). It has strong side effects on bone marrow, leading to severe anaemia and granulocytopenia (Gill et al 1987; Richman et al 1987; Walker et al 1988). Emulsomes containing phosphatidylcholine, cholesterol and either of the solid lipid tristearin or trilaurin were prepared and optimized for the lipid ratios. Stearylamine was also incorporated to impart cationic charge to the system. The developed formulations would be expected to circumvent the problems of toxicity and rapid elimination due to the presence of drug in the internal solid lipid core of the emulsomes and slow release from the same thereafter. Incorporation of cationic charge would protect the system from lysosomal degradation and ensure the intracellular localization of the system, with subsequent slow release of zidovudine from the internal solid core, and thereby might overcome the problem of multi-drug resistance. This may ultimately lead to both sustained and targeted cytosolic delivery of antiviral agent for eradication of intracellular viral infection.

Materials and Methods

Materials

Zidovudine was a kind gift from M/s Cipla (Mumbai, India). Soya phosphatidylcholine, cholesterol, stearylamine, solid lipids (tristearin and trilaurin) and Sephadex G-50 were purchased from Sigma Chemicals Company (St Louis, MO, USA). All other chemicals and solvents were of analytical grade and procured from local suppliers.

Preparation of emulsomes

Emulsomes containing zidovudine were prepared following the method of Amselem et al (1994), with slight modifications as per our laboratory setup. Solid lipids, cholesterol and phosphatidylcholine in different molar ratios (Table 1) were taken in a round-bottom flask and dissolved in a minimum quantity of chloroform containing 3 or 4 drops of methanol. To this solution, an accurately weighed quantity of zidovudine (10 mg) was dissolved. The organic solvent was evaporated until complete dryness under reduced pressure using a Buchi rotary flash evaporator to form a thin lipid film on walls of the round-bottom flask. The dried film was hydrated with phosphate-buffered saline (PBS) pH 7.4 (10 mL) and homogenized by ultrasonication (Soniweld, India) for 15 min at 40% frequency to obtain emulsomes of nanosize range. The free un-entrapped drug was removed by passing the dispersion through a Sephadex G-50 column (Fry et al 1978; New 1990). For determination of entrapment efficiency, the emulsomal vesicles were disrupted using 50% v/v n-propanol in PBS (pH 7.4) and the liberated drug was estimated spectrophotometrically at λ_{max} 266.5 nm using a Shimadzu 1601 double-beam UV/visible spectrophotometer against reagent blank. The particle size was determined by the dynamic light-scattering method (DLS) using a computerized inspection system (Malvern Zetamaster, ZEM 5002; Malvern, UK). For imparting cationic charge, the formulations using previously optimized ratio of solid lipids (trilaurin and tristearin) to cholesterol and phosphatidylcholine (1:0.5:1 mole ratio) with different mole ratios of stearylamine were prepared following the same method and entrapment efficiency and particle size were determined (Table 2).

In-vitro characterization

Shape and morphology

The shape and surface morphology of the prepared emulsomes was studied by both transmission electron microscopy (Philips, Japan) and scanning electron microscopy (JEOL, JSM-6100).

Table 1 Optimization of solid lipid, cholesterol and phosphatidylcholine ratio

S. No.	Solid lipid:cholesterol: phosphatidylcholine mole ratio	Trilaurin-based formulation		Tristearin-based formulation	
		Entrapment efficiency (%)	Vesicle size (nm)	Entrapment efficiency (%)	Vesicle size (nm)
1	1:0.5:0.25	39.5 ± 4.5	80 ± 12	41.8 ± 4.3	85 ± 14
2	1:0.5:0.5	43.7 ± 4.8	91 ± 16	46.1 ± 4.9	97 ± 18
3	1:0.5:0.75	48.2 ± 5.2	105 ± 17	51.3 ± 5.1	110 ± 19
4	1:0.5:1	53.7 ± 5.5	114 ± 12	55.3 ± 5.8	120 ± 15
5	1:0.5:1.25	51.1 ± 5.4	103 ± 23	53.5 ± 5.5	108 ± 23

Values are expressed as mean ± s.d., n = 6.

Table 2 Optimization of stearylamine ratio

S. No.	Solid lipid:cholesterol: phosphatidylcholine: stearylamine mole ratio	Trilaurin-based formulation		Tristearin-based formulation	
		Entrapment efficiency (%)	Vesicle size (nm)	Entrapment efficiency (%)	Vesicle size (nm)
1	1:0.5:1:0	53.7 ± 5.5	114 ± 12	55.3 ± 5.8	120 ± 15
2	1:0.5:1:0.05	55.4 ± 5.7	123 ± 14	57.5 ± 6.2	133 ± 21
3	1:0.5:1:0.1	57.8 ± 6.2	130 ± 18	59.7 ± 6.1	142 ± 22
4	1:0.5:1:0.15	52.1 ± 5.8	109 ± 13	54.2 ± 5.4	111 ± 18
5	1:0.5:1:0.2	45.3 ± 4.2	80 ± 12	46.8 ± 5.1	86 ± 11

Values are expressed as mean ± s.d., n = 6.

In-vitro drug release

The in-vitro drug release profiles of zidovudine from different emulsomal formulations were determined using a dialysis tube (Sigma, MO, USA) method. A 2-mL volume of the formulation was taken in the donor compartment and placed in a receptor cell containing 100 mL of PBS (pH 7.4). The assembly was placed over a magnetic stirrer and the temperature was maintained at 37 ± 1°C throughout the study. Samples were withdrawn periodically and after each sample withdrawal the medium was compensated immediately with fresh PBS. The samples were analysed for zidovudine content spectrophotometrically at 266.5 nm.

In-vivo tissue distribution study

Albino rats, 100–150 g, of either sex were divided into groups of 6 rats each. The study was carried out under the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal, Ministry of Culture, Government of India) and all the study protocols were approved by the local institutional Animal Ethics Committee. Rats were fasted overnight but allowed a free access to water. To the first group of rats, plain zidovudine injection was administered intravenously through the caudal vein. The dose of zidovudine was fixed to be 5 mg kg^{-1} body weight for each group. The other groups received various prepared emulsomal formulations in equivalent drug doses. Rats were sacrificed after 0.5, 1, 2, 4, 6 and 24 h. Blood samples were collected by cardiac puncture. Visceral organs (liver, spleen, lungs and kidney) of the dissected rats were removed, washed with Ringer's solution to remove any adhered debris and dried using a tissue paper. The isolated organs were weighed separately, minced into pieces and homogenized in 2 mL of PBS (pH 7.4) using a tissue homogenizer (York, India). The tissue homogenates were deproteinized with an equal volume of 10% v/v trichloroacetic acid in water, kept in the dark for 30 min and filtered through a 0.45-μm membrane filter. The serum was harvested from collected blood samples, deproteinized and processed in a similar way. The filtrates were analysed for zidovudine content following the HPLC method reported by Frijus-Plessen et al (1990).

Statistical analysis

All data are presented as the mean of six individual observations, with standard deviation (s.d.) of mean. One-way analysis of variance followed by post-hoc test (Tukey's test) was used for optimization of lipid ratios (effect of lipid ratios on entrapment efficiencies and mean particle size) (n = 6). Two-way analysis of variance followed by Tukey's test (n = 6) was used to evaluate the in-vitro drug release profiles of different formulations. Analysis of tissue drug levels at different time intervals following intravenous administration of different formulations was also performed using two-way analysis of variance followed by Tukey's test (n = 6). Statistical significance was designated as $P < 0.05$.

Results and Discussion

Preparation and in-vitro characterization

This study aimed at development of model antiviral drug zidovudine-loaded emulsomes, which is a lipoidal vesicular system with an internal solid fat core composed of solid lipid surrounded by phospholipid bilayers (Amselem et al 1994; Heiati et al 1996; Lowell et al 1997; Kretschmar et al 2001), in an attempt to overcome the bone marrow toxicity and rapid elimination problems of zidovudine. Antifungal drug amphotericin B-loaded emulsomes (EAmB) have been reported in the literature for efficient treatment of murine systemic infection with *Candida albicans* (Kretschmar et al 2001). It has been demonstrated that the minimum inhibitory concentration of EAmB was identical to that of the reference formulation Fungizone (deoxycholate-containing formulation; Bristol-Myers Squibb, Munich, Germany). At the same time, the toxicity of amphotericin B when incorporated in emulsomes (EAmB) was reduced considerably as compared with standard formulation Fungizone.

Emulsomes containing phosphatidylcholine and either of the solid lipids tristearin or trilaurin were prepared and optimized for the lipid ratios. The lipids were selected because of their simplicity and ready availability. Optimization of different lipid ratios has been carried out on the basis of entrapment efficiency, which is expressed as the percentage fraction of total drug incorporated in the formulation. It was observed that on increasing the mole ratio of phosphatidylcholine with respect to solid lipids and cholesterol (from 0.25 to 1.0), the entrapment efficiency and mean particle size of the formulations increased and might be attributed to the increase in the number of bilayers formed (Table 1). Drug could disperse more efficiently in the solid lipid core, as well as in the

phosphatidylcholine bilayers, and these results are in accordance with the findings of Heiati et al (1996). The maximum entrapment efficiency of 53.7 ± 5.5% and 55.3 ± 5.8%, respectively, were obtained for the trilaurin- and tristearin-based formulations at a solid lipid–cholesterol–phosphatidylcholine mole ratio of 1:0.5:1. On further increasing the phosphatidylcholine content beyond this optimum limit entrapment efficiencies reduced. The higher phosphatidylcholine content might have formed unstable vesicles with greater permeability (New 1990). It was further observed that tristearin-based emulsomes were larger in size than trilaurin-based emulsomes, while keeping the sonication time constant. The higher size may be due to the higher chain length of the tristearin molecule (Table 1).

Addition of cationic-charge-imparting stearylamine also resulted in increase in entrapment efficiency. This might be due to charge-induced repulsion between the bilayer surfaces and subsequent increase in vesicle size and capture volume (New 1990). When the concentration of stearylamine was increased beyond a particular limit, however, there was a significant reduction ($P < 0.05$) in percent entrapment of drug, with the formation of a very low number of vesicles. This may be due to the excessive charge density, which causes high repulsion between the bilayers, and thus be responsible for their eventual instability (New 1990). The optimum mole ratio of solid lipid–cholesterol–phosphatidylcholine–stearylamine was found to be 1:0.5:1:0.1, at which entrapment efficiencies of 57.8 ± 6.2 and 59.7 ± 6.1%, respectively, and mean particle size of 130 ± 18 and 142 ± 22 nm were found for the trilaurin- and tristearin-based formulations, respectively (Table 2).

The shape and morphology of the emulsome-based vesicles was determined by both transmission and scanning electron microscopy (TEM and SEM). The TEM photograph suggested that the vesicles are spherical in shape and multilamellar in nature (Figure 1). The SEM photograph also revealed the spherical shape and smooth surface of the vesicles (Figure 2). The shape of the vesicles was found to be identical irrespective of the lipid composition used in the formulations.

In-vitro release profiles of the drug from various optimized formulations of trilaurin and tristearin was determined in PBS (pH 7.4) for a period of 24 h. The cumulative percent drug released from the plain emulsomal formulations was sufficiently low and found to be 1.6 ± 0.25% and 1.1 ± 0.17%, respectively, after 2 h and 14.6 ± 1.7% and 12.5 ± 1.3%, respectively, after 24 h from the trilaurin- and tristearin-based formulations. The slow release profile might be because of the presence of the solid internal core. The stearylamine formulations, however, had no significant effect on the release profile ($P > 0.05$). Stearylamine-containing trilaurin and tristearin formulations showed a cumulative drug release of 15.3 ± 1.8% and 13.2 ± 1.6 after 24 h.

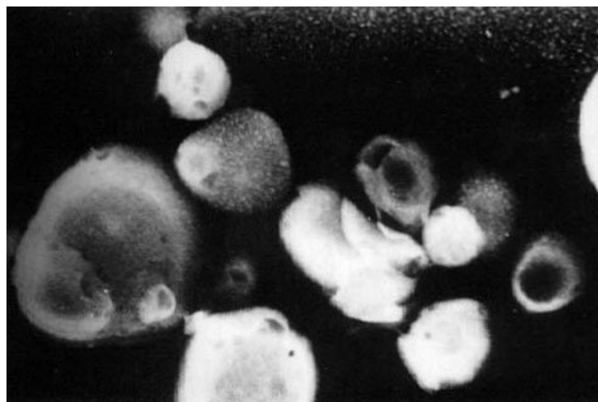

Figure 1 TEM image of emulsomes (× 40 000).

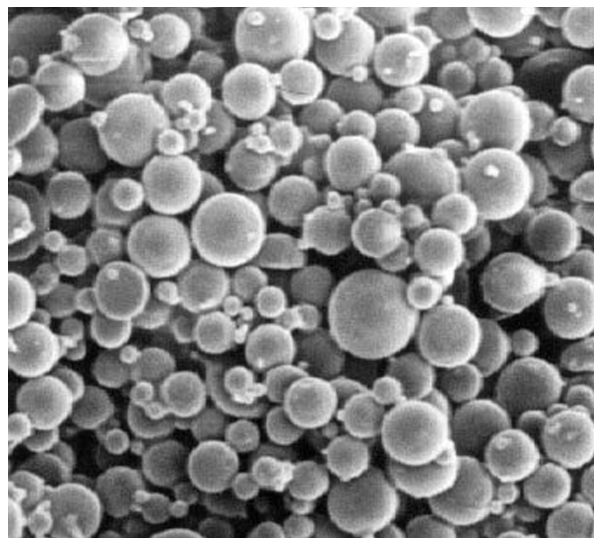

Figure 2 SEM image of emulsomes (× 28 000).

In-vivo organ distribution study

In-vivo organ distribution study of various developed emulsomal formulations was conducted in albino rats and compared with the free drug after intravenous administration (Table 3). Free zidovudine cleared rapidly from the body (blood and other tissues) and only 6.52 ± 0.75% of the injected drug dose was found to present in target organ liver after 6 h and no drug was detected after 24 h. This can be attributed to the very short biological half-life (0.9–1.2 h) of zidovudine (Blum et al 1988; Cload 1989).

In contrast, plain emulsomal formulations rapidly cleared from blood and reached the liver, leading to a high drug concentration in the liver after 1 h (54.33 ± 6.2 and 58.52 ± 6.5%, respectively, for trilaurin- and tristearin-based formulations). This may be attributed to the natural passive uptake of the colloidal carriers by the liver cells (Friend et al 1996). However, the drug content could not be sustained in liver for a longer period of time. After 6 h, the percent dose recovered from the liver after administration of trilaurin- and tristearin-based plain emulsomes was 18.39 ± 2.1 and 20.45 ± 2.3%, respectively, and the same after 24 h was 4.85 ± 0.53 and 5.61 ± 0.63%. This might be attributed to the possible lysosomal degradation of the plain emulsomes within liver cells and release of the entire drug content, which might have subsequently activated the P-glycoprotein pump system that is

Table 3 In-vivo tissue distribution profile of various developed formulations following intravenous administration to rats

Formulation	Organ	% Dose recovered after					
		30 min	1 h	2 h	4 h	6 h	24 h
Free zidovudine							
	Serum	82.32 ± 9.3	60.86 ± 7.2	35.74 ± 4.4	11.57 ± 1.8	3.82 ± 0.42	ND
	Liver	7.84 ± 0.85	15.37 ± 1.7	12.53 ± 1.4	9.64 ± 1.1	6.52 ± 0.75	ND
	Spleen	0.68 ± 0.08	2.25 ± 0.31	3.69 ± 0.39	2.51 ± 0.28	1.56 ± 0.17	ND
	Lung	0.35 ± 0.6	1.83 ± 0.22	2.61 ± 0.35	2.18 ± 0.23	1.12 ± 0.15	ND
	Kidney	0.58 ± 0.07	2.36 ± 0.31	3.54 ± 0.43	5.76 ± 0.63	4.27 ± 0.55	ND
Plain emulsomes (Trilaurin:phosphatidylcholine:cholesterol = 1:1:0.5)							
	Serum	54.56 ± 6.7	20.14 ± 2.4	12.26 ± 1.4	5.37 ± 0.65	ND	ND
	Liver	29.91 ± 3.3	54.33 ± 6.2	41.71 ± 4.8	26.46 ± 3.0	18.39 ± 2.1	4.85 ± 0.53
	Spleen	2.93 ± 0.32	5.62 ± 0.63	4.16 ± 0.47	3.35 ± 0.31	1.85 ± 0.22	ND
	Lung	2.45 ± 0.28	5.27 ± 0.61	3.73 ± 0.44	2.66 ± 0.33	1.59 ± 0.19	ND
	Kidney	ND	1.64 ± 0.19	4.19 ± 0.51	5.34 ± 0.59	3.87 ± 0.46	ND
Plain emulsomes (Tristearin:phosphatidylcholine:cholesterol = 1:1:0.5)							
	Serum	51.23 ± 5.8	17.55 ± 2.3	10.73 ± 1.4	4.18 ± 0.47	ND	ND
	Liver	32.51 ± 3.6	58.52 ± 6.5	45.43 ± 5.4	30.39 ± 3.5	20.45 ± 2.3	5.61 ± 0.63
	Spleen	3.24 ± 0.35	6.37 ± 0.67	4.92 ± 0.56	3.58 ± 0.38	2.17 ± 0.24	ND
	Lung	2.11 ± 0.24	4.53 ± 0.51	3.87 ± 0.44	2.35 ± 0.26	1.28 ± 0.16	ND
	Kidney	ND	1.46 ± 0.17	3.63 ± 0.39	4.75 ± 0.52	3.95 ± 0.43	ND
Cationic emulsomes (Trilaurin:phosphatidylcholine:cholesterol:stearylamine = 1:1:0.5:0.1)							
	Serum	57.36 ± 7.1	18.38 ± 2.3	10.77 ± 1.2	4.72 ± 0.50	ND	ND
	Liver	23.47 ± 3.2	57.96 ± 6.5	55.48 ± 6.7	46.61 ± 5.8	37.72 ± 4.3	11.57 ± 1.5
	Spleen	2.55 ± 0.28	5.88 ± 0.70	5.22 ± 0.56	4.76 ± 0.53	4.18 ± 0.46	ND
	Lung	1.81 ± 0.21	5.17 ± 0.53	4.79 ± 0.49	4.21 ± 0.45	3.51 ± 0.38	ND
	Kidney	ND	1.91 ± 0.22	3.56 ± 0.39	4.87 ± 0.54	3.43 ± 0.48	ND
Cationic emulsomes (Tristearin:phosphatidylcholine:cholesterol:stearylamine = 1:1:0.5:0.1)							
	Serum	55.25 ± 6.2	15.27 ± 1.8	8.55 ± 1.1	3.92 ± 0.45	ND	ND
	Liver	25.83 ± 2.8	61.38 ± 7.3	57.14 ± 6.4	50.65 ± 5.9	40.32 ± 4.6	14.74 ± 1.9
	Spleen	2.87 ± 3.3	6.52 ± 0.75	6.13 ± 0.73	5.28 ± 0.57	4.47 ± 0.49	ND
	Lung	1.77 ± 2.2	4.81 ± 0.55	4.24 ± 0.47	3.74 ± 0.43	3.12 ± 0.35	ND
	Kidney	ND	1.78 ± 0.21	3.21 ± 0.36	4.12 ± 0.46	3.37 ± 0.41	ND

Values are expressed as mean ± s.d., n = 6. ND, not detectable

responsible for multi-drug resistance and resulted in rapid efflux of drug (Liang 1987). Cationic stearylamine-based emulsomes could be retained in the liver cells for a prolonged period of time and 37.72 ± 4.3 and 40.32 ± 4.6% of the administered dose was recovered from liver tissues 6 h after administration of trilaurin- and tristearin-based cationic emulsomes. Even after 24 h, a significantly higher liver drug concentration as compared with respective neutral emulsomal formulations was measured ($P < 0.05$). Using trilaurin-based cationic emulsomes, 11.57 ± 1.5% of the administered dose was recovered from liver tissues after 24 h, which is about 2.4 times higher than with the respective plain neutral emulsomes. Similarly, 14.74 ± 1.9% of the injected drug dose was estimated at the target site, liver, 24 h after administration of tristearin-based cationic emulsomes, which is about 2.6 times higher than the respective plain formulation (Figure 3). Cationic emulsomes are essentially internalized by endocytosis or by direct fusion. Further, before lysosomal degradation they can destabilize the endosomal membrane because of charge–charge interaction, which causes vesicles to fuse with the endosomal membrane and be released in the cell cytoplasm where the drug is slowly released from the internal solid core matrix, thus maintaining an effective intracellular drug concentration over a prolonged period of time and avoiding the localized dose dumping that may activate the P-glycoprotein efflux pump system.

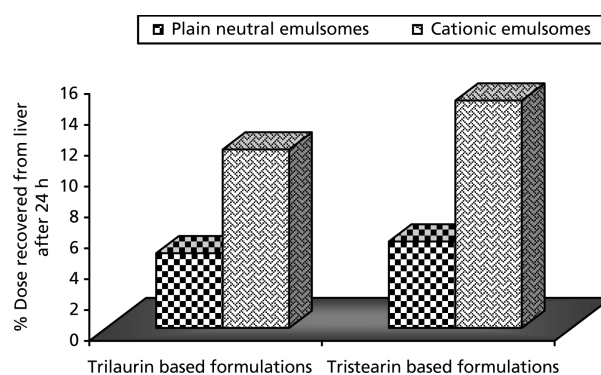

Figure 3 Relative liver drug content retained after 24 h following administration of various formulations.

Conclusion

In conclusion, the proposed cationic emulsomes-based system showed excellent potential for intracellular hepatic targeting. The formulations could significantly modify the pharmacokinetics of zidovudine, providing prolonged action at comparatively low drug doses thereby reducing the toxicity problem due to favourable localization of the drug in liver cells. Antiviral drugs could be safely and effectively delivered to treat the viral diseases of cellular tropics and the strategy could play a vital role in the effective treatment of life-threatening viral infections such as hepatitis, HIV, Epstein-Barr virus, etc. However, before such systems could clinically be realized, they need to be studied progressively to elucidate their role in multi-drug resistance and effective viral eradication. Further, more specific studies to determine the cytosolic localization of drug within liver parenchymal cells (hepatocytes) are also required. More sensitive methods, such as cytometry, specific fluorescence marker study and confocal microscopy, could give a better indication of the localization of drug–carrier composite in the cytosol of target cells.

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