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Transferrin-conjugated solid lipid nanoparticles for enhanced delivery of quinine dihydrochloride to the brain

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Abstract

Transferrin (Tf)-conjugated solid lipid nanoparticles (SLNs) were investigated for their ability to deliver quinine dihydrochloride to the brain, for the management of cerebral malaria. SLNs were prepared by an ethanol injection method using hydrogenated soya phosphatidyl choline (HSPC), triolein, cholesterol and distearylphosphatidylethanolamine (DSPE). Coupling of SLNs with Tf was achieved by incubation of Tf with quinine-loaded SLNs in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) hydrochloride in phosphate buffered saline (pH 7.4) as a cross-linker. SLNs were characterized for shape, particle size, polydispersity and percentage drug entrapment. The SLNs were 108–126 nm in size, and maximum drug entrapment was 38.4–42.7%. Average size increased on coupling with Tf but percentage drug entrapment was reduced. The in-vitro release profile was determined using a dialysis technique; non-conjugated SLNs released comparatively more drug than Tf-SLNs. Fluorescence studies revealed enhanced uptake of Tf-SLNs in brain tissue compared with unconjugated SLNs. In in-vivo performance studies, quinine plasma level and tissue distribution after intravenous administration of drug-loaded Tf-SLNs and unconjugated SLNs was compared with that of free drug. Intravenous administration of quinine dihydrochloride solution resulted in much higher concentrations of drug in the serum than with SLNs. Conjugation of SLNs with Tf significantly enhanced the brain uptake of quinine which was shown by the recovery of a higher percentage of the dose from the brain following administration of Tf-coupled SLNs compared with unconjugated SLNs or drug solution.

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Introduction

Targeting drugs to the brain is challenging because of the presence of the lipophilic blood–brain barrier, which hinders the passage of water-soluble drugs to the brain for the treatment of life-threatening diseases. In this context, ligand-anchored drug delivery systems have shown potential in achieving enhanced site-specific drug delivery and reduced uptake by the reticuloendothelial system (RES) (Pardridge 1995). The management of brain-related diseases likes cerebral malaria, brain tumour and Parkinson's disease is difficult with currently available therapeutic systems, because insufficient drug crosses the highly lipophilic blood–brain barrier; only small lipid-soluble drugs in the circulation are delivered to brain cells (Lavin 1980; Pardridge 1995). Therefore, practical strategies are required for mediating drug transport across the blood–brain barrier. Some strategies for drug delivery to the brain exploit the physiological mechanism of solute transport through the blood–brain barrier. A number of drug peptides, biological response modifiers and monoclonal antibodies and fragments have proved valuable in inhibiting a variety of malignant infectious diseases and in rectifying neurotransmitter and enzyme imbalances in tissue culture systems (Pardridge 1995). However, their therapeutic efficacy is frequently diminished in-vivo or is prevented by their inability to reach the diseased site and to be maintained there at an adequate concentration for an appropriate period of time. Peptides such as insulin, insulin-like growth factors IGF I and IGF II (Pardris 1992), transferrin (Tf; Fishman et al 1987) and leptin (Banks et al 1996) have high affinity for receptors on the brain capillaries.

Nanoparticles prepared from solid lipids at room temperature have been proposed as a new type of drug carrier system. Solid lipid nanoparticles (SLNs) have been developed as

an alternative particulate carrier system by various research groups (Almeida et al 1998; Siekmann & Westesen 1992). Recently, increasing attention has focused on these SLNs because as colloidal drug carriers they combine the advantages of polymeric nanoparticles, fat emulsions and liposomes whilst simultaneously avoiding some of their disadvantages (Sjostrom & Bergenstahl 1992; Boltri et al 1993). Under optimized conditions, SLNs can be produced to incorporate lipophilic or hydrophilic drugs, and seem to fulfill the requirements for an optimum particulate carrier system (Schwarz et al 1994; Muller et al 1995).

Tf receptors are reported to be present on the surface of diverse classes of cell types and mediate the internalization of iron-saturated Tf through receptor-mediated endocytosis (Qian et al 2002). Tf receptors are expressed on the luminal membrane of brain endothelial cells and mediate the endocytosis of Tf into these cells (Fishman et al 1987; Pardridge et al 1987). The receptor-mediated endocytosis of Tf from blood into brain is well documented (Hatakeyama et al 2004; Visser et al 2004; Soni et al 2005).

Various novel drug delivery carriers have been used for brain-targeted drug delivery, such as liposomes (Gupta et al 2005; Soni et al 2005), antibody conjugates (Wagner et al 1994) and nanoparticles (Mishra et al 2006).

Quinine dihydrochloride is the drug of choice for the treatment of cerebral malaria but requires targeted delivery to the brain. It is a low-molecular-weight, polar compound, and its transportation into the brain is limited by an efflux transport system, so very little drug reaches the brain by any currently available system. Moreover, it induces toxicities typical of the cinchona alkaloids. Therefore, attempts have been made to enhance the delivery of quinine dihydrochloride to the brain.

In this investigation we have developed Tf-conjugated SLNs (Tf-SLNs) containing quinine dihydrochloride as a carrier for drug localization into brain tissues for the treatment of cerebral malaria.

Materials and Methods

Materials

Quinine dihydrochloride was a gift from IPCA Pharmaceuticals (Ratlam, India). Hydrogenated soya phosphatidyl choline (HSPC) and distearylphosphatidylethanolamine (DSPE) were gifts from Lipoid, Ludwigshafen, Germany. Cholesterol, 6-carboxyfluorosein (6-CF), Triton X-100, Tf, Sephadex G-50 and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma Chemicals (St Louis, MO, USA). Dialysis membrane (MWCO 3500) was purchased from Himedia, Mumbai, India. All other reagents and solvents were of analytical grade and were purchased from local suppliers unless stated otherwise. Double-distilled water was used throughout the study.

Preparation of solid lipid nanoparticles

SLNs were prepared by the ethanol injection method reported by Stevens et al (2004) with minor modifications. Briefly, HSPC, triolein, cholesterol and DSPE in a w/w ratio of 9.58:9.58:3.16:3.16 were dissolved in ethanol at a concentration of 10 mg mL^{-1}, and injected into a stirring solution (1200 rpm) of phosphate buffered saline (PBS, pH 7.4) containing quinine dihydrochloride (1 mg mL^{-1}) and Tween 80 (0.5%). Both the aqueous medium and the lipid solution were prewarmed to 40°C and were kept at this temperature during the mixing. Free drug was removed by diafiltration through a tangential flow filter unit. The SLNs were then concentrated by centrifugation, and resuspended in PBS (pH 7.4). Similarly, 6-CF-loaded SLNs were prepared using 6-CF dye (10 ng mL^{-1}) in place of quinine dihydrochloride.

Conjugation of SLNs with transferrin

The Tf was covalently coupled by its carboxyl group to the amino group of DSPE present on the surface of preformed drug-loaded SLNs using EDC as the coupling agent. Drug-loaded SLNs were suspended in PBS (pH 7.4) containing Tf (lipid:Tf ratio: 90:10 w/w). EDC (10 mg per mL lipid/Tf mixture) was added, and the mixture was vortexed and thenincubated for 2 h at room temperature. Excessive unbound Tf was removed by passing the dispersion through a Sephadex G-50 column.

Particle morphology and zeta potential analysis

The shape and morphology of the SLNs were characterized by transmission electron microscopy with an accelerating voltage of 100 kV. A drop of the sample was placed onto a carbon-coated copper grid to create a thin film. Before the film dried on the grid, it was negatively stained with 1% phosphotungstic acid by adding a drop of the staining solution to the film; any excess solution was drained off with a filter paper. The grid was allowed to dry thoroughly in air, and samples were viewed under a transmission electron microscope (JEM-200 CX, JEOL, Tokyo, Japan). Particle size and size distribution was assessed using Master Sizer 2000 (Malvern Instruments Ltd, Malvern, UK). The zeta potential of the plain (unconjugated) and Tf-SLNs was determined using a zeta sizer (Zetasizer 3000; Malvern, UK).

Drug entrapment efficiency

The entrapment of quinine dihydrochloride in SLNs was determined using the method reported by Fry et al (1978). The unentrapped drug from SLN suspension was removed by passing the formulation through a Sephadex G-50 minicolumn and centrifuging at 3000 rpm for 2 min. The SLNs were then lysed using 0.1% v/v Triton-X 100, and drug content was determined spectrophotometrically at 332.0 nm.

In-vitro drug release

Drug release from the formulations was studied in-vitro using dialysis membrane (Himedia, India; molecular cut-off point 3500 Da (Gupta et al 2005). SLN suspension (1 mL) free from any unentrapped drug was placed in dialysis tubing, which was suspended in a beaker containing 20 mL PBS (pH 7.4). The contents of the beaker were stirred using a magnetic stirrer at 37 ± 2°C. Samples were withdrawn periodically and

replaced with the same volume of fresh PBS. The amount of drug was quantified spectrophotometrically at 332.0 nm.

Coupling efficiency

The amount of Tf conjugated at the surface of the SLNs was determined with the Bradford dye assay using Coomassie blue G dye (Bradford 1976), with minor modifications. Briefly, 1 mL Tf-coupled liposomal formulation was placed in a volumetric flask with 1 mL Coomassie blue G dye solution and the volume was adjusted to 10 mL with distilled water. To determine the Tf concentration, the absorbance at 595 nm was measured and compared with a blank containing the same amount of dye. Coupling efficiency is expressed as mg Tf per mmol phospholipids.

Quantitative evaluation of brain targeting by fluorescence microscopy

The brain uptake of Tf-SLNs was confirmed by fluorescence microscopy. The 6-CF was used as a fluorescent marker and was loaded into Tf-conjugated and non-conjugated SLNs. CF-loaded SLNs were administered intravenously to three albino rats at a dose of 0.5 mg 6-CF per kg body weight. The rats were killed after 60 min and the brain was removed and sliced using a microtome. Sections were fixed on slides using egg albumin solution as fixative. The sections were observed under a fluorescence microscope (TE 2000, Nikon, Japan) at excitation and emission wavelengths of 495 and 520 nm, respectively.

In-vivo distribution studies

Thirty-two albino rats of either sex, weighing 130–150 g, were divided into four groups of eight rats. Rats were fasted overnight before drug administration. One group of rats received intravenous injection of an aqueous solution of quinine dihydrochloride at a dose of 10 mg per kg body weight; one group received intravenous injection of non-conjugated SLNs; one group received Tf-SLNs at a dose equivalent to 10 mg per kg body weight in PBS (pH 7.4); the fourth group acted as controls and did not receive any drug or formulation. At selected times after injection, animals were killed and blood was collected by cardiac puncture. The major organs were excised, isolated, washed with Ringer's solution and dried using tissue paper. Organs were stored at −80°C until assay. All studies were carried out according to the guidelines of the Council for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India and approved by the University animal ethical committee, Sagar (MP), India.

Drug estimation in serum

Blood samples were centrifuged at 5000 rpm for 8 min. Serum was harvested from the supernatant. To 150 μL serum we added an equal volume of 10% (v/v) trichloroacetic acid in water. The samples were mixed by vortexing for 30 s, centrifuged at 5000 rpm for 5 min and the supernatant was filtered through a 0.45 μm filter and analysed for drug content by HPLC, as reported by Nermans-Lokkerbol et al (1989). The column used was a LiChroCART 125-4 LiChrospher 60 RP select B (5 μm) (Merck, Darmstadt, Germany). The mobile phase consisted of acetonitrile, methanol and sodium perchlorate in a v/v ratio of 6.2:17.3:6.5, pH 2.5. The flow rate was maintained at 1 mL min^{-1} at ambient temperature.

Estimation of drug in different organs

Liver, spleen, lungs, kidney and brain were dried and weighed and cut into small pieces. One gram of each organ was homogenized with 2 mL PBS (pH 7.4). In the case of organs weighing less than 1 g, the whole organ was used. To 150 μL tissue homogenate we added an equal volume of 10% (v/v) trichloroacetic acid in water. Samples were mixed by vortexing for 30 s, centrifuged at 5000 rpm for 5 min and the supernatant was filtered through a 0.45 μm membrane filter. Tissue homogenate was analysed for drug content.

Statistical analysis

The effect of the formulations on drug entrapment efficiency, particle size, zeta potential and percentage dose recovered in each organ was analysed using the Kruskal–Wallis test using SYSTAT software (v. 10.2.01), (San Jose, CA, USA). The drug concentrations in serum and brain samples were compared by one-way analysis of variance followed by Turkey's post-hoc test. Differences were considered statistically significant if P was less than 0.05.

Results and Discussion

Tf-SLNs were prepared using HSPC, triolein, cholesterol and DSPE (9.58:9.58:3.16:3.16 w/w) to enhance drug transport to the brain whilst preserving the target affinity of the surface-anchored Tf. SLNs of similar lipid composition without Tf were used for comparison.

Tf-SLNs containing quinine dihydrochloride were prepared and their surface was coupled with Tf using carbodiimide chemistry. The carboxylic group of the Tf was covalently coupled in the presence of EDC, the amine group present at the surface of the SLNs forming an amide linkage. Transmission electron microscopy revealed the spherical shape of Tf-SLNs. The mean diameter of Tf-SLNs and unconjugated SLNs was 126.4 ± 2.96 nm and 108.2 ± 2.58 nm, respectively (Table 1). A very low polydispersity index of less than 0.1 was obtained for both formulations, indicating a narrow size distribution of the nanoparticle suspensions and consequently a homogeneous distribution. The zeta potential of unconjugated SLNs and Tf-SLNs was 15.4 ± 1.4 and 3.7 ± 0.5 mV, respectively (Table 1). The zeta potential was found to be decreased by coupling the SLNs with Tf. This may be due to the masking of cationic surface charges by the Tf coating.

Drug entrapment was less in Tf-SLNs than unconjugated SLNs, which may be due to leaching of the drug during the Tf coating.

The drug release profile from the uncoupled and Tf-SLNs in PBS is shown in Figure 1. In the case of the unconjugated SLNs, drug release was 35.24 ± 1.55% after 8 h and

Table 1 Mean particle size, polydispersity index, drug entrapment efficiency and zeta potential of quinine dihydrochloride loaded plain solid lipid nanoparticles (SLNs) and transferrin-coupled solid lipid nanoparticles (Tf-SLNs)

	Plain SLNs	Transferrin-coupled SLNs
Particle size (nm)	108.2 ± 2.58	126.4 ± 2.96
Polydispersity index	0.024	0.068
Drug entrapment (%)	42.7 ± 1.34	38.4 ± 1.43
Zeta potential (mV)	15.4 ± 1.4	3.7 ± 0.5

Values are mean ± s.d. of three independent determinations. $P < 0.05$ for all properties.

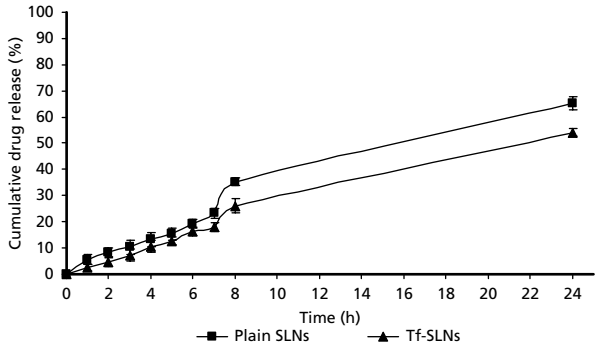

Figure 1 Drug release from plain (unconjugated) and transferrin-conjugated (Tf) solid lipid nanoparticles (SLNs). Results are mean ± s.d. (n = 3).

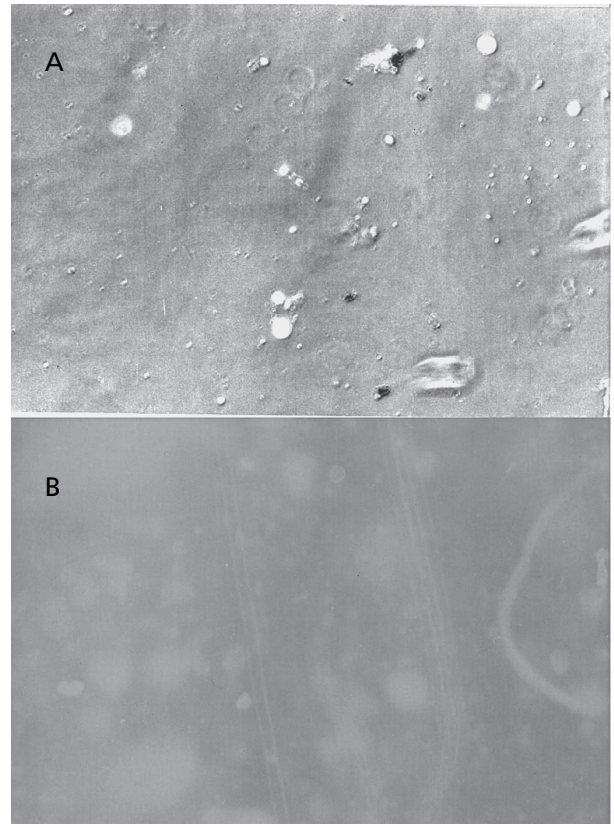

Figure 2 Fluorescence distributed in the brain tissue of the rat 1 h after intravenous injection of (A) transferrin-coupled solid lipid nanoparticles (Tf-SLNs) and (B) plain solid lipid nanoparticles (SLNs).

65.34 ± 2.42% after 24 h. Drug release from Tf-SLNs was 26.12 ± 2.56% after 8 h and 54.12 ± 1.36% after 24 h (Figure 1). Thus, coupling of Tf affects drug release from the SLNs. This may be because the structural integrity provided by Tf coupling creates a double barrier effect for drug diffusion.

The EDC concentration was optimized in order to ensure that conjugation of Tf did not result in particle–particle aggregation or extensive cross-linking. It was found that 2.5 mg EDC per mg Tf did not cause any instability to the SLNs. The coupling efficiency was found to be 46 mg Tf per mmol phospholipids, corresponding to about 28 molecules of Tf per mmol SLNs.

Fluorescence microscopy was used to observe the uptake of 6-CF loaded Tf-SLNs into the brain (Figure 2). This revealed that the Tf-SLNs targeted the brain, although very weak fluorescence was also observed in rats treated with unconjugated SLNs as well as the control group. Thus, brain targeting was related to the Tf coating on the nanoparticles. By comparing Figures 2a and 2b, it can be seen that the fluorescence remained confined to the walls of brain microvessels; it is difficult to discriminate from fluorescence microscopy whether the Tf-SLNs were adhered to the lining of the brain capillary endothelial cells or were taken up by these cells. However, it does provide conclusive evidence that brain targeting of nanoparticles was dependent on the Tf coating and involved brain capillary endothelial cells.

Equivalent doses of quinine dihydrochloride in PBS solution and in SLNs were administered intravenously to rats and the subsequent serum drug concentration profiles and tissue distribution compared. The organ distribution patterns and serum concentrations of quinine dihydrochloride with unconjugated SLNs containing quinine dihydrochloride and free quinine dihydrochloride compared with Tf-SLNs clearly establishes the superiority of the SLN formulation in reducing the concentration of free drug in serum and in increasing the accumulation of drug in the organs.

Figure 3 shows the total percentage drug recovered in serum after intravenous administration of the free drug and from the different types of SLN. In the case of free drug, 73.42 ± 3.32% of the administered dose was recovered after 1 h, whereas in the case of non-conjugated and Tf-SLNs, the maximum dose recovered after 3 h was 7.98 ± 1.45% and 6.21 ± 0.86%, respectively. Thus, there was a drastic reduction in the serum quinine dihydrochloride with SLN-encapsulated drug administration, which was further reduced by the ligand anchoring. The significant reduction in the serum concentration of free drug associated with SLN incorporation can be accounted for by the fact that the SLNs are highly partitioned into the organs compared with the blood.

Following intravenous administration of free drug, maximum uptake was by the liver (13.10 ± 0.33% and 2.54 ± 0.43% after 1 h and 24 h, respectively). With unconjugated SLNs

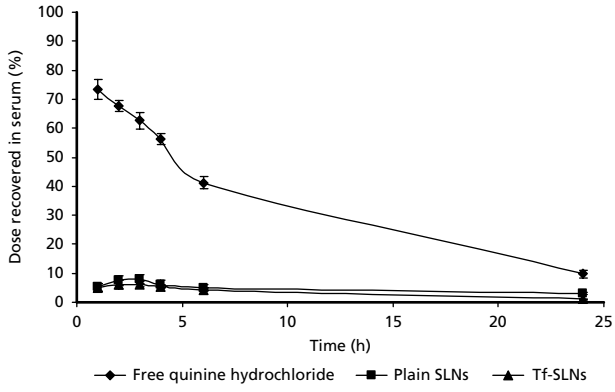

Figure 3 Serum level of quinine (as percentage of administered dose) following intravenous injection of free quinine dihydrochloride or quinine hydrochloride containing plain solid lipid nanoparticles (SLNs) and transferrin-conjugated solid lipid nanoparticles (Tf-SLNs) into rats (10 mg per kg). Results are mean ± s.d. (n = 3); $P < 0.05$. All the values at respective time points for each formulation of plain drug were used for statistical comparisons.

containing quinine dihydrochloride, maximum uptake was also by the liver (47.93 ± 0.46% and 20.12 ± 0.42% after 3 h and 24 h, respectively). Uptake by the spleen following intravenous administration of the free drug or quinine-loaded unconjugated SLNs was 4.08 ± 0.80% and 7.61 ± 0.15%, respectively, after 1 h, and 0.12 ± 0.03 and 3.32 ± 0.63%, respectively, after 24 h (Table 2). These results suggest that the liver and spleen are the major clearance organs for SLNs as well as for free drug. Active uptake mechanisms such as phagocytosis by Kupffer cells in the liver, or filtration by a meshwork consisting of reticular fibres and accompanying macrophages in the red pulp in the spleen probably account for this high clearance (Litzinger et al 1994). By contrast, uptake of the drug by the brain following intravenous administration of free drug or drug-bearing unconjugated SLNs was much lower than by the liver and spleen: 1.64 ± 0.20% and 2.90 ± 0.18%, respectively, after 1 h, and 0.13 ± 0.04% and 0.72 ± 0.02%, respectively, after 24 h. This could be attributed to limited permeability to these formulations because of the presence of tight junctions between the adjacent endothelium cells, which severely impede the movement of drug molecules. Therefore, the extent of brain distribution of SLNs is dependent on their permeability through the endothelial cells.

Administration of quinine-containing Tf-SLNs resulted in less uptake of the quinine dihydrochloride by the liver: 34.30 ± 3.32% uptake after 1 h and 15.42 ± 0.56% after 24 h, which could be due to reduction in RES uptake of the Tf-SLNs, and greater uptake of the drug by the brain: 14.90 ± 1.22% and 3.32 ± 0.56% after 2 h and 24 h, respectively (Figure 4). Brain uptake of quinine in Tf-SLNs was approximately 7 and 4 times higher than with free drug and unconjugated SLNs formulation, respectively (Figure 4). This could because Tf receptors are much less abundant in a broad variety of tissues than in the brain. In the brain, the Tf-SLNs have been shown to undergo receptor-mediated transcytosis that normally serves to mediate uptake of circulating Tf (Skarlatos et al 1995). The higher concentration of quinine in the brain persisted for a period of time because free drug liberated from SLNs remains confined in the brain tissues as a result of poor backward transport across the blood–brain barrier. Hence, drug appears to be locked in the brain compartment, leading to higher concentrations for extended periods.

Table 2 Organ distribution of quinine dihydrochloride after intravenous administration of free drug, or drug-containing plain solid lipid nanoparticles (SLNs) or transferrin-conjugated solid lipid nanoparticles (Tf-SLNs) (10 mg quinine per kg body weight) to rats

	Organs	% dose recovered					
		1 h	2 h	3 h	4 h	6 h	24 h
Quinine solution	Brain	1.64 ± 0.20	1.44 ± 0.72	1.21 ± 0.02	1.04 ± 0.15	0.54 ± 0.03	0.13 ± 0.04
	Lung	5.65 ± 0.03	4.06 ± 0.23	4.31 ± 0.21	3.29 ± 0.01	1.24 ± 0.04	ND
	Liver	13.10 ± 0.33	12.80 ± 0.01	10.48 ± 1.04	7.09 ± 0.53	6.54 ± 1.03	2.54 ± 0.43
	Spleen	4.08 ± 0.80	3.02 ± 0.32	2.54 ± 0.22	1.52 ± 0.02	0.62 ± 0.02	0.12 ± 0.03
	Kidney	4.69 ± 0.45	4.34 ± 0.24	3.85 ± 0.04	2.54 ± 0.93	2.04 ± 0.53	0.54 ± 0.02
Plain SLNs	Brain	2.90 ± 0.18	3.20 ± 1.52	3.10 ± 1.49	2.10 ± 1.56	1.84 ± 1.45	0.72 ± 0.02
	Lung	4.70 ± 0.44	4.94 ± 0.07	4.08 ± 0.22	3.12 ± 0.24	2.47 ± 0.70	0.94 ± 0.14
	Liver	41.82 ± 2.03	42.54 ± 3.04	47.93 ± 0.46	43.31 ± 0.65	37.56 ± 0.68	20.12 ± 0.42
	Spleen	7.61 ± 0.15	10.12 ± 0.65	13.24 ± 0.73	11.94 ± 0.04	8.45 ± 0.05	3.32 ± 0.63
	Kidney	4.80 ± 0.46	5.11 ± 0.52	5.98 ± 0.56	4.59 ± 0.72	3.22 ± 0.23	1.73 ± 0.14
Tf-SLNs	Brain	12.40 ± 1.67	14.90 ± 1.22	13.02 ± 1.22	10.20 ± 1.02	9.22 ± 0.99	3.32 ± 0.56
	Lung	5.70 ± 0.28	6.24 ± 0.54	5.48 ± 0.76	4.66 ± 0.56	3.87 ± 0.43	0.36 ± 0.57
	Liver	34.30 ± 3.32	38.49 ± 2.75	42.02 ± 2.85	37.86 ± 2.82	30.32 ± 0.32	15.42 ± 0.56
	Spleen	8.29 ± 0.19	9.32 ± 0.26	9.84 ± 0.68	7.31 ± 0.26	4.13 ± 0.04	1.24 ± 0.53
	Kidney	3.11 ± 0.12	4.48 ± 0.70	5.96 ± 0.15	4.20 ± 0.24	3.38 ± 0.24	0.76 ± 0.20

Values are mean ± s.d. for three independent determinations; $P < 0.05$. All the values at respective time points for each formulation of plain drug were used for statistical comparisons.
ND, not detected.

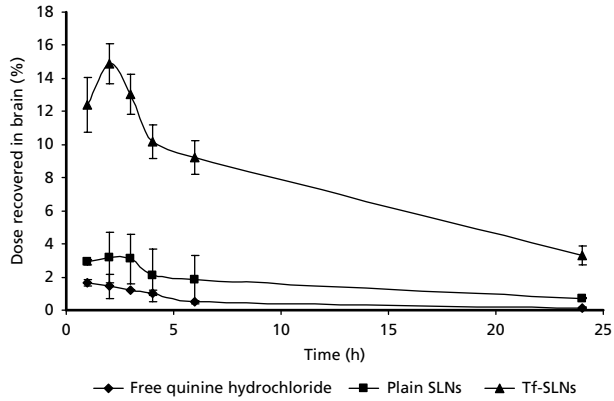

Figure 4 Level of quinine in the brain (as percentage of administered dose) following intravenous injection of free quinine dihydrochloride or quinine hydrochloride containing plain solid lipid nanoparticles and transferrin-conjugated solid lipid nanoparticles (Tf-SLNs) into rats (10 mg kg). Results are mean ± s.d. (n = 3); $P < 0.05$. All the values at respective time points for each formulation of plain drug were used for statistical comparisons.

Conclusion

This study illustrates Tf-SLNs as a potential system for the transport of drug molecules across the blood–brain barrier. It is possible that cerebral malaria could be treated effectively using quinine-containing Tf-SLNs. This study demonstrated that this system could be exploited as a potential carrier for treatments of neurological diseases such as Alzheimer's disease, Parkinson's disease, meningitis and brain tumours.

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