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Solid lipid nanoparticles as drug carriers II. Plasma stability and biodistribution of solid lipid nanoparticles containing the lipophilic prodrug 3′-azido-3′-deoxythymidine palmitate in mice

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Abstract

Solid lipid nanoparticles (SLNs) were prepared using trilaurin as the SLNs solid core and a mixture of neutral and negatively charged phospholipid. To produce SLNs with a poly(ethylene glycol) (PEG) coating, PEG was incorporated in SLNs using dipalmitoylphosphatidylethanolamine-*N*-[poly(ethylene glycol)$_{2000}$] (PE-PEG). 3′-azido-3′-deoxythymydine palmitate (AZT-P) with [^{3}H]-AZT-P as tracer were synthesized and incorporated in SLNs. Their subsequent retention in SLNs with and without PEG was determined after incubation in 50% bovine plasma. Biodistribution studies were performed in mice using free AZT-P, AZT-P incorporated in SLNs or AZT-P incorporated in PE-PEG coated SLNs (SLN-PE-PEG). The presence of PE-PEG significantly reduced the SLN zeta potential from -22 to -5 mV. Although AZT-P was rapidly released from SLNs during incubation in bovine plasma, the release rate was significantly slower in SLN-PE-PEG. AZT-P was rapidly removed from blood following i.v. injection in mice. The decrease in AZT-P blood level was biphasic and rapid, and the major excretory route of AZT-P was the kidney. Higher levels were observed after i.v. injection of AZT-P incorporated in SLNs. This effect was further increased using SLN-PE-PEG. Both SLN and SLN-PE-PEG incorporation of AZT-P significantly decreased the urinary excretion of AZT-P and increased the localization of AZT-P in the liver. The results obtained in this study indicate that using SLNs as a drug carrier increases the bioavailibility of incorporated AZT-P, and that the pharmacokinetic behaviour of the incorporated drug can be modified by changing the surface characteristics of SLNs by using the amphiphilic solvation enhancer PE-PEG.

Keywords: Solid lipid nanoparticles; Poly(ethylene glycol); Azidothymidine; Lipophilic prodrug; Biodistribution

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1. Introduction

3′-azido-3′-deoxythymidine (Zidovudine®, AZT) is an analog of deoxythymidine in which the 3′ hydroxyl has been replace by an azido (N_3) group. The drug is activated to its mono-, di-, and triphosphate forms by cellular enzymes in both human immunodeficiency virus (HIV) infected and uninfected cells (Furman et al., 1986). The active metabolite of AZT, AZT triphosphate, inhibits HIV-1 reverse transcriptase as well as mitochondrial DNA polymerase-γ (König et al., 1989). AZT was initially found to be of benefit to patients with HIV infection (Yarchoan et al., 1986), but longer treatment periods can lead to AZT-induced bone marrow toxicity (Gill et al., 1987; Pizzo et al., 1988; Kennedy et al., 1991). Several investigators have converted this nucleoside analogue into a variety of lipophilic derivatives as a mean of increasing its antiviral activity (Hostetler et al., 1990; Kawaguchi et al., 1990; Steim et al., 1990; Sqalli-Houssaini et al., 1994; van Wijk et al., 1994). The use of liposomal drug carriers has shown to reduce the toxicity of associated drugs such as doxorubicin (Rahman et al., 1985) and amphotericin B (Lopez-Berestein et al., 1987). The incorporation of AZT in liposomal carriers results in decreased bone marrow toxicity, increased bioavailibility and enhanced antiviral activity (Phillips et al., 1991; Phillips and Tsoukas, 1992), and such formulations have been evaluated clinically (Deutsch et al., 1993). Liposomal incorporation and antiviral activity of a number of lipophilic derivatives of AZT (Schwendener et al., 1994) has also been evaluated.

Phospholipid-triglyceride emulsions such as Intralipid®, which are known from parenteral nutrition studies to be non-toxic (Davis et al., 1987), have been used as carriers for a variety of drugs (Caillot et al., 1993; Higashi et al., 1995; Tibell et al., 1995; Yamaguchi et al., 1995). Solid lipid nanoparticles (SLN), which are comprised of a high melting point triglyceride (TG) as the solid core and a phospholid (PL) coating (Heiati et al., 1996), have been already used as a drug carrier for lipophilic drugs (Schwarz et al., 1994; Müller et al., 1996; Heiati et al., 1997). One of the characteristic of colloidal drug carriers such as liposomes and fat emulsion is their rapid clearance from the blood stream by the mononuclear phagocyte system (MPS), characterized primarily as uptake by liver Kupffer cells and splenic macrophages. (Gregoriadis and Neerunjun, 1974; Kimelberg et al., 1976). Such carrier systems have been employed for the passive targeting of drugs (Haynes and Cho, 1988; Scieszka et al., 1988; Bender et al., 1994). The profound involvement of the macrophage in the pathogenesis of HIV infection (Meltzer et al., 1990) presents a unique opportunity for evaluating cell-specific drug targeting via the use of colloidal carriers.

It has been shown that PEG conjugated to phosphatidylethanolamine (PE) can modify the circulation time of liposomes and fat emulsions if the conjugate is incorporated into the PL bilayers (Klibanov et al., 1990; Lundberg et al., 1996) The ability of PE-PEG to prolong liposome circulation time appears to be greater than that of GM_1 gangloside on a molar basis (Klibanov et al., 1990).

In a previous communication from our laboratory we reported the incorporation and the in vitro retention of the lipophilic prodrug, AZT palmitate (AZT-P) in SLNs (Heiati et al., 1997). The objective of the present study was to investigate the in vitro release of AZT-P in the presence of bovine plasma and to determine the biodistribution of AZT-P in mice after intravenous injection of free AZT-P and SLNs containing AZT-P with and without a PE-PEG coating.

2. Materials and methods

2.1. Materials

Trilaurin (TL), palmitoyl chloride, 3′-azido-3′-deoxythymidine-methyl-^{3}H (8.18×10^{10} Bq/mmol), carbonic anhydrase (from bovine erythrocytes), mouse IgM and gentamycin (1000 × solution) were purchased from Sigma (St. Louis, MO). Dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylglycerol, sodium salt (DMPG), and dipalmitoylphosphatidylethanolamine-*N*-[poly(ethylene glycol)$_{2000}$] (PE-PEG) were obtained from Avanti (Alabaster, AL). Sephadex

G-25M PD-10 columns and Sepharose 2B were purchased from Pharmacia Biotech (Uppsala, Sweden). Bio-Rad protein assay reagent was from Bio-Rad (Richmond, CA). TLC silica gel 60 F254 plates were obtained from Merck (Darmstadt, Germany). Silica gel (60–200 mesh) was from Baker (Phillipsburg, NJ). 3′-azido-3′-deoxythymidine (Zidovudine®, AZT) was supplied by GlaxoWellcome, (Research Triangle Park, NC). Bovine albumin fraction V and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were purchased from Boehringer Mannheim (Mannheim, Germany). Bovine plasma containing citrate phosphate/dextrose/adenine solution USP (CPDA-1 USP, 0.14 ml/ml blood) as the anticoagulant agent, was supplied by the Faculté de Medicine Veterinaire, Université de Montréal (Québec, Canada). The buffer used in the experiments was 10 mM HEPES buffer at pH 7.4, prepared with saline/gentamycin solution (sterile 0.9% saline containing 0.1 μl/ml gentamycin 1000 × solution as the preservative agent). Hyamine hydroxide, 1 M solution in methanol was purchased from ICN (Irvine, CA). All materials were used without further purification, and all other chemicals were of analytical or USP grade. Spectroscopic determinations were carried out using an HP 8452A diode array spectrophotometer (Hewlett Packard, CA). Radioactivity was counted in a liquid scintillation cocktail (CytoScint™ ES, ICN, Costa Mesa, CA) using a LKB-Wallac Rackbeta (Turku, Finland) at an efficiency of 40% with automatic quench and chemiluminescence correction. Female CD-1 mice, 5–7 weeks old, weighing approximately 25–30 g were supplied by Charles River (St. Constant, Quebec, Canada).

2.2. Methods

2.2.1. Synthesis of AZT-P

AZT-P and [^{3}H]-AZT-P were synthesized according to the procedure of Kawaguchi et al. (1990) with the modifications described in our previous report (Heiati et al., 1997).

2.2.2. Preparation of SLN

To prepare SLNs, 400 mg TL, 160 mg DPPC, 16 mg DMPG and 40 mg AZT-P (spiked with 16.2×10^7 Bq ^{3}H-AZT-P) were dissolved in chloroform (20 ml) in a sterile round bottom flask at room temperature. SLNs with a PE-PEG$_{2000}$ coating (SLN-PE-PEG) were prepared by adding 10% (mol ratio) PE-PEG (PE-PEG: PL) to the lipid mixture. After removal of the organic phase by rotary evaporation in vacuo at 50 ± 0.5°C, an oily lipid film was obtained on the flask wall. The lipid film was hydrated with 20 ml of saline/gentamycin solution and rotated for 5 min at 50 ± 0.5°C. The emulsion obtained (mean diameter 1–2 μm) was homogenized for ten cycles at 60–70°C and 15000 psi, using a high pressure homogenizer (Emulsiflex®-30, Avestin, Ottawa, Canada) to produce SLNs. In order to minimize bacterial contamination, the high pressure homogenizer was washed with 70% ethanol and rinsed with saline/gentamycin solution prior to use. SLN preparations were collected in sterile polyvinyl containers and allowed to cool to 20 ± 0.5°C over a period of 1 h. A saline/gentamycin suspension of AZT-P was prepared using only AZT-P.

2.2.3. Particle size measurement and zeta (ζ) potential

The mean particle size of the samples was determined using photon correlation spectroscopy (N4 Plus, Coulter, Hialeah, FL). SLNs were diluted with saline/gentamycin solution to give a particle count rate between 5×10^4 and 1×10^6 counts/s. Mean particle diameter was calculated in size distribution processor mode (SDP) using the following conditions: fluid refractive index 1.33; temperature 20°C; viscosity 0.93 centipoise; angle of measurement 90.0°; sample time 10.5 μs, and sample run time 90 s.

The ζ potential was measured using a Delsa 440SX (Coulter, Hialeah, FL) using the following conditions: current 0.7 mA; frequency range 500 Hz; temperature 20°C; fluid refractive index 1.33; viscosity 0.93 centipoise; dielectric constant 78.3; conductivity 16.7 ms/cm; on time 2.5 s, off time 0.5 s, and sample run time 60 s.

2.2.4. Determination of incorporated AZT-P by gel permeation chromatography

Retention of AZT-P in SLNs was determined by gel permeation chromatography on cross-linked dextran (Sepharose 2B, Pharmacia Biotech, Uppsala, Sweden). Preliminary calibration of the Sepharose 2B column (13 × 1 cm bed volume) was carried-out using SLN or SLN-PE-PEG, AZT-P and AZT. SLN preparations (0.2 ml) were applied to the Sepharose 2B column and eluted with HEPES buffer (0.5 ml/min). Fractions (0.5 ml) containing SLNs were collected, to which 4.5 ml scintillation cocktail was added prior to β-counting. The percentage of AZT-P incorporated in the SLNs was calculated relative to the radioactivity of 0.2 ml of SLN suspension before gel permeation chromatography.

2.2.5. AZT-P retention in SLNs in the presence of 50% bovine plasma

The Sepharose 2B column was calibrated with plasma and with protein molecular weight markers as follows: Plasma (0.2 ml) or protein solution (0.2 ml containing albumin fraction V, 10 mg/ml; IgM, 1 mg/ml or carbonic anhydrase, 1 mg/ml) were applied separately to the Sepharose 2B column and eluted with HEPES buffer (0.5 ml/min). Fractions of 0.5 ml were collected. To each fraction 2 ml HEPES buffer and 0.2 ml Bio-Rad protein assay solution was added. The samples were immediately vortexed, and the absorbance determined at 595 nm.

SLNs with or without a PEG coating were incubated in bovine plasma (50:50, V/V) at 37 ± 0.5°C. At timed intervals samples (0.2 ml) were applied to the Sepharose 2B column and the amount of AZT-P associated with the SLNs was determined as described above.

2.2.6. Biodistribution studies

Female CD-1 mice were injected with 0.2 ml of each preparation via a lateral tail vein. The animals were sacrificed at timed intervals, blood and urine taken and the organs removed and weighed. Tissue samples (100–200 mg) were digested in hyamine hydroxide (2 ml) for 5 days at 55°C before determination of radioactivity. The radioactivity of the samples (0.1–0.2 ml) was determined by adding 4.5 ml scintillation liquid. Blood samples were centrifuged at 23000 × *g* for 20 min. Serum was collected and 4.5 ml of scintillation liquid was added to 0.1 ml of each serum sample prior to radioactivity counting. The radioactivity of the urine samples was determined by adding 4.5 ml scintillation liquid to 0.1 ml of urine.

3. Results

3.1. AZT-P incorporation in SLNs

The incorporation of AZT-P in SLNs was not affected by the presence of PE-PEG. The incorporation of AZT-P was 98 ± 2% in SLNs or SLN-PE-PEG. Although the PE-PEG coating did not significantly change the mean diameter of the SLNs, a significant reduction in SLN ζ potential was observed (Table 1). The SLNs and SLN-PE-PEG containing AZT-P were physically stable during all experiments, and no particle aggregation or changes in ζ potential were observed following their incubation with plasma.

Table 1
Mean diameter and ζ potential of SLN and SLN-PE-PEG in the presence and absence of plasma[a]

	ζ potential (mV)		Diameter (nm)	
	HEPES buffer	Plasma	HEPES buffer	Plasma
SLN	−22 ± 4	−20 ± 5	181 ± 26	183 ± 48
SLN-PE-PEG	−5 ± 3	−6 ± 4	152 ± 42	182 ± 44

[a] SLN preparations were incubated with bovine plasma (50:50, V/V) for 30 min. At 37 ± 0.5°C. Mean ± S.D. of three experiments.

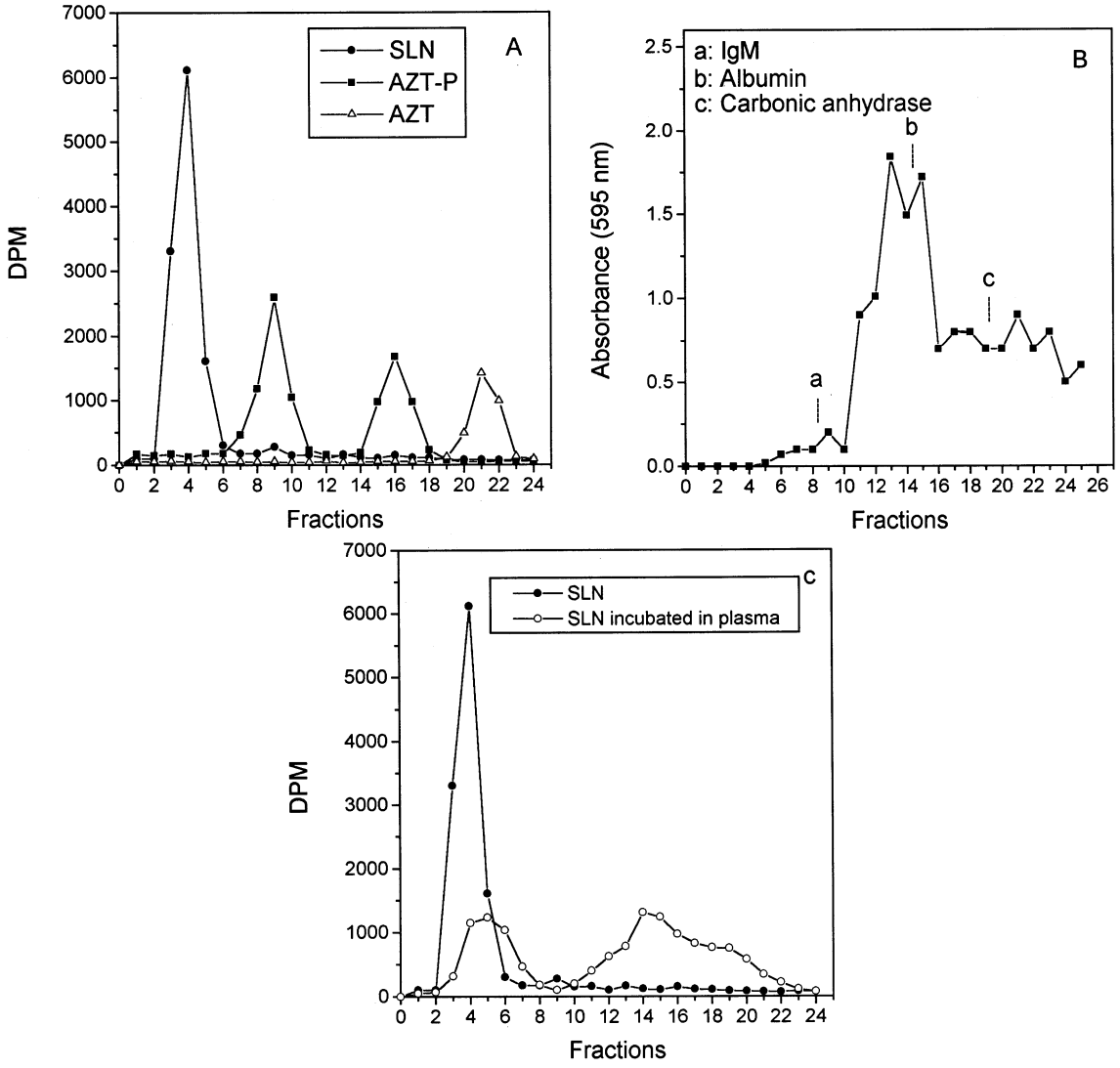

Fig. 1. Sepharose 2B column elution profiles of SLN, AZT-P and AZT (a), plasma and protein standards (b) and SLNs containing AZT-P or SLNs containing AZT-P incubated with plasma (c). M_W of the protein standards were as follows: IgM, 800000 Da; albumin fraction V, 67000 Da; and carbonic anhydrase, 29000 Da.

3.2. AZT-P retention in SLNs in the presence of 50% bovine plasma

Fig. 1a,b show the calibration of the Sepharose 2B chromatography column with SLN containing AZT-P, AZT-P, and AZT (Fig. 1a) or plasma and protein molecular weight markers (Fig. 1b). SLNs were rapidly eluted in the void volume between 1.5 and 3 ml, AZT-P in two fractions, between 3.5 and 5.5 ml and between 7 and 9 ml, and AZT was eluted between 10 and 11.5 ml. The apparent molecular weight (M_W) of the small and large AZT-P micelles in the two fractions were determined using the calibration curve obtained from the protein standards (Fig. 2). The gel chromatography profile of plasma showed a broad peak starting from fraction 9 (Fig. 1b). Incubation of SLNs containing AZT-P with plasma for 4 h

resulted in a reduction in the SLN peak followed by a broad peak of radioactivity having a maximum corresponding to that of the plasma protein albumin (Fig. 1c).

AZT-P was rapidly released from SLNs during incubation with bovine plasma (Fig. 3). However, the retention of AZT-P in the presence of plasma was significantly enhanced using PEG coated SLNs.

3.3. Blood clearance and tissue distribution studies

Blood levels and tissue distribution of AZT-P were calculated on the radioactivity found in blood or the respective tissue. Higher blood levels of AZT-P were observed after the administration of SLNs containing AZT-P compared to the administration of free AZT-P (Fig. 4). This effect was further increased by using SLNs with a PEG coating. Tissue distribution studies showed that following the i.v. administration of free AZT-P, a high level of radioactivity was found in the kidney, urine and spleen (Fig. 5). Administration of AZT-P incorporated in SLNs or SLN-PE-PEG

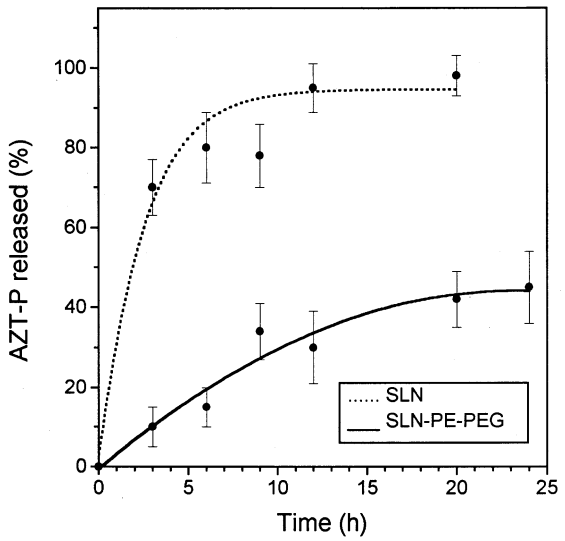

Fig. 3. AZT-P retention in SLNs and SLN-PE-PEG in the presence of 50% bovine plasma at 37°C. Mean ± S.D. of three experiments.

significantly increased the radioactivity level in liver and significantly decreased the radioactivity levels in kidney. No significant difference was observed in tissue radioactivity after the adminis-

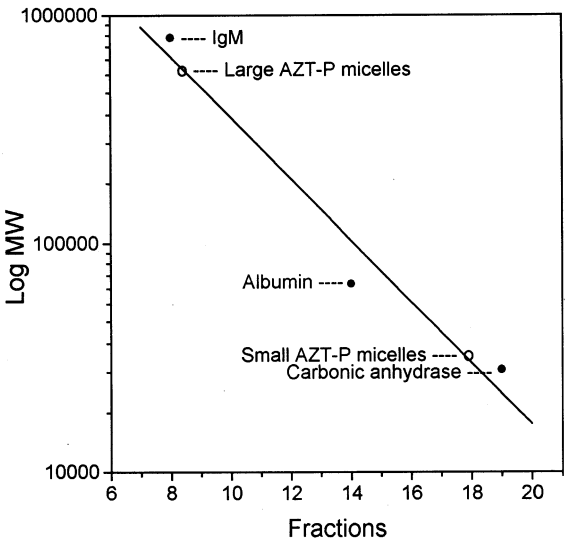

Fig. 2. Calibration curve of Sepharose 2B obtained with IgM, albumin fraction V and carbonic anhydrase. The apparent M_W of the micelles was calculated from the two fractions corresponding to AZT-P.

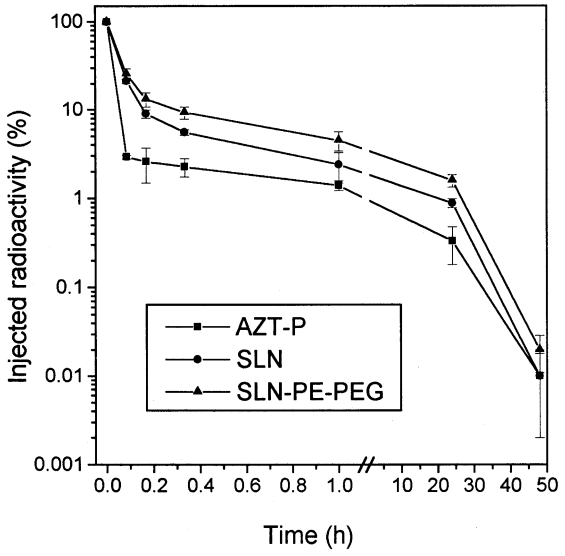

Fig. 4. Percentage of the initial injected radioactivity found in blood after i.v. administration of free AZT-P, SLNs containing AZT-P and SLN-PE-PEG containing AZT-P in mice. Mean ± S.D. (three mice).

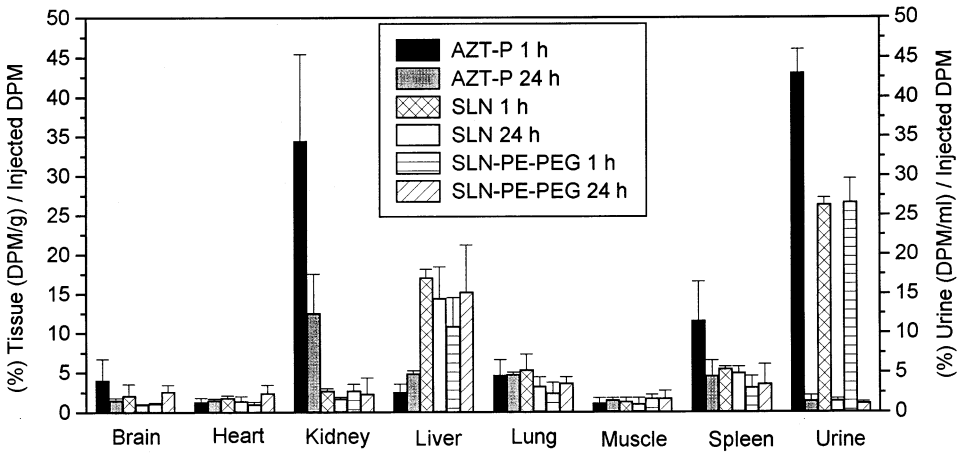

Fig. 5. Radioactivity found in different organs after i.v. administration of free AZT-P, SLNs containing AZT-P and SLN-PE-PEG containing AZT-P in mice. Mean ± S.D. (three mice).

tration of AZT-P incorporated in SLNs or SLN-PE-PEG.

4. Discussion

4.1. Gel chromatographical profiles

Because of their high M_W, SLNs were eluted rapidly from the Sepharose 2B column. Palmitic acid ester drugs are amphiphilic (Benameur et al., 1993). The palmitoyl fatty acid chain and the AZT head group of AZT-P may give amphiphilic characteristics to AZT-P, resulting in the formation of micelles in aqueous medium which are in equilibrium with AZT-P monomers (Heiati et al., 1998). The two different molecular weights (M_W) ranges observed in the gel chromatographical profile of AZT-P (approximate M_W of 30000 and 700000 Da respectively) (Fig. 1a and Fig. 2) may therefore correspond to large AZT-P micelles composed of approximately 1000 molecules and small AZT-P micelles of approximately 60 molecules (Fig. 2). These peaks were not the result of hydrolysis of AZT-P, as AZT was eluted between 10 and 11.5 ml, after the elution of AZT-P (Fig. 1a). Plasma proteins eluted from fraction 9 with a peak concentration in fraction 13 (Fig. 1b). The plasma protein peak maximum between fractions 13–15 represents albumin, which is present at a concentration of about 50 mg/ml in plasma (Fig. 1b). The reduced radioactivity of the SLN peak after the incubation with plasma was concomitant the appearance of a broad peak of radioactivity and is consistent with a release of incorporated AZT-P from the SLNs and subsequent binding to plasma proteins (Fig. 1c). This supposition is supported by the similarity between the large peak seen in the SLN/plasma gel chromatographical profile and the plasma protein profile. The results also indicate that free AZT-P is mainly drug bound to plasma albumin.

4.2. Retention of AZT-P in the presence of bovine plasma

Data obtained from the in vitro experiments is useful in predicting the behavior and the physical characteristics of drug carriers in vivo (Amselem et al., 1993). Because of the potential interaction with plasma components, the behavior of drug carriers is not always the same as it is in buffered aqueous solutions. Several studies have demonstrated that drug release from PL-based drug carriers such as liposomes is substantially different in plasma than in buffer (Gaber et al., 1995; Nagayasu et al., 1995). In a previous experiments we reported excellent retention of AZT-P in SLNs incubated in buffer at 37°C over a prolonged period of time (Heiati et al., 1997). The present

study shows that incubation of SLNs in plasma results in rapid release of AZT-P (Fig. 3). This may be due to enzymatic degradation of the PL membrane surrounding the SLNs core (Miller et al., 1992), to an interaction with specific plasma proteins such as complement factors in plasma (Devine et al., 1994; Liu et al., 1995) or to an interaction with lipoproteins (Chonn et al., 1992; Hernández-Caselles et al., 1993), followed by the subsequent release of AZT-P from the SLNs.

The ability of PEG to act as a hydrophilic shield surrounding the SLN was demonstrated by the observation that the ζ potential of the SLN-PE-PEG was significantly lower than that of the SLNs (Table 1). The ability of amphiphilic PEG to act as a hydrophilic shield in drug carriers has been shown in many studies (Klibanov et al., 1990; Tröster et al., 1992; Gaber et al., 1995; Lundberg et al., 1996). The release of AZT-P in the presence of plasma was slower in SLN-PE-PEG than in SLNs (Fig. 3). These results indicate that PEG is capable of sterically preventing the approach of destabilizing plasma components towards the surface of the SLNs, thus reducing their specific interaction with the PL membrane and increasing the retention of AZT-P.

4.3. Biodistribution studies

Fig. 4 shows that the removal of all preparations from the circulation was biphasic, with a rapid initial phase (0–60 min) following by a much slower rate of elimination. The rapid loss of AZT-P is consistent with clearance via the kidney and a minimal degree of plasma protein binding. However, higher blood level radioactivity was observed after the administration of SLNs containing AZT-P compared to free AZT-P. This effect was further increased using SLNs with a PEG coating. A 6-fold increase in blood radioactivity was observed between free AZT-P and SLN-PE-PEG containing AZT-P. The steric barrier presented by the PEG is consistent with a reduction in the uptake of SLNs by tissue-fixed macrophages, thus resulting in a higher blood level of radioactivity following the administration of SLN-PE-PEG. The major route of AZT excretion is the kidney (Howe et al., 1992). The tissue distribution obtained in the present study demonstrated that the major excretory route of AZT-P is also the kidney (Fig. 5). A significant proportion of the initial dose of AZT-P (42%) was found in the urine 1 h following i.v. injection. Both SLN- and SLN-PE-PEG-incorporation of AZT-P significantly decreased the urinary excretion of AZT-P, and increased the localization of AZT-P in liver. These results indicate that although the presence of PEG does result in higher blood levels, it does not ultimately prevent the uptake of SLNs containing AZT-P by this reticulendothelial organ.

5. Conclusion

This study demonstrates that the surface property of SLN drug carriers can be significantly modified following of the incorporation of amphiphilic PEG. The release of the amphiphilic prodrug AZT-P from SLN carriers in the presence of plasma was significantly retarded by the presence of amphiphilic PEG. The incorporation of AZT-P in SLNs increased the blood level of the prodrug. PEG coating of the SLNs increases further the blood level of AZT-P comparing to the non-coated SLNs. Free AZT-P is excreted rapidly from the kidney; however incorporation of AZT-P in SLNs (with or without PEG) significantly reduces the urinary excretion. Incorporation of AZT-P within SLNs resulted in a rapid accumulation of the drug in the liver, which may be used as a means of targeting this drug to the reticulendothelial organs.

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