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Submicron lipid emulsions containing amphipathic polyethylene glycol for use as drug-carriers with prolonged circulation time

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Abstract

Submicron-sized lipid emulsions possess many favourable properties as drug-carriers like biocompatibility, physical stability and ease of preparation. The use of colloidal carriers is, however, hampered by their rapid clearance from the circulation. In the present study, this problem was tackled by coating the emulsion droplets with polyethylene glycol modified phosphatidylethanolamine (PEG-PE). Physical stability was obtained by using a mixture of phosphatidylcholine and polysorbate 80 as emulsifying agent. Sonicated preparations with the standard composition; triolein (TO): dipalmitoyl phosphatidylcholine (DPPC): polysorbate 80: PEG-PE at mass ratios of 2: 1: 0.4: 0.1 exhibited a mean particle size of 44 nm (by quasi-elastic light scattering) and an excellent physical stability. In vivo plasma clearance data were obtained by intravenous injection of emulsions into mice. Coating of the emulsion droplets with PEG-PE gave a considerable increase in circulation lifetime. A further notable effect was obtained when the cosurfactant polysorbate 80 was introduced into the system, apparently as a result of decreased particle size. Lipid emulsions with the standard composition showed first order kinetics during 6 h with a circulation half-life of about 3 h. Phospholipid transition temperature and emulsion particle size were found to be important factors while the clearance rate was essentially independent of lipid dose.

Keywords: Lipid emulsion; Drug carrier; Hydrophilicity; In vivo clearance; Organ uptake

1. Introduction

Submicron-sized oil-in-water (o/w) lipid emulsions are appealing alternatives as drug delivery systems (for reviews see Davis et al., 1987; Lundberg, 1991). The basic structure of a lipid emulsion particle is a neutral lipid core (i.e. liquid triglyceride) stabilized by amphipathic lipids (i.e. phospholipids). Such emulsions can solubilize considerable amounts of lipophilic drugs in the hydrophobic domain of the oil core and have potential therapeutic applications as carriers for lipophilic drugs.

Submicron lipid emulsions meet most requirements for a good parenteral delivery system since

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they are biocompatible, biodegradable, physically stable and can easily be produced in a large scale. The utilization of lipid emulsions as drug-carriers is, however, hampered by two major drawbacks; the fast clearance of colloidal particles by the mononuclear phagocyte system (MPS) and the restricted access to the extravascular space from the blood stream. Regarding the extravasation problem, the transfer of lipid emulsions may be improved by use of particles with a small size, especially since the microvascular permeability is increased in sites of inflammation and neoangiogenesis (tumours) (Jain, 1989; Illum et al., 1989). In vivo experiments with small, long-circulating liposomes have, in fact, shown that a particle size smaller than 100 nm can be favourable for tumour uptake, leading to an enhanced tumour accumulation and antitumour activity (Mayhew et al., 1992). A previous study showed that the combination of a phospholipid emulsifier with a non-ionic cosurfactant enabled the production of stable lipid emulsions with a particle size of about 50 nm (Lundberg, 1994a).

The fast clearance of particulate drug-carriers like liposomes and nanoparticles, primarily by uptake by liver Kupffer cells and spleen fixed macrophages, has impeded their effective use as drug-carriers. The finding that coating of the particles with a hydrophilic polymer gave a slow clearance rate resulted in a major improvement of the utility of such particles (Illum and Davis, 1984, Blume and Cevc, 1990). The hydrophilic coating is thought to mask the surface from opsonins marking the particle for uptake by MPS.

The objective of this study was to design a submicron lipid emulsion with slow clearance rate suitable for a carrier of lipophilic drugs. The concept was to prolong the circulation lifetime of the emulsion droplets by coating them with a hydrophilic polymer. PEG-PE was the coating material of choice since this compound has been shown to extend the shelf-life of liposomes (Woodle and Lasic, 1992) and also to prolong the circulation time of emulsion droplets (Wheeler et al., 1994).

2. Materials and methods

2.1. Materials

Dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylethanolamine (DPPE) was purchased from Avanti Polar-Lipids Inc. (Birmingham, AL). High-purity 3-*sn*-phosphatidylcholine (EPC) and sphingomyelin (SM) from egg yolk and polysorbate 80 were obtained from Fluka Chemie AG (Buchs, Switzerland), and cholesterol (CH), cholesteryl oleate (CO), triolein (TO) and tristearin (TS) from Nu Chek Prep, Inc. (Elysian, MN). Pluronic F-68 was from ICI Chemicals and Polymers Ltd. (Cleveland, U.K.). [^{3}H]Cholesteryl oleoyl ether (CO ether) and glycerol tri [^{14}C]oleate were obtained from Amersham International plc (Amersham, U.K.). The synthesis of the TO ether was made as described by Morgan and Hofmann (1970). PEG-PE was synthesized by a method based on the reaction of PEG2000 with carbonyldiimidazole, followed by addition of DPPE (Allen et al., 1991). PEG-PE was purified by adding a small amount of water to the evaporated reaction residue and the resulting PEG-PE micelles were dialyzed against water using a Spectra/Por® CE 300 000 MWCO dialysis bag (Spectrum Medical Industries, Inc. Houston, TX) for 1 week and then lyophilized (Maruyama et al., 1992). The purity of the product was checked by silica gel TLC.

2.2. Preparation and characterization of emulsions

The preparation of emulsions with the required compositions and particle sizes were performed by sonication according to a method described in details elsewhere (Lundberg, 1994a). In short, the emulsion components were dispersed into a conic test tube from stock solutions, the solvent was evaporated under a stream of nitrogen and the samples lyophilized overnight. Then, 2 ml of phosphate-buffered saline (PBS) was added to the lipids (total mixture about 4 mg), the sample heated to 45°C, vortexed for 20 s and then sonicated (3 × 20 s) with a Sonifier® cell disruptor B-30 (Branson Sonic Power Co.) using the continuous mode and an output control of 1.5. The

sonicated preparations were filtered through a sterile Acrodisc® 0.2 μm filter (Gelman Sciences, Ann Arbor, MI). The loss of material by filtration was routinely checked and usually less than 5%. For comparative studies, emulsion droplet size was varied by changes of the ratio between core and surface material, holding constant the sonication intensity and time.

The emulsion droplet size was measured by quasielastic laser light scattering using a BI-90 particle sizer (Brookhaven Instruments Corp., Ronkonkoma, NY) and by electron microscopy. Emulsions were stained on formvar coated grids with 2% phosphotungstic acid and viewed on a Jeol 2000 FX transmission electron microscope.

The physical stability of the emulsions during prolonged storage was determined by repeated measurements of particle size by the laser particle sizer and by quantitation of recovery of radioactive label by scintillation counting after centrifugation for 15 min at 10000 × *g*.

2.3. Injection studies

For measurements of the plasma disappearance rate and the organ distribution, emulsions labelled with radioactive core lipids were injected into a tail vein of non-anesthetized 8-week-old female BALB/c inbred mice weighing about 20 g (Animal Resources Centre, Canning Vale, W.A.). The emulsions, double labelled with [^{3}H]CO ether and [^{14}C]TO, were injected as a bolus of 100 μg core lipids/mouse in 100 μl PBS, if not otherwise stated. Blood samples were taken by retroorbital bleeding under light anesthesia at 1, 3, 6 and 24 h after injection of the emulsion. Individual mice received about 4 × 10^5 and 2.5 × 10^5 cpm of ^{3}H and ^{14}C, respectively. After separation by centrifugation, the collected plasma was measured for radioactivity by taking 40 μl and adding 5 ml Emulsifier™ Safe (Packard Instrument B.M.-Chemical Operations). For removal of liver and spleen, the mice were killed by ip administration of pentobarbital. The livers were perfused with cold PBS to remove the blood. The organs were extracted with 30 volumes of chloroform/methanol (2:1, v/v), then aliquots were taken, the solvents were evaporated and the radioactivity was measured by liquid scintillation spectrometry in 10 ml of scintillant.

3. Results and discussion

3.1. Emulsion characteristics

A previous paper described the preparation of stable submicron lipid emulsions with EPC as phospholipid component and with polysorbate 80 as cosurfactant (Lundberg, 1994a). In the present study, DPPC was chosen as the principal surface component since this phospholipid has been shown to give a relatively slow clearance rate (Redgrave et al., 1992). However, preparation of TO emulsions with DPPC as the sole emulsifier (TO/DPPC 2:1 w/w) resulted in coarse unstable emulsions with a mean diameter of 128 nm and a fast aggregation when stored at 4°C. In a recent paper, Wheeler et al. (1994) reported the preparation of a stable phospholipid-corn oil emulsion with PEG-PE as the sole coemulsifier. In the present study, the addition of PEG-PE at a mass ratio of 0.1 to DPPC resulted in a reduced particle size of 96 nm and an improved stability compared to DPPC alone, but after 1 week of storage, 58% of the dispersed material was lost at centrifugation. However, when the emulsion was further stabilized with polysorbate 80 at a mass ratio of 0.25 to DPPC, the particle sizer gave a value of 44 ± 2 nm (mean ± S.E., n = 5) and the preparation appeared optically transparent. The size distribution of a typical preparation with a polydispersity of 0.136 is shown in Fig. 1A. The emulsion showed an excellent colloidal stability and after 3 months of storage at 4°C, no significant change in diameter was noted and 93% of the core material remained in suspension after centrifugation. On the basis of these initial experiments, we selected for further studies a standard emulsion composition of TO — DPPC — polysorbate 80 — PEG-PE at the mass ratios of 2: 1: 0.4: 0.1.

Examination of emulsions with the standard composition by negative-staining electron microscopy revealed round droplets ranging in size from 20 to 40 nm, with few contaminating vesicles

or disc-like structures (Fig. 1B). The mean particle size was calculated to be about 30 nm and thus considerably smaller than the value of 44 nm obtained by the laser particle sizer. Storage of an emulsion for 3 months had no discernible effect on the electron microscopic appearance.

3.2. Blood clearance and tissue uptake

The in vivo behaviour of lipid emulsions was studied by iv injection into mice. The standard dose of lipid was 100 μg of TO. The fate of the lipid emulsions was investigated by tracing [^{14}C]CO ether and [^{3}H]TO incorporated during preparation. The CO ether is a lipid marker that is not exchanged or metabolized in plasma (Derksen et al., 1987) and will thus give values for the disappearance of whole emulsion particles from the blood stream. Removal of the TO, labelled in the fatty acid portion, reflects the sum of both lipolysis and uptake of particles. The effects of PEG-PE and cosurfactants on the clearance of whole lipid emulsion globules, with the basic composition of TO/DPPC (2:1 w/w), are shown in Fig. 2A. Emulsions with DPPC as the sole emulsifier were rapidly cleared from the blood after intravenous injection, with only about 11% of the CO ether label left in circulation after 1 h. This finding is in agreement with previous studies using lipid emulsions as chylomicron models (Redgrave et al., 1992). The conclusion was that in order to obtain prolonged circulation the surface properties of the emulsion droplet had to be changed. PEG-PE, with a molecular weight of 2000 for the PEG part, was selected for the hydrophilic coating of the droplets because this compound has been shown to impart an effective steric barrier when used with liposomes (Mori et al., 1991; Woodle et al., 1992). The data presented in Fig. 2A clearly show that addition of PEG-PE gave a prolonged clearance rate especially during the first 3 h. A further addition of the cosurfactants polysorbate 80 or Pluronic F-68 resulted in a marked extension of the circulation lifetime and the curves show an essentially exponential disappearance of the CO ether label (first order kinetics) during the first 6 h. The $t_{1/2}$ value for clearance of emulsions droplets containing polysorbate 80 was estimated to be about 3 h. This is considerably faster than the 20 h reported for PEG-liposomes (Woodle and Lasic, 1992), but slower than that of about 1 h obtained with polystyrene-PEG2000 particles (Dunn et al., 1994). The notable effects of Pluronic F-68 and polysorbate 80 can apparently be attributed mainly to the decrease in droplet size, although an additional influence by increased hydrophilicity may not be ruled out.

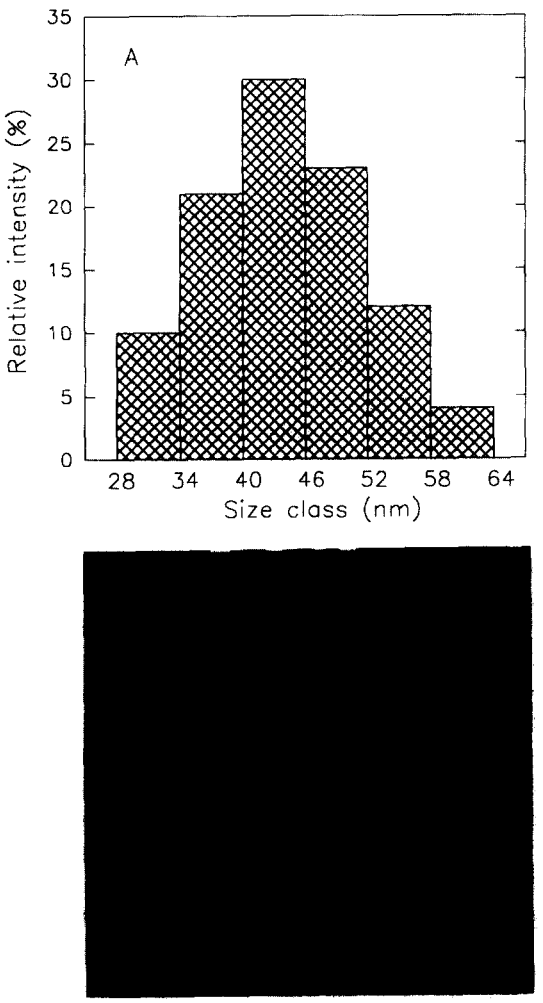

Fig. 1. Particle size distribution (A) and negative staining electron microscope appearance (B) of the standard lipid emulsion system; TO: DPPC: polysorbate 80: PEG-PE (2: 1: 0.4: 0.1). The scale bar represents 40 nm.

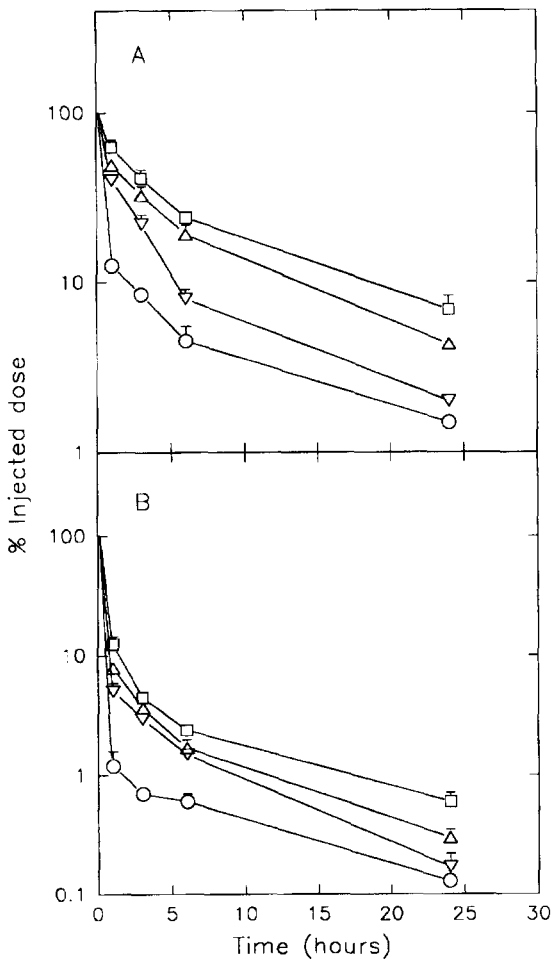

Fig. 2. Effects of emulsion surface constituents on the clearance from plasma of CO ether (A) and TO (B) labels as a function of time after intravenous administration in mice of emulsions composed of TO: DPPC (2:1) (○), TO: DPPC: PEG-PE (2: 1: 0.1) (▽), TO: DPPC: Pluronic F-68: PEG-PE (2: 1: 0.4: 0.1) (△) and TO: DPPC: polysorbate 80: PEG-PE (2: 1: 0.4: 0.1) (□). Results are given as mean ± S.E., $n = 3$.

The disappearance of TO from the circulation was much faster than that of the CO ether (Fig. 2B). With DPPC as sole emulsifier, only 1.2% of the TO was left after 1 h, while coating with PEG-PE and polysorbate 80 increased the value to 12.4%. These data can be compared to the corresponding values of 12.5 and 62.5% for the CO ether. The apparent conclusion is that the major part of the TO is removed from the blood by lipolysis and not by uptake of whole emulsion droplets.

The effects of phospholipid component and cholesterol are shown in Fig. 3. Replacing DPPC with EPC resulted in a faster initial clearance and hydrolysis, while SM gave values similar to those obtained with DPPC. The similar behaviour of emulsions stabilized with DPPC and SM could be due to the fact that both, different from EPC, are

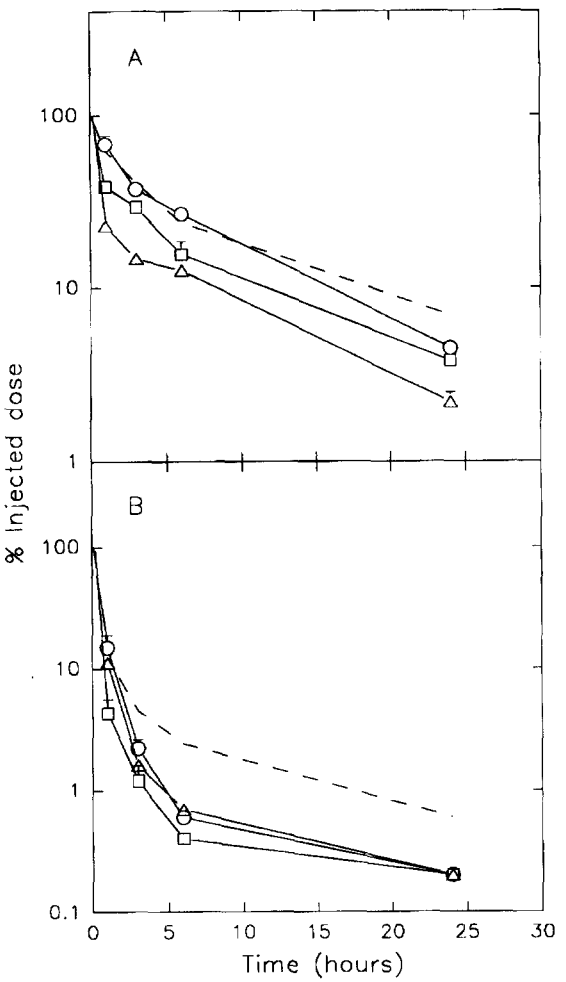

Fig. 3. Effects of phospholipid component and CH on the clearance rates from plasma of CO ether (A) and TO (B) labels as a function of time after intravenous administration of emulsions composed of TO: SM: polysorbate 80: PEG-PE (2: 1: 0.4: 0.1) (○), TO: EPC: polysorbate 80: PEG-PE (2: 1: 0.4: 0.1) (□) and TO: DPPC: CH: polysorbate 80: PEG-PE (2: 1: 0.3: 0.4: 0.1) (△). Emulsions composed of TO: DPPC: polysorbate 80: PEG-PE (2: 1: 0.4: 0.1) are shown as comparison (broken line). Values are mean ± S.E., $n = 3$.

high phase transition phospholipids, as a result of more saturated fatty acyl chains. These results are in general agreement with data obtained with lipid emulsion droplets without PEG-PE and polysorbate 80 (Redgrave et al., 1992), but differ from those obtained with PEG-liposomes where the shelf-life is more independent of bilayer fluidity (Allen and Hansen, 1991). Addition of cholesterol at a 3:1 mass ratio to DPPC clearly increased the CO ether clearance rate, while the disappearance of TO remained essentially unchanged. These results are in accordance with those obtained with chylomicron-like lipid emulsions showing that cholesterol promotes their uptake by the liver (Mortimer et al., 1995).

A factor which may have a considerable effect on the metabolic fate of lipid emulsion droplets is the composition of the neutral lipid core. Fig. 4 shows the effects of substituting TO with other neutral lipids. Replacing 20% of the core TO with CO had only a minor effect on the clearance rate of the CO ether label, while the removal of the TO label was somewhat faster. A droplet with a core composition of 30% TO and 70% TS gave a reduced lifetime for the CO label, but somewhat slower disappearance of the TO label. Based on these results, it seems that the possibility of extending the circulation time by replacing TO with other natural neutral lipids does not seem promising. A working-hypothesis was that a reduced lipolysis rate of the TO portion of the emulsion particle could prolong the circulation lifetime. In order to clarify this question, TO was substituted for the non-degradable TO ether (Morgan and Hofmann, 1970). The results (Fig. 4) were surprising and inconsistent with the working hypothesis; up to 1 h both clearance and hydrolysis were markedly slow with about 90% of the [^{14}C]CO ether and 40% of trace amounts of [^{3}H]TO label still in the circulation. However, for times exceeding 1 h, the disappearance of both the labelled compounds was very fast. These data suggest that factors other than the rate of TO hydrolysis are more decisive regarding the clearance of small lipid emulsion droplets.

The effects of dose and size of the emulsion droplets on the clearance rate are shown in Fig. 5. An increased dose from 100 μg up to 400 μg TO

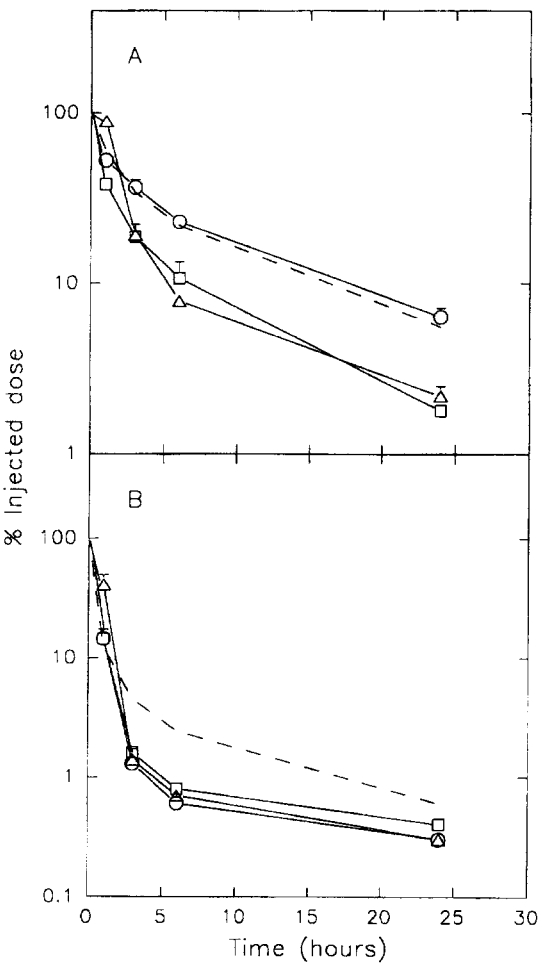

Fig. 4. Effects of emulsion core composition on the clearance rates from plasma of CO ether (A) and TO (B) labels as a function of time after intravenous administration of emulsions composed of TO: CO: DPPC: polysorbate 80: PEG-PE (1.6: 0.4: 1: 0.4: 0.1) (○), TO: TS: DPPC: polysorbate 80: PEG-PE (0.6: 1.4: 1: 0.4: 0.1) (□) and TO ether: DPPC: polysorbate 80: PEG-PE (2: 1: 0.4: 0.1) (△). Values given are mean ± S.E., n = 3.

had only a small effect on the clearance rate during the first 6 h. Such dose-independent pharmacokinetics, also noted for PEG-liposomes, offer an advantage for the therapeutic use of lipid emulsions as drug-carriers. In contrast to the minimal effect of dose, the effect of droplet size (Fig. 5B) was quite profound; an increase in particle diameter from 50 to 100 nm increased the rate of clearance dramatically, especially during the first

hour after injection. A further increase to 175 nm gave only a small increase in clearance rate up to 3 h. An enhanced in vivo uptake with increased particle size seems to be a general feature of colloidal carriers, although the effect noted for liposomes was less marked (Woodle and Lasic, 1992).

The organ uptake of emulsion droplets was measured for selected preparations. An initial experiment, including liver, spleen, fat, lung and carcass, showed that the quantitatively most significant uptake could be ascribed to the liver. Another organ of special interest is the spleen because it gives information about the uptake by macrophages. Therefore, further experiments were restricted to liver and spleen. With DPPC as the sole emulsifier the liver uptake was fast, in accordance with earlier reports (Redgrave et al., 1992). PEG-PE reduced the liver uptake, but for the CO ether label it was still 60 and 75% at 6 and 24 h, respectively. Polysorbate 80 gave a further decrease in the liver uptake, with corresponding values of 38 and 42%. An interesting detail is the modest additional uptake by liver and spleen between 6 and 24 h, while there is still a considerable blood clearance at the same time (see Fig. 2A). The tissues, besides liver and spleen, responsible for the removal of emulsion droplets during this time period were not identified. The liver values for the TO label were much lower and amounted to 8 and 1.6% for PEG-PE-emulsions at 6 and 24 h, respectively, while the addition of polysorbate 80 reduced these values to 4 and 1%. The low liver values for TO compared with those for the CO ether indicated that most of the TO was hydrolyzed, probably by lipoprotein lipase, before the droplets were removed by the liver. The uptake of emulsion droplets by the spleen was relatively slow with CO ether values of 1.5 and 2.1% during 6 and 24 h, respectively. These low values for the spleen indicate that uptake by macrophages was not the major clearance mechanism. The organ uptake data clearly show that the blood residence time for the emulsions to a great extent is determined by the rate of liver uptake. Further efforts to prolong the circulation lifetime of lipid emulsions should thus aim at the reduction of the liver uptake.

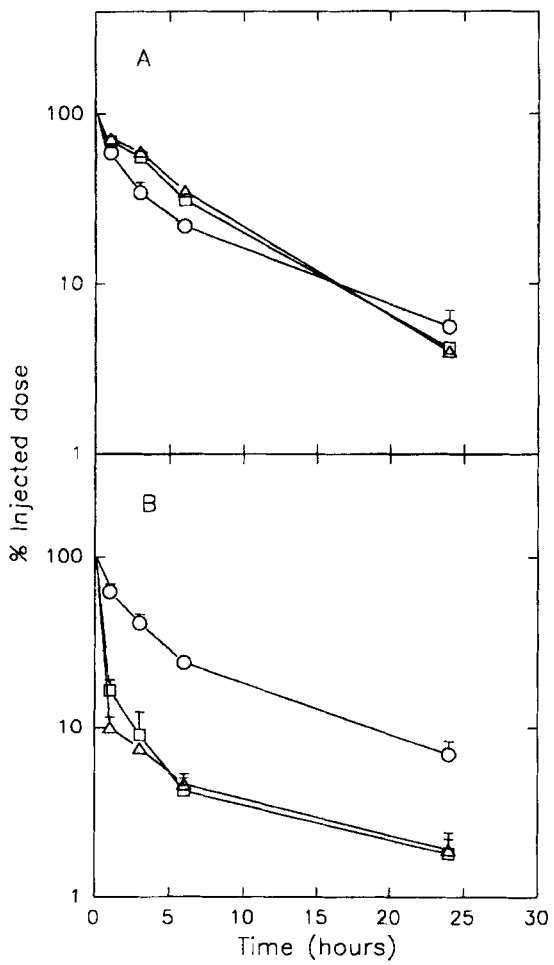

Fig. 5. Effects of emulsion dose (A) and particle size (B) on the clearance of CO ether label from plasma as a function of time after intravenous administration into mice. The injected doses of TO in the form of emulsions composed of TO: DPPC: polysorbate 80: PEG-PE (2: 1: 0.4: 0.1) was 100 μg (○), 200 μg (□) and 400 μg (△). The particle sizes of the emulsions injected were 50 nm (○), 100 nm (□) and 175 nm (△).

4. Conclusions

In a previous paper, submicron lipid emulsions proved to be suitable carriers for lipophilic anticancer drugs, with preserved cytotoxic activity against cancer cells in vitro (Lundberg, 1994b). The present study demonstrates the in vivo utility of such drug-carrier emulsions. Coating of the

droplet surface with a hydrophilic polymer (PEG) results in a considerable extension of the circulation lifetime. Optimum particle diameter was obtained by combination of DPPC with the non-ionic surfactant polysorbate 80. The importance of the small particle size for pharmaceutical applicability is underlined by the prolonged residence in circulation and likely optimisation of extravasation into tumours during neoangiogenesis. The limiting factor for a long circulation lifetime of lipid emulsions is the liver uptake, which is the dominating mechanism of removal during the first 6 h after injection. Therefore, the reduction of liver uptake is an important target for further development of long-circulating lipid emulsions. Future applications for long-circulating lipid emulsions could include both passive and active targeting. The potential for site-specific drug delivery, by ligands for cellular receptors attached to the surface of the lipid emulsion droplet, has been demonstrated with apolipoprotein B (Lundberg, 1987) and apolipoprotein E (Rensen et al., 1995) as ligands.

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