Fat Emulsions Based on Structured Lipids (1,3-specific triglycerides): An Investigation of the *in Vivo* Fate

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Purpose. Structured lipids (1,3-specific triglycerides) are new chemical entities made by enzymatic transesterification of the fatty acids in the 1,3 positions of the triglyceride. The purpose of this study is to characterize structured lipids with either short chain fatty acids or medium chain fatty acids in the 1,3 positions with regard to their hydrophobicity, and investigate the *in vivo* fate in order to evaluate the potential of structured lipids as core material in fat emulsions used as parenteral drug delivery system.

Methods. The lipids were characterized by employing reversed phase high performance liquid chromatography. The biodistribution of radioactively labeled emulsions was studied in rats.

Results. By employing high performance liquid chromatography a rank order of the hydrophobicities of the lipids could be given, with the triglycerides containing long chain fatty acids being the most hydrophobic and the structured lipid with short chain fatty acids in the 1,3 positions the least. When formulated as fat emulsions, the emulsion based on structured lipids with short fatty acids in the 1,3 positions was removed slower from the general blood circulation compared to emulsions based on lipids with long chain fatty acids in the 1,3 positions.

Conclusions. The type of core material influences the *in vivo* circulation time of fat emulsions.

KEY WORDS: structured lipids; emulsions; *in vivo* fate; particulate drug delivery systems.

INTRODUCTION

The use of fat emulsions as particulate drug delivery systems for lipophilic drugs has been reviewed by others (1–2). Particulate drug delivery systems have been envisaged as a means to direct drugs to a specific target, to protect drugs from degradation or to prolong the circulation time of drugs *in vivo* (3). Prolonging the circulation time of particulate drug delivery systems *in vivo* has been accomplished by modifying their surface properties (4–5). Recently, it was demonstrated that it is possible to increase the circulation time of a fat emulsion based on vegetable oils by modifying its surface properties employing a block copolymer (5). In the field of fat emulsions used as drug delivery systems, only little work has explored whether the type of core material in fat emulsions, i.e. the nature of the oils as the dispersed phase in fat emulsions has any influence on the circulation time *in vivo*. With the advent of biotechnology, it is now possible to modify vegetable oils enzymatically giving 1,3-specific triglycerides, i.e. structured lipids. SLS and MLM oils (Fig. 1) are both examples of such structured lipids, and they are made from soybean oil employing 1.3-specific lipases. Our hypothesis is that the differences in the chain lengths of structured lipids will influence the overall hydrophobicity, which in turn may influence the incorporation of drugs into the emulsions and drug release rate, as well as the rate of hydrolysis and *in vivo* fate of the emulsions.

In the present study, we have investigated if different kinds of core material (either structured lipids or soybean oil) impose any influence on the *in vivo* elimination of the emulsions in rats.

MATERIALS AND METHODS

^{14}C-cholesteryl oleate and ^{3}H-triolein were supplied by Dupont, USA. The structured lipids (SLS and MLM) were synthesized from soybean oil and ethyl esters of short and medium chain fatty acids using immobilized 1,3-specific lipase (Lipozyme, Novo Nordisk A/S, Denmark). Soybean oil and glycerol were purchased from Unikem A/S, Denmark. The oils had following specifications with regard to: (1) acid value, (2) peroxide value, and (3) iodine value; SLS: (1) 0.8 (2) 6.59 (3) 113; MLM: (1) 0.2 (2) 3.91 (3) 84; Soybean oil (LLL) (1) 0.1 (2) 0.36 (3) 133. Lipoid E80 was obtained from Lipoid A/G, Germany. Sodium pentobarbital was from Nycomed DAK, Denmark. Catheters (i.d. 0.58, o.d. 0.96 ref. 800-100-200-100) were from Astra Tech., Denmark. Soluene-350, Ultima Gold and Hionic Fluor were from Packard, The Netherlands. Propionitrile and methanol of HPLC grades were from RoMil, UK. All other chemicals were of a reagent grade.

Characterization of the Lipids

The hydrophobicity of the lipids was determined using high-performance liquid chromatography (HPLC) (6). Briefly, the HPLC system consisted of a solvent delivery system (Shimadzu, Japan), a rheodyne injection loop (loop volume 20 μl), a refractometer (SHOWA DENKO K.K., Japan), a chart recorder (Servogor, Austria) and a column oven (Waters, USA). HPLC of the lipids was done on a Spherisorb column ODS (500 × 4,6mm, 5μm particle size) at 27.5°C with a flow rate of 0.7 ml/min. Pure propionitrile or a mixture of propionitrile:-methanol (50:50) was used as the mobile phase. Standards contained 1.5 mg triglyceride dissolved in 5 μl toluene. The mean retention time was defined as the time where 50% of the HPLC profile was eluted.

Preparation and Characterization of the Radioactive Fat Emulsions

The overall composition of the emulsions was: 20% lipids, 1.2% Lipoid E 80, 2.1% glycerol and water to a final volume

Fig. 1. Schematic drawing of structured lipids (SLS and MLM) and a long-chain triglyceride (LLL), where S is short chain fatty acids (C_4), M is medium chain fatty acids (C_{8-10}), and L is long chain fatty acids (C_{16-18}).

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of 10 ml. The radioactive markers, ^{14}C-cholesteryl oleate and ^{3}H-triolein, were dissolved in the lipids (SLS oil, MLM oil or soybean oil, i.e., LLL oil) and heated to 75°C. Lipoid E 80 and glycerol was dispersed in ultrapure water and heated to 75°C prior to the addition of the oil phase. The two phases were emulsified using an Ultra-Turrax (J&K, Germany) for 10 min at 13,500 RPM. Water was added to a final volume of 10 ml. The premix was then treated in portions of 2.5 ml with an ultrasound probe (Branson, USA) equipped with a microtip for 10 min at a output value of 3 using icecold water for cooling. To assure thorough treatment the emulsion was shaken periodically. The pH was adjusted to 7.5 using 0.01 M sodium hydroxide. In order to obtain similar size and remove titanium particles the emulsions were centrifuged for 10 min at 700 xg (LLL), 85 xg (SLS), and 25 xg (MLM) employing a Biofuge A (Heraeus, Germany). An aliquot of emulsion per portion was removed and used for the *in vivo* experiments. To determine the final fat content of the emulsions, the radioactivity before and after centrifuging was assessed. The emulsions were stored under nitrogen.

To assure that the particles were of similar size, the particle size was determined in Milli Q water using a Zetasizer 4 (Malvern, UK). The emulsions were characterized by their zeta potential in 0.001 M phosphate buffer pH = 7.4 employing a Zetamaster (Malvern, UK).

Control of the Integrity of the Emulsions

The integrity of the radioactive emulsions was assessed by size exclusion chromatography. Briefly, the emulsion was incubated with an aliquot of 140mM NaCl, 10mM TRIS pH = 7.4 buffer (1:9) and 100 µl of the incubation mixture was loaded on a Sepharose CL-2B column (Bio-Rad), eluted with TRIS-NaCl buffer and fractions of 0.25 ml were collected. Each fraction was transferred to a scintillation vial and the scintillation cocktail was added.

Animal Experiments

Male Sprague-Dawley rats (Mollegaarden, Denmark) (370g ± 50g) were fasted overnight prior to the experiment, which adhere to the "Principles of Laboratory Animal Care". Anaesthesia was introduced by an intraperitoneal injection of sodium pentobarbital (50 mg/kg). A venous catheter was inserted near the junction of the left jugular and subclavian veins. The tip was advanced to lie in the superior vena cava. The catheter was maintained with 4% sterile citrate buffer, pH = 7.4 and without heparin (7). A bolus injection of 70 mg triglyceride/kg body weight of the radioactive emulsion was given into a lateral tail vein. Blood samples of 300 µl were withdrawn from the venous catheter after 3, 5, 8, 12, 15, 20, 25, and 30 min in heparinized Eppendorf tubes. Prior to sampling, the catheter was emptied of citrate buffer. Each blood sample was replaced by an equal volume of 0.15M NaCl. After the last sample, the animals were sacrificed by injecting an overdose of sodium pentobarbital. The liver, spleen, heart, lungs and muscle were rapidly removed and rinsed three times with ice-cold saline and blotted between paper-tissue. The organs were weighed and, if not further processed, the samples were immediately frozen.

Analytical Methods

The organs were minced and 2 ml of Soluene-350 was added per 100 mg organ and placed for 3–5 h at 50°C. The digested samples were decolorized by adding 0.3 ml of hydrogen peroxide and placed for 30 min at 50°C. Finally, 10 ml of Hionic-Fluor liquid scintillation cocktail was added. All samples were kept in the dark overnight before counting in a Minaxi scintillation counter (Packard, USA). The blood samples were centrifuged at 15,000 g for 10 min in a Heraeus Biofuge A. An aliquot of plasma was transferred to a scintillation vial and 10 ml of Ultima Gold liquid scintillation cocktail was added. The percent of injected dose in plasma was calculated assuming a total blood volume of 7.5% of the bodyweight (8) and a haematocrit of 48% (7).

RESULTS AND DISCUSSION

In order to document experimentally that the lipids are different with respect to hydrophobicity, their affinities for an RP-18 column were investigated. When using propionitrile as the mobile phase the LLL and MLM oils had a mean retention time of 25 min and 13 min, respectively, whereas the SLS oil was found in the solvent front. Using a mixture of propionitrile and methanol as the mobile phase the mean retention time of the LLL, MLM and SLS oils was >30 min, 19.5 min and 19 min, respectively. By employing two different mobile phases a rank order of their relative hydrophobicities could be given, with the LLL oil being the most hydrophobic and the SLS oil the least.

The physical integrity of the emulsions was examined by gel chromatography. Figure 2 shows that the two radioactive markers are co-eluting; hence, we have established the physical integrity of the emulsions. Furthermore, the coelution of the radioactive markers with the lipids was assessed by analysing each fraction with regard to the content of triglycerides employing an enzymatic kit (data not shown).

The data presented in Table I show that the zeta potential of the LLL emulsion was more negative than the zeta potential of the SLS and MLM emulsions. This may be explained by a higher content of free fatty acids in the LLL emulsion. However,

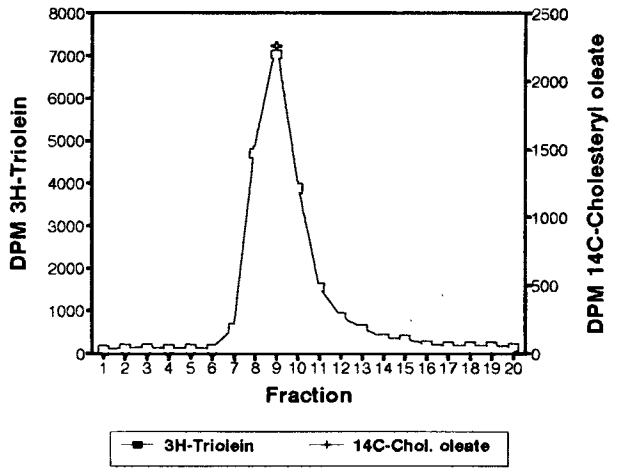

Fig. 2. Characterization of a representative emulsion (SLS) preparation by Sepharose CL-2B size exclusion chromatography. Elution profiles of the two radioactive markers, ^{14}C-cholesteryl oleate and ^{3}H-triolein.

Table I. Size, Polydispersity Index, and Zeta Potential (in 0.001 M Phosphate Buffer pH = 7.5) of LLL, MLM, and SLS Emulsions

Emulsion	size (nm ± s.d.)	polydispersity index	zeta potential (mV)
LLL	404 ± 26	0.42 ± 0.05	−57.2 ± 1.1
MLM	407 ± 35	0.43 ± 0.09	−28.1 ± 1.9
SLS	373 ± 11	0.31 ± 0.06	−33.9 ± 1,4

the acid value of the SLS oil (0.8) was slightly higher than both the MLM oil (0.2) and the LLL oil (0.1). The more negative zeta potential may therefore be explained by the fact that the free fatty acids of the LLL oil (C_{16-18}) have a lower solubility in water than those of both SLS and MLM oils (C_4 and C_{8-10}, respectively). Hence, the free fatty acids of the LLL oil (C_{16-18}) remain in the fat emulsion droplets being the main reason for a more negative zeta potential.

The elimination profiles of the radioactive marker, ^{3}H-triolein, show that the SLS emulsion has a longer half-life when compared to the MLM and LLL emulsions (Fig. 3). The elimination constants of SLS were found to differ significantly from those of LLL ($p < 0.001$) and MLM ($p < 0.01$) (Table II). When comparing the elimination constants of the MLM and LLL emulsions there was a statistically significant difference for the ^{3}H-triolein marker ($p < 0.01$), but no statistically significant difference for the ^{14}C-cholesteryl oleate marker. In another study also performed in fasted rats (9), there was no statistical significantly difference in the elimination of the MLM emulsion compared to the LLL emulsion.

It is well known that the core of triglyceride-rich particles in plasma is reduced in size through the action of lipoprotein lipases yielding remnants which are removed by the liver (10). Additionally, it has been speculated that the size of the fat droplets is an important regulatory factor in the clearance of fat emulsions. The size is the variable that determines the total interfacial area which is accessible to the lipoprotein lipases and, thus, determines the speed at which the triglyceride is hydrolyzed by the lipases (11). In the present study, the three emulsions were of similar particle size (Table I); hence, differences in *in vivo* elimination could not be ascribed to differences in particle size.

Recently, Deckelbaum et al. (12) have suggested that the surface concentration and mobility of the triglycerides at the surface of the phospholipid-stabilized emulsions might be of major importance for their hydrolysis by lipases. It was found in the case of MMM emulsions that there was a greater solubility of MMM at the emulsion-water interface and this increase in solubility was associated with a decrease in lipase affinities for MMM emulsions compared to that of LLL emulsions. In another *in vitro* study, it was shown that random structured lipids were hydrolyzed slightly slower than MMM and LLL (7).

It has been suggested that the elimination of fat emulsions droplets from plasma is a result of lipolysis as well as removal of the fat emulsion droplets by the reticuloendothelial system (13). In this study, we have employed two radioactive markers, ^{3}H-triolein, which is susceptible to the action of lipoprotein lipases and ^{14}C-cholesteryl oleate, which is not. Hence, the removal of ^{3}H-triolein represents the triglyceride metabolism,

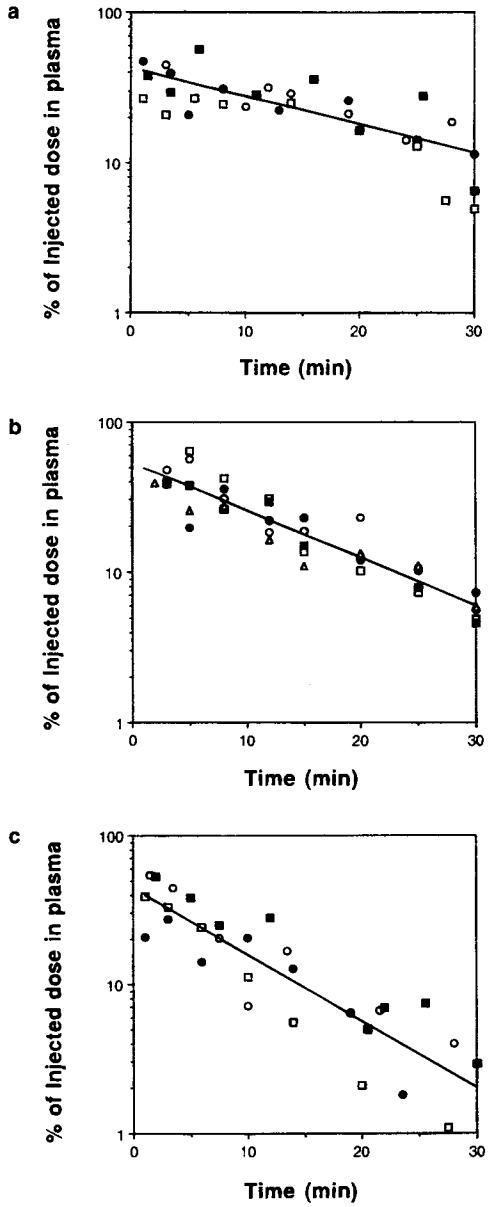

Fig. 3. Percentage of injected dose in plasma as a function of time of ^{3}H-triolein (a) SLS, n = 4, (b) MLM, n = 5, and (c) LLL, n = 4. Each type of symbol represents one rat.

Table II. Elimination Constants (k_e) of the Radioactive Markers

	^{3}H-triolein	^{14}C-cholesteryl oleate
LLL	0.103 ± 0.005[a]	0.086 ± 0.004[c]
MLM	0.072 ± 0.002[b]	0.071 ± 0.002[d]
SLS	0.043 ± 0.004	0.039 ± 0.003

[a] $P < 0.001$ versus the SLS ^{3}H elimination constant.
[b] $P < 0.01$.
[c] $P < 0.001$ versus the SLS ^{14}C elimination constant. (t-test).
[d] $P < 0.01$.

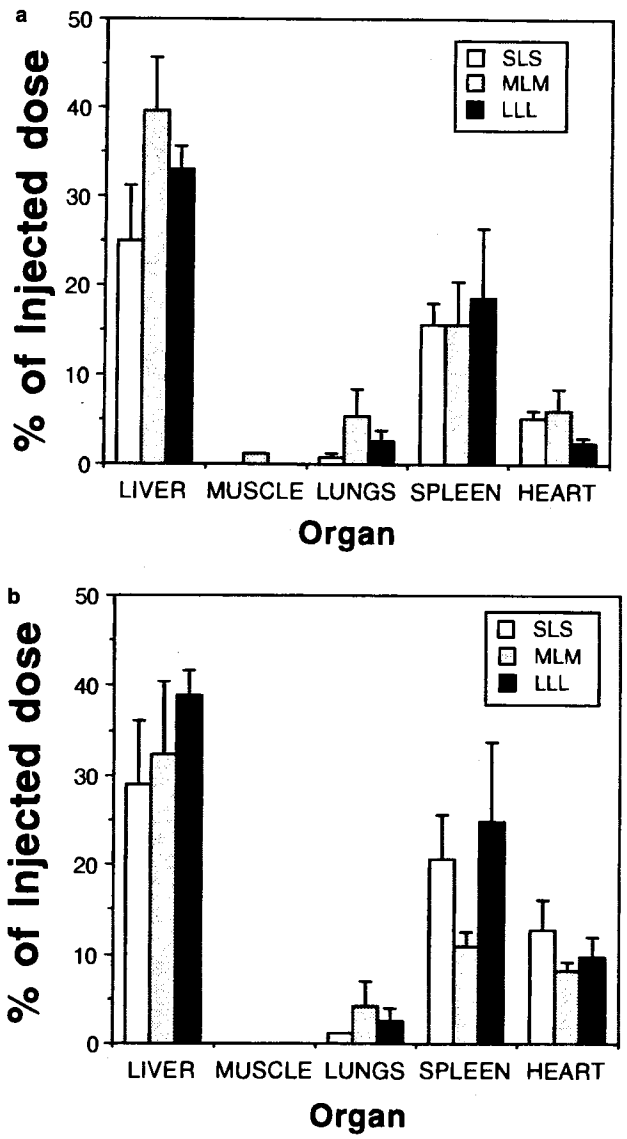

Fig. 4. Organ distribution of (a) ^{3}H-triolein and (b) ^{14}C-cholesteryl oleate.

whereas the other marker is a core marker of particle removal by organ uptake (9). We have speculated whether the difference in elimination rates for the two radioactive markers incorporated in the LLL emulsion, which is not observed for the elimination constants of the MLM and SLS emulsions, may reflect a higher affinity of the lipoprotein lipase for the LLL emulsion compared to the MLM and SLS emulsions and therefore a higher degree of lipolysis. Since the enzymatic hydrolysis of fat emulsions by lipoprotein lipases is mediated by the uptake of apolipoprotein CII and because even small differences in the composition of the interface of the fat droplets can have marked effects on the binding of lipases and related proteins (12), we are currently undertaking investigations to see if there are differences in the interaction of the fat droplets with various plasma components.

As seen with other particulate drug delivery systems (4, 5), the radioactive markers are mainly sequestered by the liver and spleen (Fig. 4). There is a tendency of the SLS emulsion to accumulate less radioactive marker in the liver compared to the MLM and LLL emulsions.

In conclusion, elimination of the SLS-emulsion was slower than that of the MLM and LLL emulsions, and the emulsions were eliminated from the circulation mainly by the liver. The significance of this slower elimination is low if the aim is a prolonged circulation time; however, by modifying surface properties employing block copolymer as shown by others (5), it may be possible to improve the circulation time. We believe that, with the differences in elimination rates and hydrophobicity shown in this report, structured lipids may have a potential as core material in fat emulsion-based drug delivery systems as well as increase the possibilities with regard to the incorporation of a wider range of lipophilic drugs within fat emulsions as compared to traditional emulsions.

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