Formulation of Polyiodinated Triglyceride Analogues in a Chylomicron Remnant-Like Liver-Selective Delivery Vehicle

Marc A. Longino,[1,2,4] Douglas A. Bakan,[2,3] Jamey P. Weichert,[1] and Raymond E. Counsell[2]

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Purpose. A formulation methodology for the incorporation of polyiodinated triglyceride (ITG) analogues into a protein-free chylomicron remnant-like emulsion was developed to provide a vehicle for the selective hepatic delivery of these agents for contrast-enhanced X-ray computed tomography (CECT).

Methods. Triglyceride emulsions (10% w/v) were prepared at various processing pressures, temperatures and times with a Microfluidizer® 110-S using different emulsion component proportions to establish processing and compositional parameters in order to afford stable ITG emulsions (ITG-LE) approaching 200 nm mean diameter.

Results. Preliminary data indicated that with a formulation composed of 2.4% dioleoyl PC with a cholesterol:DOPC mole ratio of 0.4 emulsified at 14,700 psi, 35°C for 10 min routinely afforded ITG-LE in the desired size range. The elimination of salt and amino acid from the bulk phase enhanced the stability of the ITG-LE. Incorporation of cholesterol into the monolayer was of critical importance in generating a stable emulsion near the targeted size, with a C:DOPC mole ratio of 0.4 producing a size minimum relative to higher or lower C:DOPC values.

Conclusions. The ITG analogues can be readily incorporated into stable remnant-like emulsions of relatively uniform particle size. Combination of the unique ITG contrast agent with the remnant-like delivery vehicle demonstrates a high degree of hepatic selectivity in biodistribution studies and offers significant potential for selective hepatic CECT.

KEY WORDS: polyiodinated triglyceride analogues; chylomicron remnant-like emulsion; liver-selective delivery vehicle; contrast-enhanced computed tomography; Microfluidizer®.

INTRODUCTION

Tissue- or cell-selective delivery of therapeutic, pharmacologic and diagnostic agents has the potential to improve the efficacy of numerous pharmaceutical agents. For parenteral administration, delivery vehicles that accommodate both the physicochemical characteristics of the specific agent and exploit uptake mechanisms of the target tissue have been developed. Liposomes, modified antibodies, polymeric complexes, nanoparticles and various forms of emulsions are among the many strategies employed to enhance targeted delivery (1). Synthetic lipid emulsions designed to mimic the transport and delivery properties of endogenous lipoprotein particles for the selective hepatic accumulation of lipophilic drugs have also been evaluated (2–4).

A number of stable polyiodinated triglyceride (ITG) analogues have been developed in our laboratory as potential contrast agents for X-ray computed tomography (CT) of the liver (5). For hepatic CECT, these compounds offer significant improvements in selectivity and tolerance at lower total iodine doses than the non-specific, water-soluble urographic agents in current clinical use. The success of these new lipophilic agents depends upon the ability to selectively deliver the ITG's to the liver in an emulsion vehicle which mimics endogenous chylomicron remnants, the class of lipoprotein particles responsible for providing the liver with significant amounts of dietary triglyceride on a daily basis (6).

Selected ITG analogues, along with sufficient carrier triglyceride, were incorporated into the core of emulsion particles stabilized by a surface monolayer of phospholipid and cholesterol to produce remnant-sized particles. The effects of changes in the ratios of emulsion surface components and emulsification conditions (temperature, pressure and duration) on emulsion formation and size were evaluated.

MATERIALS AND METHODS

Cholesterol (99+%), α-tocopherol (>97%) and L-phenylalanine were acquired from Aldrich (Milwaukee, WI). Triolein was purchased in sealed ampoules from Sigma (St. Louis, MO). The synthetic phospholipid, 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC, in $CHCl_3$ or C_2H_5OH) was obtained from Avanti Polar Lipids (Alabaster, AL). The iodinated triglyceride analogues 1,3-di-(3-[3-amino-2,4,6-triiodophenyl]-2-ethylpropionyl)-2-oleoyl-sn-glycerol (DIOG) and 1,3-di-(7-[3-amino-2,4,6-triiodophenyl]-heptanoyl)-2-oleoyl-sn-glycerol (DHOG) (Scheme 1) were synthesized, purified and characterized by literature methods (5). Chloroform and ethyl acetate were of analytical reagent grade. USP ethanol was purchased from Midwest Grain Products (Weston, MO). USP glycerol was purchased from J. T. Baker (Phillipsburg, NJ). Sterile water and sterile saline for injection were obtained from Abbott Laboratories (North Chicago, IL).

In a typical formulation, triolein and ITG (1:1 w/w, 10%w/v), cholesterol (0.27–0.7 mol/mol DOPC) and α-tocopherol (0.6% w/v) were weighed sequentially into a tared glass tube. DOPC (1.2%–3.6% w/v) in $CHCl_3$ or C_2H_5OH solution was transferred into the tube with a gas tight syringe (Hamilton, Reno NV). The tube was gently agitated to dissolve all the lipid components and the tube was rinsed down with $CHCl_3$ or EtOAc:C_2H_5OH (2:1, v/v). The tube containing the lipid mixture was connected to a Buchi rotary evaporator (Brinkmann, Westbury, NY) and solvents were evaporated at 40°C under vacuum. After completion of solvent removal the tube was stored under nitrogen at 8°C overnight.

The tube was warmed to 37°C and reconnected to a vacuum line for 1 h to remove the final traces of organic solvent. USP glycerol (500 mg) was added to the lipid mixture and emulsified under nitrogen with a Polytron homogenizer (Model PT 10/35,

[1] Department of Radiology, University of Michigan, 1514 MSRB-I, Ann Arbor, Michigan 48109-0662.
[2] Department of Pharmacology, University of Michigan, Ann Arbor, Michigan 48109.
[3] Current address: Molecular Biosystems, Inc., 10030 Barnes Canyon Road, San Diego, California 92121.
[4] To whom correspondence should be addressed.

ABBREVIATIONS: ITG: polyiodinated triglycerides; ITG-LE: ITG lipid emulsion; CECT: contrast-enhanced X-ray computed tomography; PCS: photon correlation spectroscopy.

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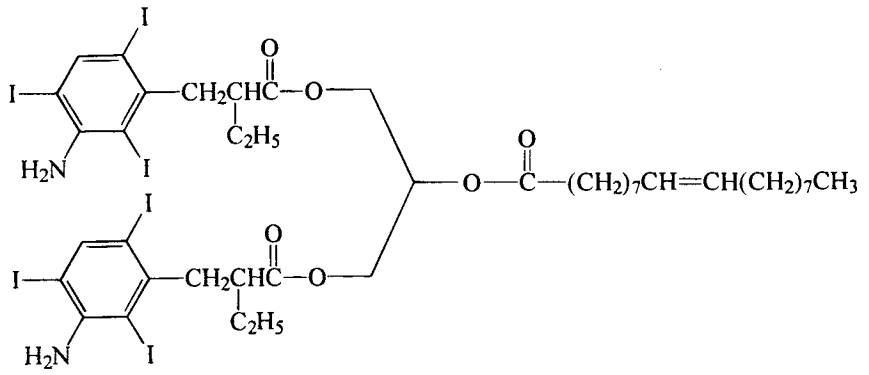
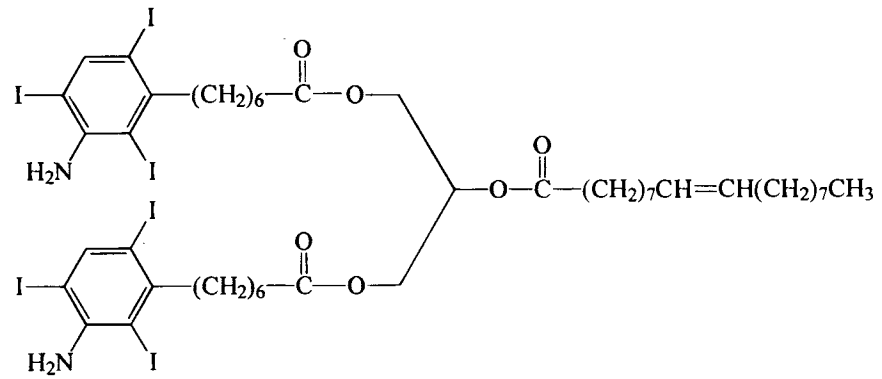


DIOG



DHOG

Scheme 1. Structures of the polyiodinated triglyceride analogues 1,3-di-(3-[3-amino-2,4,6-triiodophenyl]-2-ethylpropionyl)-2-oleoyl-sn-glycerol, DIOG, and 1,3-di-(7-[3-amino-2,4,6-triiodophenyl]heptanoyl)-2-oleoyl-sn-glycerol, DHOG.

PTA 10 generator, Kinematica GmbH, Kriens-Luzern, Switzerland) at approximately 12,500 rpm for 5 min at <55°C. A crude oil in water emulsion (o/w) was generated by the addition of a 5–6 ml aliquot of sterile saline or water and emulsification at 25,000 rpm under identical conditions for 5 min. The final volume was adjusted to 10 ml with additional bulk phase.

The rough emulsion was transferred to a Microfluidizer® (Model 110-S, Microfluidics Corp., Newton, MA) for emulsification at 14,700 psi (pressure amplification factor 140) for 10–30 min between 24.6–74°C utilizing the manufacturer's accessory cooling coil immersed in a Lauda MS-6 constant temperature bath (Brinkmann Instruments, Westbury, NY). The resulting emulsion was removed from the Microfluidizer® and passed sequentially through sterile 0.45 μm and 0.20 μm Acrodisc filter units (Gelman Sciences, Ann Arbor, MI) into a multidose vial (Miles Inc., Spokane, WA). The emulsions were stored overnight at room temperature before particle size analysis using laser photon correlation spectroscopy (Nicomp 370 particle sizer, Nicomp Particle Sizing Systems, Santa Barbara, CA).

RESULTS

Early attempts to prepare ITG emulsions by ultrasonication failed to provide reproducible emulsions free from liposomes. Therefore, generation of emulsions was achieved by the sequential use of a Polytron and a Model 110-S Microfluidizer®. The emulsion bulk phase initially consisted of 5% glycerol in sterile 0.15 M saline with 0.1% L-phenylalanine as a stabilizer. However, replacement of the L-phenylalanine-saline solution with sterile water resulted in an improved emulsion.

In order to optimize the emulsion vehicle for selective hepatic delivery of the ITG's, it was necessary to evaluate the effects of changes in processing conditions and compositional variations on emulsion size and stability. Thus, alterations of processing time, pressure and temperature as well as changes in the content of phosphatidylcholine and cholesterol were evaluated without and with ITG in the lipophilic core.

The effect of Microfluidizer® processing duration on the mean particle size of a 10% triolein emulsion with 2.4% DOPC was evaluated by PCS particle sizing of emulsion aliquots (50 μl) removed at 5 min intervals up to 30 min total processing.

The 30 min emulsion was sampled before and after sterile filtration through 0.45 μm and 0.20 μm filters. The PCS sizing data indicated that the mean particle diameter of <300 nm did not change significantly beyond 5 minutes of Microfluidizer® emulsification, although, the size range became more uniform about the mean. Thus, a processing time of 10 min was selected as the time necessary to yield an acceptably uniform emulsion.

Microfluidizer® interaction chamber processing pressure significantly influences the size of the resulting emulsion. Therefore, the effect of processing pressure variations between 6,300 psi and 18,200 psi on mean particle diameter was examined. A 10 μl aliquot of a 10% TO-LE was removed from the Microfluidizer® for size analysis before each increase in operating pressure. Processing pressure was incremented 2,800 psi for the first four steps, but only 700 psi for the final step, as the upper operating limit of the 110-S had been reached. Mean particle size declined from 318 nm at 6,300 psi to 274 nm at 11,900 psi processing pressure. No further decreases in mean emulsion size were observed as pressures increased from 14,700 psi to 18,200 psi (274–279 nm). All emulsions were subsequently prepared at 14,700 psi.

The effect of change of surfactant quantity on mean emulsion particle diameter was examined by varying the DOPC concentration between 1.2% and 3.6% w/v in 10% TO-LE prepared at 14,700 psi, 55°C for 10 min. The results indicate that 3.6% DOPC afforded the smallest mean emulsion diameter, although the size change between 2.4% and 3.6% DOPC was relatively small (~50 nm) compared to the nearly 2-fold reduction in emulsion diameter observed with an increase from 1.2% to 2.4% DOPC (Fig. 1). Negative stain transmission electron microscopy of the 3.6% DOPC emulsion showed major contamination by liposomes which were not observed in the 2.4% DOPC formulation (data not shown).

Attempts to reduce DOPC to 1.2% w/v without reducing the cholesterol content accordingly, resulted in the formation of a white paste during Polytron emulsification. The creamy mixture was unsuitable for processing in the Microfluidizer®.

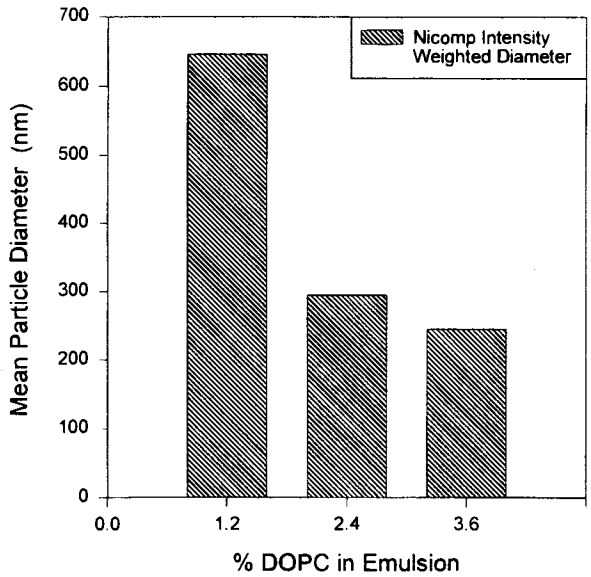


Fig. 1. The effect of increasing DOPC concentration (% w/v) on the mean particle size of 10% triolein-DOPC emulsions.

Examination of the molar ratio of cholesterol to DOPC in these formulations revealed that the 3.6% and 2.4% DOPC compositions had ratios below 0.7 while the 1.2% prep had a ratio of 1.35. Reduction in cholesterol content to a C:DOPC ratio of 0.67 afforded a stable 1.2% DOPC emulsion. Stable emulsions were also prepared at 1.2% and 2.4% DOPC with a C:DOPC molar ratio of 0.5, however, at a molar ratio of 1.0 the 2.4% DOPC preparation produced a similar white cream.

A series of 10% triolein emulsions containing 2.4% DOPC were prepared with variation of the C:DOPC molar ratio between 0.3–0.7. PCS analysis of the resulting emulsions revealed that an emulsion size minimum was reached at a C:DOPC ratio of between 0.4–0.5 in 5% glycerol/saline/phenylalanine bulk phase. The substitution of sterile water for the L-phenylalanine-saline solution yielded an emulsion at C:DOPC of 0.4 that was even smaller than the corresponding L-phenylalanine-saline preparation (Fig. 2). Additional sterile water formulations verified the reproducible production of a smaller, more stable emulsion.

In order to assess the effect of temperature on emulsion particle diameter, the temperature of the emulsion mixture was regulated by immersing the Microfluidizer® cooling coil in a thermostated water bath. Temperatures of 25°C, 35°C, 55°C and 74°C were evaluated for the preparation of 10% TO emulsions at 14,700 psi for 10 min. The individual emulsions were sized by PCS after overnight equilibration at room temperature. The data demonstrated that the 35°C preparation had the smallest mean diameter, 174.6 nm, while the emulsions prepared at the other temperatures sized between 192–236 nm. This observation was supported by the sizing results for subsequent 10% TO emulsions prepared at 35°C.

Preliminary attempts to incorporate an ITG in a 10% emulsion with 2.4% DOPC, C:DOPC = 0.0–0.4, and 0.6% α-tocoph-

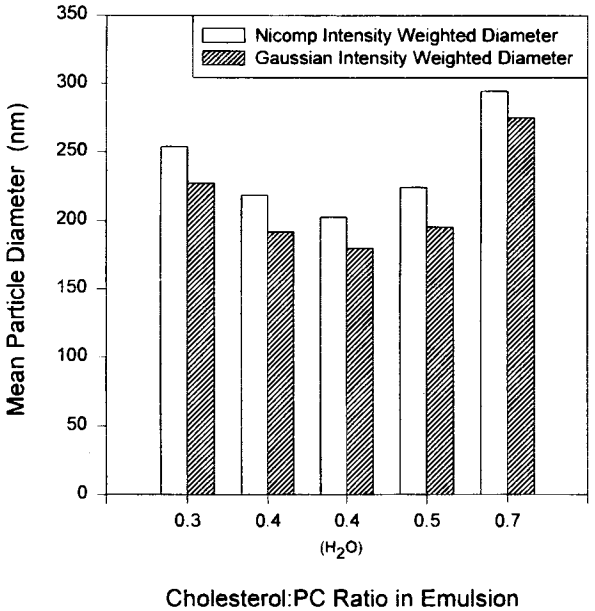


Fig. 2. The influence of cholesterol:DOPC mole ratio on mean particle diameter of 10% triolein-DOPC emulsions. The bar graph for 0.4 (H_2O) represents the mean diameter of an emulsion prepared in 5% glycerol-sterile water rather than 5% glycerol in a solution of L-phenylalanine-sterile saline.

erol as an antioxidant were performed with DIOG at 14,700 psi and 35°C. While the mean particle diameter of these emulsions was approximately 270 nm, emulsions prepared by substituting saline for sterile water yielded formulations with a mean diameter in excess of 300 nm. Although these preparations were useful in demonstrating that an ITG emulsion could be generated under these conditions, DIOG is a viscous oil at 37°C and is very slowly eliminated from the body. Thus, the more fluid ITG analogue, DHOG, was used in subsequent studies.

DHOG-TO emulsions (DHOG:TO, 1:1, w/w) were prepared as before in 2.4% DOPC without cholesterol or at C:DOPC ratios of 0.1, 0.4, 0.6 and 0.8. The most stable and uniform ITG-LE size was observed for the 0.4 C:DOPC formulation (Fig. 3). Emulsions with a C:DOPC of 0.6 displayed reduced stability to room temperature storage compared to the 0.4 C:DOPC formulations. Attempts to prepare DHOG-TO emulsions at C:DOPC > 0.7 resulted in the formation of mixtures unsuitable for final emulsification.

Substitution of ethyl acetate-ethanol (2:1, v/v) for chloroform to dissolve the lipid components proved to be a useful change, reducing evaporation time and eliminating potential contamination of the emulsion with residual chloroform.

DISCUSSION

The selective delivery of iodinated contrast agents to the liver has been actively pursued since the introduction of X-ray body computed tomography nearly two decades ago. In order to overcome the inefficiencies of urographic contrast agents for hepatic CT, researchers have attempted to encapsulate these water-soluble contrast agents in liposomes (7) or have prepared emulsions of iodinated aliphatic esters such as EOE-13 (8). These preparations are rapidly sequestered by hepatic Kupffer cells and splenic macrophages. Product stability, compound stability in the case of the iodinated aliphatic esters and the inability to sterilize the final formulation have precluded the successful application of any of these preparations to hepatic-selective CECT(8). More advanced liposomal preparations are currently under investigation, although these formulations remain RES-targeted (7).

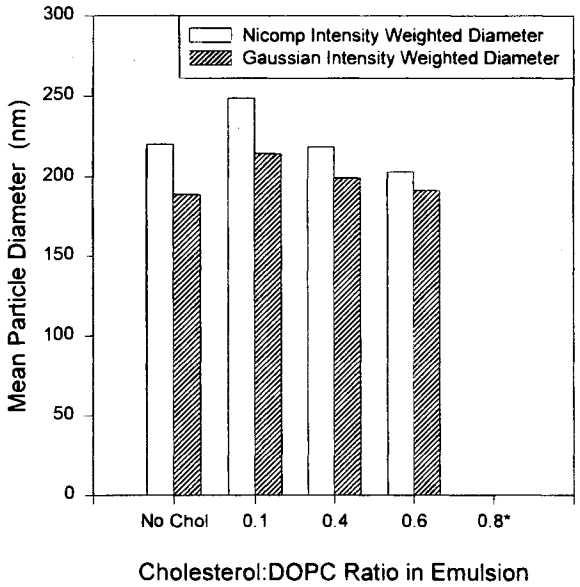


Fig. 3. The effect of cholesterol:DOPC mole ratio on the mean particle size of 10% DHOG-TO-DOPC lipid emulsions.

Ivancev and coworkers reported the preparation of a small, well-defined contrast emulsion, Intraiodol, which contains the iodinated aliphatic ester mixture, Lipiodol, emulsified with egg lecithin (9). These elegant studies demonstrated the importance of emulsion size in realizing transport to and uptake of the emulsion by hepatocytes. However, Intraiodol contains alkyl iodide species which are known to undergo facile deiodination *in vitro* and *in vivo* and thus required inclusion of an amino acid additive to stabilize the emulsion. The ITG analogues developed in our laboratory offer the increased stability of aryl iodides over alkyl iodides as well as the defined nature of the ITG relative to an undefined iodinated oil mixture found in Intraiodol or EOE-13 (5,9–11).

The potential application of lipoproteins and lipoprotein-like delivery vehicles for the transport and selective delivery of lipophilic drugs or diagnostic agents has been thoroughly reviewed (12,13). The ITG's were designed to exploit selective delivery to hepatocytes in a chylomicron remnant-like emulsion. This high capacity delivery system should permit sufficient hepatic accumulation of ITG for CECT.

Although production of CM remnant-like emulsions by ultrasonication is used routinely by Redgrave, et al., the procedure requires isolation of the remnant-like emulsion by ultracentrifugation (14). Although acceptable for enzyme, transport and uptake studies, the preparation of emulsions for parenteral administration is impractical by this method. The preparation of a parenteral feeding emulsion with a Microfluidizer® was reported by Washington and Davis (15). ITG-containing emulsions have been routinely prepared in our laboratory by preliminary emulsification coupled with final processing in a Model 110-S Microfluidizer®.

Optimization of emulsification conditions and emulsion composition was carried out with triolein as the sole core component prior to incorporation of the ITG analogues into the emulsion. This procedure permitted the incorporation of 10% (w/v) ITG:TO into the core of an emulsion stabilized by a DOPC (2.4%, w/v) monolayer containing cholesterol at a mole ratio of 0.4 relative to DOPC. The final emulsification is performed at 14,700 psi for 10 min at 35°C in a Microfluidizer®. Following sterile filtration through 0.45 μm and 0.2 μm membrane filters, the emulsions routinely displayed mean diameters in the range of 200 nm ± 50 nm compared to the TO emulsion sizes of 170 nm ± 30 nm. Inclusion of cholesterol in the phospholipid monolayer resulted in a more stable and smaller emulsion in agreement with literature observations that cholesterol enhances the organization of phospholipids in membranes resulting in closer packing and greater stability (16,17).

The increased fluidity of DIOG and DHOG at 37°C relative to their saturated aliphatic analogues enhanced the ease of emulsification. Biodistribution studies in female rats with ^{125}I-labeled DIOG and DHOG emulsions demonstrated that hepatic uptake at 30 min post-injection was similar. However, by 24 hours the hepatic DHOG levels had declined to less than 10% dose/organ while the DIOG levels remained essentially unchanged from the 30 min level (5). Preliminary metabolic studies are highly suggestive that hepatobiliary excretion is the primary route of elimination for DHOG-TO emulsion.

CONCLUSIONS

This study demonstrates the feasibility of incorporating a polyiodinated triglyceride analogue into the neutral lipid core of a chylomicron remnant-like lipid emulsion. The inclusion of cholesterol in the phospholipid monolayer produced smaller, more stable emulsion particles than in the absence of cholesterol. The addition of α-tocopherol to the emulsion was compatible with emulsification and afforded protection to the other lipid components. Processing pressure, duration and temperature were optimized for preparing 10% ITG-TO emulsions with a Model 110-S Microfluidizer®. Previously reported biodistribution studies clearly demonstrate that the combination of the unique ITG contrast agents with a remnant-like delivery vehicle offers a high degree of hepatic selectivity. This ITG selectively targeted with this remnant-like delivery vehicle has significant potential for providing both anatomical and functional information in liver-selective CECT imaging.

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