Comparison of the Lymphatic Transport of Halofantrine Administered in Disperse Systems Containing Three Different Unsaturated Fatty Acids

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Purpose. To compare the influence of the degree of fatty acid unsaturation (oleic [C18:1], linoleic [C18:2], or linolenic acid [C18:3]), with the intestinal lymphatic transport of halofantrine free base from disperse systems in anesthetized rats.
Methods. The mesenteric lymph duct was cannulated in anesthetized rats. Lipid vehicle containing halofantrine was administered by intraduodenal infusion. The concentration of halofantrine in blood and lymph samples was analyzed.
Results. The rank order of the lymphatic transport of halofantrine was C18:2 > C18:1 > C18:3. Comparison of the area under the curve (AUC) from the three fatty acids showed no statistically significant differences between the AUCs from the lymph cannulated rats. In terms of rank order effects, the plasma concentrations of halofantrine were highest for the rats dosed C18:2 followed by C18:3 and C18:1.
Conclusions. Using C18:2 as a vehicle increased the lymphatic transport of halofantrine 16.6-fold over that observed for the system containing C18:3. The extent of lymphatic transport for the C18:1 system did not differ from the other two formulations, but the combined lymph and plasma data indicated that the C18:2 was the most suitable lipid vehicle for the oral delivery of halofantrine.

KEY WORDS: lymphatic transport; lipid; fatty acid; unsaturation; halofantrine.

INTRODUCTION

For most orally administered drugs, the absorption via the portal blood is the major contributor to the bioavailability. However, for some very lipophilic compounds, including dietary lipids, cholesterol, and lipid soluble vitamins, the major way of absorption is transport by the intestinal lymphatic system. Lipophilic chemical compounds such as DDT (1–5), halofantrine (6,7), mepitionstan (8,9), ontazolast (10), penclomedine (11), and MK-386 (12) are all examples of xenobiotics transported by the intestinal lymphatic system in an amount that contributes significantly to bioavailability. The pharmaceutical advantages associated with intestinal lymphatic transport comprise the avoidance of hepatic first-pass metabolism and the potential to selectively target drugs to the lymphatic system.

The physicochemical properties of the compound have been shown to affect the total extent of lymphatic transport. Charman and Stella (4) have suggested that the compound must have a high partition coefficient (log $P > 5$) and a high solubility in the triglyceride lipid (solubility > 50 mg/ml) for transport via the intestinal lymphatic to be significant.

Generally, compounds transported by the intestinal lymph are absorbed in association with the lipid core of lipoproteins (13), and as such require coadministered lipid to stimulate lipoprotein formation. Short- and medium-chain fatty acids are transported to the systemic circulation by the portal blood and are not incorporated to a greater extent in chylomicrons (14), whereas long-chain fatty acids and monoglycerides are re-esterified within the intestinal cell after digestion and absorption. The triglycerides are thereafter incorporated into chylomicrons and secreted from the intestinal cell by exocytosis into the lymph vessels. Consequently, the long-chain triglycerides are most suitable for enhancement of lymphatic transport of drugs, as confirmed by Palin and Wilson (15) and Noguchi *et al.* (16).

Cheema *et al.* (17) examined the rate and output of chylomicrons, very low density lipoprotein (VLDL) and low density lipoprotein (LDL) in the mesenteric lymph of anesthetized rats after intraduodenal administration of either oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), or arachis oil. It was found that with each increase in the degree of fatty acid unsaturation, the lag-time in the chylomicron formation was reduced, compared with NaCl dosed rats. It probably reflects the more rapid rate of absorption with increasing degree of unsaturation. The arachis oil gave the longest lag-time, which reflects the need for hydrolysis of the triglyceride before absorption. At steady-state lymph chylomicron concentration, linoleic acid produced the highest concentration followed by linolenic and then oleic acid. This led Cheema and co-workers to suggest that linoleic acid was the most suitable of the investigated lipid vehicles for delivery of drugs to the lymphatic system, but further investigations of this have to our knowledge never been published. However, a direct extrapolation between trends in transport data of administered lipids of varying degrees of unsaturation, and the likely effect lipids would have on the transport of coadministered lipophilic drugs (18) may not necessarily be expected. The objective of this study was therefore to investigate whether this positive effect in chylomicron transport after administration of the different unsaturated free fatty acids also has an influence on the lymphatic transport of halofantrine free base, which previously has been shown to be transported by the intestinal lymphatic system (6,7).

MATERIALS AND METHODS

Materials

Halofantrine hydrochloride and the internal standard 2,4-dichloro-6-trifluromethyl-9{1-[2-(dibutylamino) ethyl]}phenathrenemethanol hydrocloride were donated by SmithKline Beecham Pharmaceuticals (Worthing, UK). Oleic acid 99% (C18:1, n-9) and linoleic acid 99% (C18:2, n-9,12) were obtained from Sigma Chemicals (St. Louis, MO); linolenic acid 99% (C18:3, n-9,12,15) was obtained from Larodan Fine Chemicals (Malmö, Sweden); and Tween 80 was obtained from AppliChem (Darmstadt, Germany). All free fatty

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acids and the emulsifier were used without further purification.

Acetonitrile, tert-butylmethylether, and dichloromethane were high-performance liquid chromatography (HPLC) grade, and sodium dodecyl sulphate was electrophoresis grade. The water used in all experiments was obtained from a Milli-Q-water purification system (Milipore, Molsheim, France). All other chemicals were analytical reagent grade.

Male Sprague-Dawley rats (280–320 g) were purchased from M&B (Lille Skendsved, Denmark) and maintained on standard food and water *ad libitium* in our laboratory for at least 1 week prior to entering the experiment.

Preparation of Halofantrine Free Base

The amorphous form of halofantrine free base was prepared from the halofantrine hydrochloride salt as described previously by Porter *et al.* (6), to increase the solubility in the lipid vehicle.

Solubility

A glass centrifuge tube with a Teflon-lined cap containing halofantrine free base and free fatty acid in nitrogen atmosphere was placed on a shaking water bath, maintained at 25°C for 3 days. Each vial was then centrifuged at 1,000 g for 10 min, and a sample of the fatty acid was taken, diluted with acetonitrile, and analyzed by a HPLC with ultraviolet detection (19). The day after, another sample was taken, to ensure equilibrium was reached. The evaluation of the amorphous form of halofantrine solubility in each of the three fatty acids was performed in duplicate.

The HPLC used for analysis included a Hewlett Packard 1100 system with a Hewlett Packard DAD detector. The separation of halofantrine from the biological matrix was accomplished by using a reversed-phase Luna C_8 column (4.6 × 250 mm 5 μm ODS(2)), with a Phenomenex C8, ODS 4 × 3.0 mm guard column. The data were analyzed using Hewlett Packard ChemStation software for LC (Hewlett Packard, Waldbroom, Germany).

Halofantrine Formulations

The concentration of halofantrine in the formulations was determined by HPLC (19), and the formulations were visually inspected for physical changes.

Disperse System

The systems were prepared by slowly introducing an 8% (w/v) Tween 80 in saline under rapid stirring to the free fatty acid and halofantrine free base. Subsequently, this system was diluted with a 0.9% saline solution. This gave a disperse system containing 2 mg halofantrine base and 200 μl lipid in the final dose volume of 3.0 ml, stabilized with 2% Tween 80 (w/v). The micellar systems were prepared maximum 24 h prior to dosing, due to possible interaction between the hydroxy-group on halofantrine and the carboxylic acid on the free fatty acids.

Intravenous Formulation

Halofantrine was incorporated into an emulsion by using the method of El-sayed and Repta (20). Halofantrine free base was dissolved in dimethylformamide (125 mg/ml). In aseptic conditions, 0.25 ml of this solution was then slowly added to 20 ml of a rapidly stirred Intralipid® 10% emulsion (containing 10% soybean oil, 1.2% lecithin, and 2.25% glycerol in water). The formulation was thereafter filtered, in aseptic conditions, through first a 0.45 μm filter, and then through a 0.22 μm filter into a sterilized glass bottle with a rubber membrane and a crimp lid.

Surgical Procedures

All surgical and experimental procedures were reviewed and approved by the Danish Animal Experimentation Ethics Committee, and the study complied with the NIH Guide for the Care and Use of Laboratory Animals. All rats used in the experiments were fasted 24 h prior to each experiment with free access to water. The animals were anesthetized for the duration of the experiment with intraperitoneal 50 mg/kg sodium pentobarbitone. Additional injections were given as needed.

The mesenteric lymph duct was cannulated using a slight modified method as previously described by Noguchi *et al.* (2) with polyethylene tubing (PE50, Clay Adams, NJ). Any auxiliary lymph ducts to the right of the mesenteric artery were cut to ensure all lymph flow was into the main mesenteric duct. The lymph cannula was secured with cyanoacrylate adhesive (B. Braun, Germany) and the auxiliary lymph duct was sealed with cyanoacrylate adhesive.

The duodenum was cannulated approximately 2 cm below the pylorus with a piece of polyethylene tubing (PE50) and secured with instant cyanoacrylate adhesive. The intraperitonal cannula was inserted between sutures after closing the animal's abdominal muscle and skin layer.

Tracheotomy was performed with a 3 cm piece of polyethylene tubing (7445-PE 205, Clay Adams, NJ) to ensure patency of the airways during the experimental procedures, and the jugular vein was cannulated with polyethylene tubing (PE50). The cannula was placed at the level of the right atrium.

Experimental Procedures

After completion of the surgical procedures, the animals were transferred to a heated pad maintained at 37°C (Fine Science Tools, Heideberg, Germany). A continuous intraduodenal infusion of saline at a rate of 1.5 ml/h was initiated via a constant rate pump (Ismatic, Switzerland) to maintain hydration and intestinal lymph flow. The animals were stabilized for 3 h before dosing the formulations. The animals were randomly assigned to receive one of the disperse systems. The animals received 3.0 ml of the disperse system in the duodenum at a rate of 1.5 ml/h for 2 h. Thereafter, the infusion of normal saline was continued at the same rate.

In all the experiments, including both the animals dosed intraduodenal and intravenous, the lymph was continuously collected into a 5 ml blood collection tube containing 100 μl EDTA solution and changed 1, 2, 3, 4, 5, 6, 8, 10, and 12 h after the start of drug administration. The lymph fractions were stored at –20°C after collection until further processing.

Blood samples (0.25 ml) were taken at –5 min, and 2, 4, 6, 8, 10, and 12 h after the start of drug administration into a blood collecting tube containing 20 μL of 200 units/ml hepa-

rin solution. Plasma was immediately harvested by centrifugation.

Rats receiving the intravenous halofantrine formulation were dosed with 0.3 ml of the emulsion (containing 0.5 mg of halofantrine base) as a bolus injection via the right jugular vein. Rehydration via the intraduodenal cannula was maintained during and after dosing. Blood samples (0.1 ml) were taken at −5, 10, 20, 60, and 120 min, and 0.25 ml blood samples were taken at 4, 6, 8, 10, and 12 h postdosing.

At the end of the experiment, the animals were sacrificed by an overdose of sodium pentobarbitone given in the jugular catheter, and the integrity of the lymphatic and duodenal cannulas was verified.

Analysis of Halofantrine in Lymph and Blood

From the lymph samples, 100 μl of lymph was added to 1 ml acetonitrile and 100 μL IS (2 μg/ml in acetonitrile). The sample was vortexed for 2 min and the insoluble protein-based components were removed by centrifugation for 10 min. The supernatant was removed and 20 μl was injected and analyzed by HPLC. Standard curves were linear in the spiked lymph concentration range 40 to 6000 ng/ml (r^2 >0.99). The average recoveries (mean ± SD, $n = 12$) for halofantrine were 96.2 ± 2.0% across the concentration range.

The blood samples were analyzed by a validated HPLC method previously described by Humberstone *et al.* (19).

Analysis of Lymph Triglyceride

Lymph triglyceride concentrations were determined by an enzymatic colorimetric method (Boehringer Mannheim, Mannheim, Germany; cat. no. 701912) and assayed on a Cary 1Bio Uv-vis spectrophotometrer (Varian Instruments, Mulgrave, Victoria, Australia).

Lymphatic triglyceride transport due to exogenously administered lipid was determined by subtracting the endogenous lipid component from the mass of triglyceride lipid determined in each collected lymph sample. Endogenous lymphatic transport, which was zero order, was calculated to be 1.22 ± 0.38 mg/h determined from the slope of the regression of mean cumulative lymphatic triglyceride transport measured over 12 h ($r^2 = 0.9988$) in six rats.

Pharmacokinetic Analysis

Plasma concentration-time data for halofantrine in individual rats were analyzed by noncompartment estimations using the WinNonlin software version 2.1. The area under the curve (AUC) for halofantrine after intravenous administration was determined using a two-compartment model in WinNonlin, and the area for the last measured point to infinity was calculated by the linear trapezoidal rule.

Statistical Analysis

Statistical analysis was performed by one-way analysis of variance using Statgraphics version 7.0, and the Student-Newman-Keuls multiple comparison test was applied for analyzing potential difference between the formulations. The results were considered significant if $P < 0.05$.

RESULTS AND DISCUSSION

Solubility

The solubility of amorphous halofantrine free base (mean ± SD, $n = 3$) in the three different free fatty acids was found to be 305 ± 11 mg/ml (oleic acid), 311 ± 4 mg/ml (linoleic acid), and 282 ± 7 mg/ml (linolenic acid). These values were considered equal and, therefore, the solubility of halofantrine in the re-esterified triglycerides was also assumed to be equal. Difference in lymphatic transport was therefore expected not be ascribed due to difference in solubility.

Intestinal Lymphatic Transport of Halofantrine and Triglyceride

The results of the lymphatic transport of halofantrine free base in lymph cannulated rats, plotted as the cumulative percentage of the administered dose, are shown in Fig. 1. The rank order trend of halofantrine transported was linoleic > oleic > linolenic acid. Statistically, linoleic acid gave a significant higher ($p < 0.05$) cumulative amount of halofantrine free base transported in the intestinal lymphatics compared with linolenic acid. There was no significant difference between the oleic acid vehicle and the two other vehicles, but a trend toward a higher lymphatic transport compared with linolenic acid appears. Porter and co-workers (6) examined the lymphatic transport of halofantrine after intraduodenal administration to anesthetized rats in an emulsion or a micellar system comprising of 50 μl oleic acid/glyceryl monoolein (2:1 w/w) with either 0.2% (v/v) or 4% (w/v) Tween 80. The lymphatic recovery of the halofantrine dose was found to be 11.8 ± 1.7% and 17.2 ± 2.7, respectively, which led the authors to conclude that lymphatic transport of drugs may be improved by employing disperse lipid systems. In this study 2% (w/v) Tween 80 was used to solubilize 200 μl free fatty acids, which makes Porter and co-workers' 0.2% (v/v) Tween 80 formulation the most relevant for comparison. This implies that there is no difference in the lymphatic transport of halofantrine in the two studies, and thereby indicates that there is no influence from the dosed monoglycerides. However, Char-

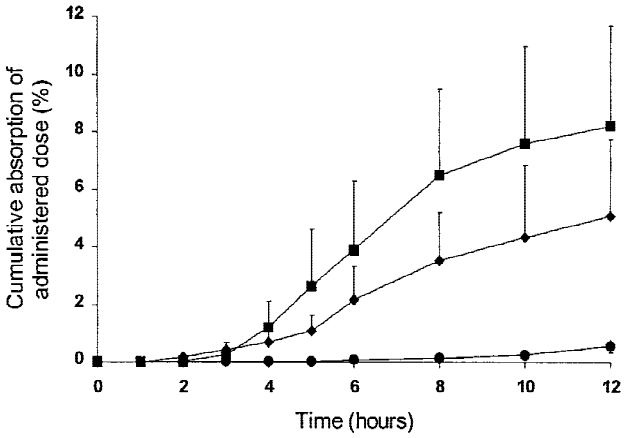

Fig. 1. The cumulative appearance of halofantrine (mean ± SE) in mesenteric lymph as a function of time after intraduodenal administration of 2 mg halofantrine in micellar systems stabilized with 2% (w/v) Tween 80 in anesthetized rats. The formulations contained oleic acid ($n = 5$, ◆), linoleic acid ($n = 7$, ■), and linolenic acid ($n = 7$, ●).

man and Stella (3) found a trend toward a higher lymphatic transport of DDT when dosed intraduodenal to anesthetized rats in a oleic acid vehicle compared with a oleic acid/glycerol monoolein (2:1 w/w) vehicle containing equal amounts of Tween 80.

The cumulative transport of triglycerides into the mesenteric lymph after intraduodenal dosing of the three lipid vehicles is shown in Fig. 2. A clear difference in the mean values of the cumulative triglyceride transport after 12 h among the three formulations exists, but no significant difference was found. The amount of triglyceride found in the lymph before endogenous correction was at the same level as previously reported by Porter *et al.* (6), but a higher lipid dosed was administration in this study. Why the higher lipid input was not reflected in the lymph triglyceride output when dosed as free fatty acids was not addressed in this study, and needs further investigation. Linolenic acid induced a smaller mass of triglycerides transported through the lymph than the two other examined fatty acids. The lower absorption of linolenic acid was also found in a recent study by Porsgaard and Høy (21), who examined the intestinal absorption of rapeseed oil with a low and a high content of linolenic acid. Administration of low linolenic rapeseed oil led to higher values of triglyceride transport (63.3 and 97.7% at 8 and 24 h, respectively) than high linolenic rapeseed oil (40.7 and 78.4%), but at what biochemical step in the lipid processing the differences arose was not addressed.

The cumulative halofantrine versus cumulative triglyceride transport into mesenteric lymph was linear correlated (Fig. 3), as previously indicated by Hauss and co-workers (10), showing that the efficiency by which the free fatty acid promotes lymphatic triglyceride transport affects the transport of drug into the lymph. The slope of the lines from the three fatty acids shows a pronounced difference in the amount of halofantrine transported in the mesenteric lymph per milligram triglyceride for linolenic acid, compared with oleic and linoleic acid. The lower proportion of halofantrine transported into the lymph when dosed with linolenic acid relative to the animals dosed with oleic and linoleic acid was

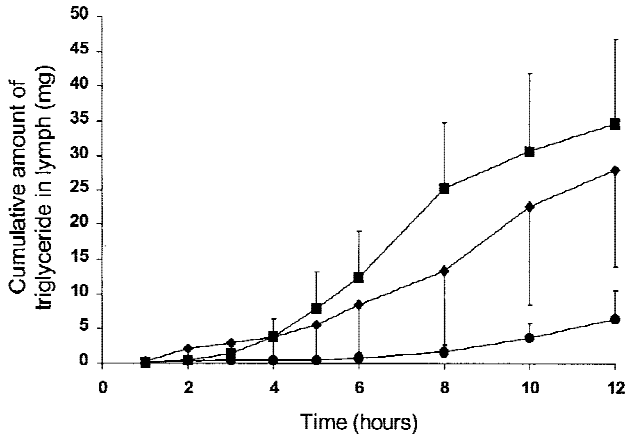

Fig. 2. The cumulative transport of triglyceride (mean ± SE) in mesenteric lymph as a function of time and formulation after intraduodenal administration of a micellar system stabilized with 2% (w/v) Tween 80 in anesthetized rats. The lipid vehicles contained oleic acid (n =5, ◆), linoleic acid (n = 7, ■), and linolenic acid (n = 7, ●).

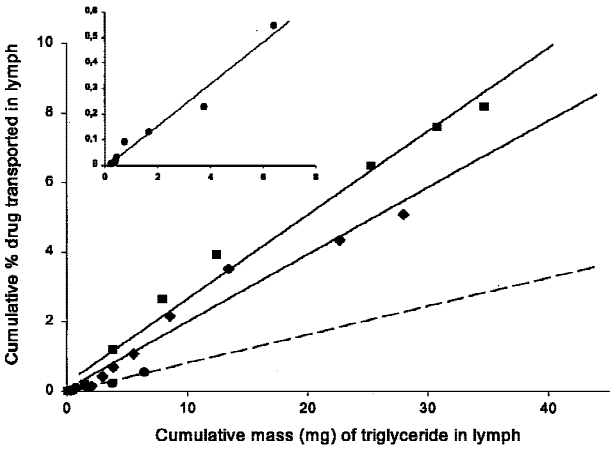

Fig. 3. The cumulative transport of halofantrine into the mesenteric lymph as a function of cumulative triglyceride transport into mesenteric lymph and formulation. The enlarged figure shows the linear relationship for linolenic acid alone. The formulations contained oleic acid (slope = 0.193, r^2 = 0.96, ◆), linoleic acid (slope = 0.242, r^2 = 0.99, ■), and linolenic acid (slope = 0.0821, r^2 = 0.97, ●).

therefore caused by a low triglyceride transport, combined with a lower amount of halofantrine incorporated into the lymph lipoproteins per mg triglyceride when dosed with linolenic acid.

The rank order of transport of halofantrine found in this study is not completely consistent with the observations reported by Cheema *et al.* (17). They found the highest chylomicron formation with linoleic acid followed by linolenic and then oleic acid. Cheema and co-workers measured the amount of chylomicrons with high performance size-exclusion chromatography, and the absorbance of the elution at 290 nm was monitored. The area under the elution peaks after lipid administration was used to determine the proportion of the chylomicrons in the lymph, normalized toward the area under the elution peak from rats receiving phosphate buffered saline. The poor drug transport properties of linolenic acid compared to oleic and linoleic acid were not detected by this method, and as such may explain some of the observed difference in lymph transport order from this study. Secondly, Cheema *et al.* (17) collected and analyzed lymph for only 240 min. This relatively short collecting period combined with the slow lymph appearance time found for halofantrine could also have contributed to the observed difference.

The time-dependent rates of halofantrine transported by the lymph as a function of formulation are shown in Fig. 4. Very similar peak transport times are achieved for the three fatty acids (5–6 h). Porter *et al.* (6) found the peak transport of halofantrine to occur earlier (3–4 h), in a study where anesthetized rats were dosed over 2 h with 2 mg halofantrine in 2.88 ml micellar solution, containing 50 μl molar mixture 2:1 (w/w) of oleic acid:glycerol monooleate stabilized by 4% Tween 80. Charman and Stella (3) have shown that increasing the volume of lipid coadministrered with DDT to anesthetized rats from 50 to 200 μl increased the lymph appearance time, consistent with previous observations (22). The higher volume of lipid dosed in this study compared with that of Porter *et al.* (6) may include some of the explanation of the difference in peak transport times between these two studies.

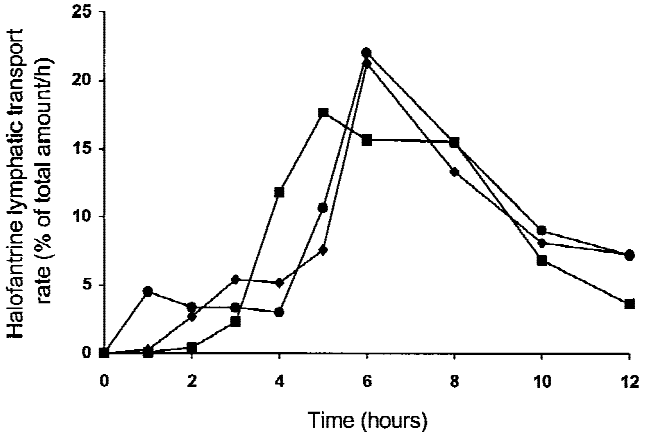

Fig. 4. Rate of lymphatic transport of halofantrine, normalized for the total amount transported over 12 h, as a function of time and formulation. The formulations contained oleic acid (◆), linoleic acid (■), and linolenic acid (●).

Systemic Blood Absorption of Halofantrine

The pharmacokinetic profile and lymphatic transport of halofantrine administered intravenously were determined to interpret the plasma profiles after intraduodenal administration, and to estimate the amount of halofantrine entering the intestinal lymphatic system directly from the vascular compartment. Transfer of halofantrine from the blood to the mesenteric lymph following intravenous administration was found to be negligible, because less than 10 ng halofantrine was found in the lymph after 12 h ($n = 6$). This result confirms that the halofantrine found in the lymph originates from there, and not from a distribution through the systemic circulation. The calculated pharmacokinetic parameters (mean ± SE, $n = 6$) were: $\beta = 0.040 \pm 0.08\ h^{-1}$, MRT = 19.3 ± 3.6 h, AUC = 5695 ± 443 ng·h/ml, Cl_{pl} = 84.10 ±17.6 ml/h. These results were consistent with the data previously published (6), in an experimental set-up very similar to the one used in this study.

Fig. 5 presents the plasma concentration versus time profiles from intraduodenal administration of halofantrine in the free fatty acid vehicles. It was not possible to show any statistical difference between the AUC for the three formulations. The drug plasma levels could not be followed for longer periods due to the limitations in the anesthetized model and because of the slow systemic elimination ($t_{½}$ = 17.3 ± 1.5 h) it is difficult to draw any firm conclusions from the data presented here, but the rank order of the halofantrine plasma concentrations combined with lymph data indicate that the linoleic acid promotes increases in the bioavailabilty for halofantrine compared to oleic and linolenic acid.

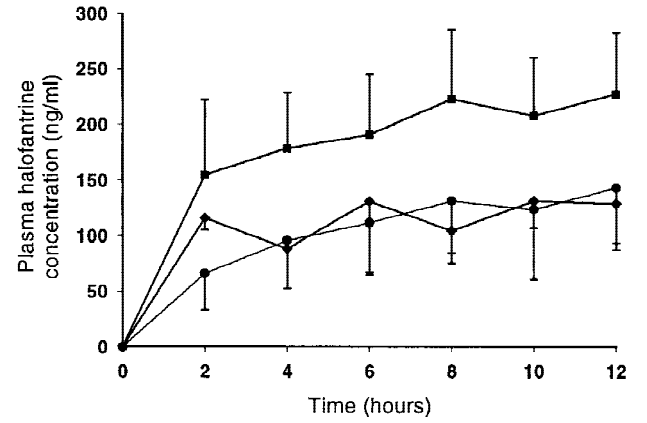

Fig. 5. Plasma halofantrine concentration-time profiles (mean ± SE) as a function of formulation, following intraduodenal administration of 2 mg halofantrine in micellar systems containing oleic acid ($n = 5$, ◆), linoleic acid ($n = 7$, ■), or linolenic acid ($n = 7$, ●) stabilized with 2% (w/v) Tween 80 in anesthetized rats. The AUC^{0-12h} (mean ± SE) for oleic acid was 1299 ± 549 ng·h/ml, 2040 ± 634 ng·h/ml for linoleic acid and 1247 ± 459 ng·h/ml for linolenic acid.

CONCLUSIONS

In conclusion, the present investigation illustrates the importance of an appropriate choice in the lipid selection phase during the development of a new lipid formulation. The question of whether an increase in fatty acid unsaturation affects the lymphatic transport and the portal absorption of halofantrine was examined in the present study. In the anesthetized rat model used in the present examination, where the drug was administered intraduodenally, the data showed that the type of free fatty acid coadministered in a disperse formulation affects the lymphatic transport of halofantrine free base, and the total bioavailability may be improved for halofantrine (or other drugs where the lymphatic transport contributes to the total bioavailability), by choosing oleic or linoleic acid instead of linolenic acid. A linear relationship was found between the cumulative transport of halofantrine in the mesenteric lymph and the cumulative transport of triglycerides in the mesenteric lymph, but a difference was seen in the amount of triglycerides transported and in the efficiency of the three fatty acids to transport halofantrine. Administration of linolenic acid led to lower amounts of triglyceride found in the lymph and to a lower amount of halofantrine incorporated into the lymph lipoproteins per mg triglyceride compared with oleic and linoleic acid. Further work will assess if these findings are also valid for triglycerides with a different degree of unsaturation in conscious rats.

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