Lymphatic transport of methylnortestosterone undecanoate (MU) and the bioavailability of methylnortestosterone are highly sensitive to the mass of co-administered lipid following oral administration of MU

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Non-standard abbreviations: AUC, area under the plasma concentration versus time curve; AUC_{0-\infty}, AUC extrapolated to infinity; AUC_{\infty-0}^{iv}, AUC extrapolated to infinity following intravenous dosing; AUC_{\infty-0}^{oral}, AUC extrapolated to infinity following oral dosing; BA, bioavailability; CL, clearance; C_{max}, maximum plasma concentration; D_{iv}, intravenous dose; D_{oral}, oral dose; LLQ, lower limit of quantitation; M, 7α-methyl-19-nortestosterone; MU, 7α-methyl-19-nortestosterone undecanoate; t_{max}, time to reach C_{max}; TU, testosterone undecanoate; V_{D}, apparent volume of distribution

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ABSTRACT

The contribution of lymphatic transport to the oral bioavailability of methylnortestosterone (M) following oral administration of the lipophilic prodrug methylnortestosterone undecanoate (MU) has been evaluated and the sensitivity of lymphatic MU transport to lymphatic lipid transport investigated. M and MU were administered intravenously and orally to greyhound dogs to determine absolute bioavailability following oral dosing of MU. MU was also administered orally with differing quantities of food (lipid) to lymph duct-cannulated greyhound dogs to investigate the relative roles of lymph vs blood transport on M bioavailability and the effect of lipid load on systemic exposure. The relationship between lymphatic lipid and MU transport was further investigated in anesthetized rats. The oral bioavailability of M after administration of MU was found to be highly dependent on co-administration of food and the bioavailability of M increased approximately 700% in fed vs fasted animals. In both cases, lymph diversion resulted in negligible systemic exposure of M, indicating almost complete dependence on lymphatic transport of MU for systemic exposure of M. Lymphatic transport of MU was even more highly dependent on the quantity of co-administered lipid and increased over 50-fold with increasing lipid load. Increasing the quantity of food or lipid co-administered with MU therefore stimulated a significant increase in the lymphatic transport of MU and systemic exposure of M. The lipid sensitivity of lymphatic transport of MU is significantly higher than previously observed for more metabolically stable compounds, suggesting a role for co-administered lipid in promoting avoidance of enterocyte based cleavage of MU.
INTRODUCTION

7α-methyl-19-nortestosterone (M) is a potent and selective synthetic androgen with potential for the treatment of testosterone insufficiency (Anderson et al., 2003) and control of male fertility (von Eckardstein et al., 2003). M is a more potent agonist at androgen receptors than testosterone (Kumar et al., 1999) but unlike testosterone, M is not a substrate for 5α-reduction (Agarwal and Monder, 1988; Kumar et al., 1992) and is therefore considered to be a prostate-sparing androgen. M has previously been administered as a slow release oily depot or subdermal implant of a prodrug ester (typically the acetate) (Anderson et al., 1999; Noe et al., 1999). Discomfort arising from implant insertion, however, has been reported and patient complaints that the location of the implants (medial aspect of the upper arm) interferes with normal physical activity have also been noted (Noe et al., 1999). Implants also suffer from the disadvantage that they must be surgically implanted by a medical practitioner. An oral dosage form of M which allowed for self administration would therefore offer advantages in terms of patient acceptability.

Whilst the oral bioavailability of M has not been reported, like testosterone it is expected to be low due to extensive pre-systemic metabolism during transit across the gut wall and on first pass through the liver. The use of prodrugs is one approach that has been used successfully to increase the oral bioavailability of highly metabolised compounds (Stella et al., 2007). In the case of testosterone, previous studies have shown that oral administration of testosterone esters may be utilized to facilitate effective testosterone exposure (Coert et al., 1975; Noguchi et al., 1985) and an oral formulation for the administration of testosterone undecanoate (Andriol testocaps) is currently marketed in several countries. The bioavailability of testosterone following oral administration of testosterone undecanoate (TU) is low and predominantly attributable to lymphatic transport of the lipophilic ester prodrug followed by post-absorptive cleavage to release the active testosterone (Shackleford et al., 2003). Consistent with the dependence on lymphatic TU transport for testosterone bioavailability, the systemic exposures of TU and its active metabolites testosterone and 5α-dihydrotestosterone exhibit pronounced food effects as a consequence of enhanced lymphatic transport, and exposure to all three species is significantly higher following oral administration of TU with
food (Frey et al., 1979; Bagchus et al., 2003; Schnabel et al., 2007). This has led to the clinical recommendation that Andriol Testocaps be taken with a meal. A recent study by Schnabel et al. has further demonstrated in human subjects that the exposure to TU and its metabolites is not only dependent on the presence of food but that the lipid content of the meal also plays an important role in bioavailability (Schnabel et al., 2007).

Oral testosterone therapy, however, is limited both by the potential for androgenic prostate effects and the relatively low potency of testosterone which necessitates the administration of large doses of TU (up to 160 mg per day). Due to its higher potency and altered metabolic profile M may therefore offer advantages in terms of dose and prostate-sparing capacity. Analogous to testosterone, the undecanoate ester of M (MU) exhibits oral activity and this activity has been hypothesized to arise from lymphatic transport of the ester and subsequent systemic hydrolysis to the free M, effectively by-passing first pass metabolism. Preliminary evidence in human subjects supports this hypothesis and has shown increased MU and M exposure following oral administration of MU with food (data on file, Schering Plough, Oss, NL). To this point, however, the absolute bioavailability of MU and M has not been established, and the contribution of lymphatic transport of MU following oral administration to the bioavailability of M has not been evaluated. The sensitivity of MU and M exposure to the quantity of co-administered food has also not been examined, but is potentially critically important to understanding in-use variability and to the definition of labeling requirements.

In the current study, therefore, the absolute bioavailability of MU and M was evaluated in fed and fasted greyhound dogs and the impact of co-administration with different quantities of food (lipid) on the lymphatic transport of MU examined. The studies suggest that systemic exposure of M is almost entirely dependent on lymphatic transport of MU and that this is highly dependent on the quantity of co-administered food (lipid). Comparison to data previously reported for the lymphatically transported antimalarial, halofantrine, suggests that the quantity of co-administered lipid required to support lymphatic transport is compound specific and is related (at least in part) to the enterocyte-based metabolic lability of the drug.
METHODS

Materials.

M and MU were supplied by Schering Plough (Oss, NL). Intralipid (10%) was purchased from Baxter Healthcare (Sydney, Australia). Castor oil, oleic acid, L-α-lysophosphatidylcholine, sodium taurocholate and sodium taurodeoxycholate were purchased from Sigma Chemical Co. (St Louis, USA). Lauroglycol FCC was supplied by Gattefossé (Cedex, France). Tween 80 was purchased from BDH chemicals (Kilsyth, Australia). Purified egg yolk lecithin (containing approximately 60% phosphatidylcholine) was obtained from Pharmacia LKB (Uppsala, Sweden). All other chemicals were of analytical grade and solvents were of HPLC grade. Water was obtained from a Milli-Q (Millipore Corporation, Bedford, MA) water purification system unless otherwise specified.

Methods.

In vivo greyhound dog studies

Oral and intravenous formulations of M and MU.

MU for oral administration in the dog studies was formulated in soft gelatin capsules containing castor oil/lauroglycol FCC (60:40 % w/w). Each individual capsule contained approximately 80 mg of MU in 500 mg of castor oil/lauroglycol FCC. An aliquot of MU solution was retained at the time of capsule filling for accurate measurement of the MU concentration. Capsules were weighed before and after filling to allow the mass of MU administered to be calculated.

Intralipid emulsions containing MU and M for intravenous administration to greyhound dogs were prepared via a modification of a previously described method (Shackleford et al., 2003). Briefly, 22.5 mg of MU or M was dissolved in N,N-dimethylacetamide/triacetin [3:5 (v:v), 600 μL] which was added dropwise into 20 mL of Intralipid. Incorporation was achieved by sonicating the emulsion for 2 min after the addition of each drop of MU or M solution using a probe sonicator (XL-Series, Misonix, Inc., Farmingdale NY). The emulsion was cooled throughout the incorporation/sonication process by immersion in an ice water bath. After complete addition of the MU or M solution the concentration of MU or M was determined.
before and after centrifugation to determine the extent of incorporation into oil droplets. The resulting emulsions were sterilized prior to administration by filtration through a 0.22 μm filter (Millipore Corporation, Bedford, MA). An aliquot of the filtered emulsion was retained immediately after administration for quantification of the exact MU or M concentration at the time of dosing. Intralipid formulations were diluted at least 100-fold in acetonitrile prior to analysis by HPLC.

*Bioavailability and lymphatic transport studies in greyhounds.*

All surgical and experimental procedures were approved by the local Institutional Animal Experimentation Ethics Committee. For studies involving the collection of lymphatic fluid, the thoracic lymph duct of healthy adult male greyhound dogs (30 – 41.2 kg) was cannulated under general anesthesia as previously described (Khoo et al., 2001). Following surgery dogs were allowed to recover unrestrained for approximately 16 h during which time they resumed normal ambulatory movement. An intravenous catheter was inserted into the cephalic vein immediately prior to drug dosing to allow for serial blood sampling throughout the study. For studies involving intravenous dosing, catheters were inserted into both left and right cephalic veins such that dosing and blood sampling could be conducted via separate cannulas.

Oral doses of MU were administered as a single soft gelatin capsule (followed by 50 mL of tap water administered via a syringe to the side of the mouth) to lymph-cannulated and non lymph-cannulated greyhound dogs following an overnight fast (14-16 h). In fasted state studies dogs remained fasted throughout the 8 hour sample collection period post-dosing. In fed state studies dogs were fed a weighed quantity of commercial dog food (100-680 g) containing approximately 5% fat, 30 to 45 min prior to dosing. Three mL blood samples were collected via the cephalic vein cannula immediately prior to oral dosing and at 15, 30, 45, 60, 75, 90, 105, 120, 180, 210, 240, 360, 480 min and 24 h post-dosing. All blood samples were collected into individual tubes containing 5 mg of dipotassium EDTA. Plasma was separated by centrifugation (10 min, 1600 x g) and stored frozen at -80°C until analysis.
Intravenous doses of MU and M were administered by slow infusion over 5 min via the cephalic vein cannula to non lymph-cannulated greyhound dogs. The cephalic cannulas were flushed immediately with heparinised saline to ensure the entire dose was administered. Three mL blood samples were taken from the contra-lateral cephalic vein cannula prior to dosing (pre-dose), immediately following the dose infusion (time = 0) and at 10, 20, 30, 40, 50, 60, 75, 90, 105, 120, 150, 180, 210, 240, 360 and 480 min post-dosing. Blood samples were collected into individual tubes and the plasma separated as described above.

Lymph was collected continuously from lymph-cannulated greyhound dogs, for 10 h post-dose, into polypropylene tubes containing 75 mg disodium EDTA. Lymph collected for each hourly time point was pooled and the total volume of lymph collected per hour determined gravimetrically (assuming a specific gravity of 1 g mL\(^{-1}\)). Aliquots of the pooled lymph samples were dispensed into Eppendorf tubes and stored at 4-8°C until analysis (usually within 24 h). To limit possible dehydration due to continuous drainage of lymph a 25 mL bolus of normal saline was administered via the cephalic vein cannula every hour. Water was also available ad libitum throughout the study.

Analysis of MU in lymph and in formulation samples. To prepare lymph samples for analysis, 200 \(\mu\)L of acetonitrile was added to 50 \(\mu\)L of lymph and vortexed for 60 seconds. Samples were then centrifuged at 1600 x \(g\) for 2 min to pellet lymph proteins. Four mL of acetonitrile was then added and vortexed for 2 min to dissolve lymph lipids. Following centrifugation (1600 x \(g\) for 5 min) the entire supernatant was transferred to clean tubes and the solvent removed by evaporation under nitrogen gas. The resulting sample was then re-dissolved in 100 \(\mu\)L acetonitrile for analysis. Formulation samples were diluted at least 100-fold in acetonitrile prior to analysis by LC-UV.

The HPLC system consisted of a Waters 717plus Autosampler connected to a Beckman Programmable Solvent Module 126 and a Beckman Programmable Detector Module 166 set at 240 nm. A 25 \(\mu\)L volume of sample was injected onto a Symmetry C\(_8\) column (Waters Corp., Milford, MA, dimensions 3.9 x 150 mm, I.D. 5 \(\mu\)m). Mobile phase A consisted of 95%
(v/v) water and 5% (v/v) acetonitrile, mobile phase B consisted of 5% (v/v) water and 95% (v/v) acetonitrile. The initial proportion of mobile phase B was 10% which was linearly increased to 100% over 10 min and held for 6 min before returning to 10% over the following 2 min. Mobile phase was then held at 10% B for a further 6 min to allow complete washout and re-equilibration prior to subsequent injection. The total run time was 25 min at a flow rate of 1 mL min⁻¹. The LLQ for MU in lymph and in formulation samples using UV detection was 0.11 μM.

**Analysis of M in lymph and analysis of MU and M in plasma.** 180 μL of acetonitrile was added to a 20 μL aliquot of lymph or plasma, shaken for 10 min then centrifuged at 5700 rpm (6120 x g) for 20 min. To a 50 μL of the diluted sample was added 50 μL DMSO and 200 μL acetonitrile containing internal standard (3-benzoyl-2-naphthoic acid, 500 nM) and concentrations of M and MU were quantified by LC-MS. The HPLC system was an API 4004: SPE (Symbiosis Pharma)-LC-MS (Applied Biosystems, MDS Sciex). A 40 μL sample was injected onto a Polaris C₁₈ column, 60 x 3.0 mm, maintained at 40°C. Mass spectrometry was performed with positive-ion APCI. The needle current was 3 μA and the nebuliser temperature 450°C. The MS detector was operated in the MRM (multiple reaction monitoring) mode. Mobile phase A consisted of water/0.1% formic acid/1.0% isopropyl alcohol and mobile phase B consisted of methanol/0.1% formic acid/1.0% isopropyl alcohol. A 4 min gradient was used in which the mobile phase composition varied (linearly) from 75% A, 25% B (v/v) to 10% A, 90% B (v/v) followed by 100% B for one minute, at a flow rate of 0.7 mL min⁻¹. The LLQ for M in lymph was 6 nM, and for MU and M in plasma samples was 2.4 nM and 6 nM respectively.

**Analysis of triglyceride concentration in lymph.** Lymph triglyceride concentrations were measured in individual lymph samples using a Roche Cobas Mira clinical chemistry analyzer (Basle, Switzerland) and a commercial enzyme-based colorimetric assay kit (Boehringer Mannheim, Germany). Lymph samples were diluted with Milli-Q water prior to analysis if required.
Data analysis. $C_{\text{max}}$ and $t_{\text{max}}$ were determined from inspection of individual plasma concentration versus time plots. The systemic exposure to MU and M following oral and intravenous administration was determined by calculating the area under the plasma concentration versus time curve (AUC) using WinNonlin version 4 (Pharsight, Apex, NC) and the linear trapezoidal method. Where the terminal elimination phase was apparent the area was extrapolated from the last measured plasma concentration to infinity to give AUC$^{0-\infty}$. Plasma clearance of MU and M following intravenous administration was calculated as the ratio of the intravenous dose to the AUC$^{0-\infty}$, i.e.,

$$CL_{\text{IV}} = \frac{D_{\text{IV}}}{AUC_{\text{IV}}^{0-\infty}} \quad (1)$$

The oral bioavailability of MU and M following oral administration of MU were determined by calculating the ratio of dose (D) normalised AUC$^{0-\infty}$ following oral administration to the dose normalised AUC$^{0-\infty}$ following intravenous administration, i.e.,

$$BA_{\text{oral}} = \left( \frac{AUC_{\text{oral}}^{0-\infty}}{D_{\text{oral}}} \right) \cdot \left( \frac{D_{\text{IV}}}{AUC_{\text{IV}}^{0-\infty}} \right) \cdot 100\% \quad (2)$$

The apparent systemic conversion of MU to M was determined following intravenous administration of MU by dividing the dose normalised AUC$^{0-\infty}$ of M following intravenous administration of MU by the dose normalised AUC$^{0-\infty}$ of M obtained following intravenous administration of M as follows:

$$\% \text{ conversion} = \frac{AUC_{\text{M following IV MU}}^{0-\infty}}{AUC_{\text{M following IV M}}^{0-\infty}} \cdot 100\% \quad (3)$$

The proportion of the administered MU dose transported via the lymph (%) LT was calculated by determining the cumulative mass of MU recovered in lymph over the entire lymph collection period and comparing this to the orally administered dose i.e.,

$$\% \text{ LT} = \frac{\text{cumulative mass of MU transported in lymph}}{D_{\text{oral}}} \cdot 100\% \quad (4)$$
In vivo rat studies

Oral formulations of MU

The formulations utilized in the in vivo rat studies were based on those previously shown to result in different levels of lymphatic triglyceride transport and the methods used in the current study are the same as those described previously (Trevaskis et al., 2005). Briefly, 1 mg of MU was dissolved in either 4 mg (low lipid load), or 40 mg (high lipid load) of oleic acid or castor oil/lauroglycol FCC (60:40 w/w) which was then emulsified in a solution of either 0.2% (w/v) Tween 80 in normal saline (pH 7.4), or in a bile salt (BS) solution comprising 3.6 mM L-α-lysophosphatidylcholine (LPC) and 5 mM sodium taurocholate in phosphate buffer (pH 6.9). A lipid-free formulation was prepared by dissolving 1 mg MU directly in 0.2% (w/v) Tween 80. The final volume of all oral formulations was 5.6 mL. Oral formulations were emulsified by sonication using a probe sonicator (XL-Series, Misonix, Inc., Farmingdale NY).

Lymphatic transport determinations in rats

The surgical and experimental procedures have been described in detail previously (Edwards et al., 2001; Trevaskis et al., 2005) and were approved by the local Institutional Animal Experimentation Ethics Committee. Briefly, anesthetized, fasted, male, Sprague-Dawley rats (280 - 320 g) had a J-shaped polyethylene cannula inserted into the duodenum, 1 cm below the pylorus, and a second cannula inserted into the mesenteric lymph duct. Both cannulas were secured by a drop of instant cyanoacrylate adhesive (Selleys Supa Glue®, Padstow, Australia). A third cannula was inserted into the trachea to maintain a patent airway throughout the experiment. Animals remained anesthetized throughout the experiment as described previously (Trevaskis et al., 2005) and body temperature was maintained by placing the anesthetized, cannulated animal on a heated pad set at 37°C. Animals were rehydrated by infusing normal saline via the duodenal cannula at a flow rate of 2.8 mL·h⁻¹ for at least half an hour after the completion of the surgery. For administration of MU, the saline infusion was switched to the lipid formulation which was infused intraduodenally at a rate of 2.8 mL·h⁻¹ for 2 h (5.6 mL total). Normal saline was then re-introduced and infusion continued at 2.8 mL·h⁻¹ for the remainder of the experiment. One group of animals received a pre-dose infusion of a very high-dose lipid formulation (100 mg oleic acid in bile salt solution [3.6 mM
LPC / 5 mM sodium taurocholate in phosphate buffer (pH 6.9)) for 1 hr prior to administration of a high lipid MU formulation (1 mg of MU in 40 mg oleic acid emulsified in the same bile salt solution) in an attempt to maximize the availability of lymph lipoprotein precursors in the enterocytes. Lymph was collected continuously over 8 h via the lymph duct cannula, into tared polypropylene tubes containing heparin (100 IU) to prevent clotting. Lymph from each 60 min period was pooled and the volume determined gravimetrically (assuming a specific gravity of 1 g mL\(^{-1}\)). Aliquots of the pooled lymph samples were dispensed into Eppendorf tubes and stored at 2-8°C until analysis (usually within 24 h). At the conclusion of the experiment rats were killed humanely by a lethal IP dose of 1 mL sodium pentobarbitone (100 mg mL\(^{-1}\)).

Analysis of MU in formulations and lymph and analysis of triglyceride content in lymph was conducted using the methods described for the dog studies.

**Solubility determinations.**

The solubility of MU and M in various media was determined at 25°C as MU melts at temperatures above 29°C. An excess of either MU or M was weighed into a glass test tube and the solvent added. The solvents investigated were water, bile (obtained from beagle dogs) diluted 1:1 (v/v) with phosphate buffered saline (pH 7.4), soybean oil (a representative long chain triglyceride), and micellar solutions containing either 20 mM sodium taurodeoxycholate/ 5 mM purified egg lecithin or 5 mM sodium taurodeoxycholate/ 1.25 mM purified egg lecithin in buffer (50 mM Tris maleate, 5 mM CaCl\(_2\), 150 mM NaCl, pH 7.5). Tubes were rotated at 30 rpm for 7 h at 25°C. After incubation each sample was centrifuged for 20 min at 7200 \(x\) g to separate undissolved MU or M from the solution. The supernatant was analysed for measurement of MU and M concentrations.

**Analysis of solubility samples.**

The HPLC system consisted of a Hewlett Packard 1100 system equipped with a diode array detector and temperature controlled column compartment. Mobile phase A was water, mobile phase B was acetonitrile and mobile phase C consisted of tetrahydrofuran. The initial
composition of the gradient was 60% A, 40% B and 0% C which changed to 10% A, 90% B and 0% C over 10 min. This composition was held for 20 min after which the composition was changed to 0% A, 50% B and 50% C and held for a further 5 min. The composition then returned to 60% A, 40% B and 0% C for re-equilibration. The flow rate was 1.0 mL min\(^{-1}\) and the total run time was 45 min. Twenty μL samples were injected onto a Symmetry C\(_{18}\) column (Waters Corp., Milford, MA, dimensions 150 x 3.9 mm, I.D. 5 μm) maintained at 40°C. Detection was achieved at 240 nm.
RESULTS

Pharmacokinetics of MU and M following intravenous administration to greyhound dogs

Mean plasma concentration versus time profiles for MU and M following intravenous administration to dogs are shown in Figure 1. The calculated pharmacokinetic parameters for MU and M following intravenous administration to greyhounds are summarised in Table 1. After intravenous infusion of MU the plasma concentration of MU declined monoexponentially with an apparent elimination half-life ($t_{1/2}$) of 23.6 min. MU clearance (CL) and volume of distribution ($V_d$) following intravenous administration were low (2.2 mL-min$^{-1}$·kg$^{-1}$ and 71.8 mL·kg$^{-1}$ respectively). In contrast CL and $V_d$ of M following intravenous administration were significantly higher (54.9 mL-min$^{-1}$·kg$^{-1}$ and 2530 mL·kg$^{-1}$ respectively) although the terminal $t_{1/2}$ was similar (34.4 min) compared with MU. The apparent elimination $t_{1/2}$ of M was not significantly different after intravenous administration of M and MU (34.4 ± 6.5 min vs. 40.2 ± 6.5 min). In this study the systemic conversion of MU to M was estimated according to equation (3) and was calculated to be approximately 117%. This comparison assumes that the systemic clearance of M is the same following conversion from MU as it is after intravenous administration of M. It also assumes linear pharmacokinetics. The greater than 100% systemic conversion of MU may reflect a deviation from either of these assumptions. Alternatively it may simply reflect variability in the data acquired across different animals. Nonetheless approximately complete conversion appears likely.

Pharmacokinetics and bioavailability of MU and M following oral administration of MU to fed and fasted dogs

The mean systemic plasma concentration versus time profiles for MU and M following oral administration of MU to non-lymph-cannulated greyhounds are presented in Figure 2. Dogs were either fasted or were fed a meal containing approximately 34 g of lipid 30 – 45 min prior to dosing. The calculated pharmacokinetic parameters following oral administration are summarised in Table 2. $C_{max}$ following oral administration of MU was approximately 40-fold higher in fed animals compared with fasted animals and the systemic exposure of MU (AUC$^{0-\infty}$) increased 35-fold upon administration with food. The absolute bioavailability of MU when
administered orally to fed and fasted greyhounds was 2.8% and 0.08%, respectively. Food also increased the systemic exposure to M following oral MU administration approximately 7-fold; from 1.7% oral bioavailability in the fasted state to 12.1% following post-prandial administration.

**Lymphatic transport of MU and M in dogs**

The total mass of MU transported in the lymph of fed and fasted greyhound dogs over 10 h represented 5.8% and 0.11% of the oral dose, respectively, (Fig. 3) indicative of a greater than 50-fold increase in lymphatic transport in the presence of food. The plasma concentrations of MU and M in lymph-cannulated greyhounds were below the LLQ for all time points in fasted animals and in fed animals only 5 individual time points (out of a total of 42) contained quantifiable levels of M or MU, all other data was below the LLQ. Lymph diversion therefore resulted in negligible systemic exposure to MU and M, suggesting almost complete dependence on lymphatic transport for systemic exposure following oral dosing.

The cumulative percentage of the oral dose of MU transported in the lymph of animals fed varying quantities of food (and hence lipid) increased with total lymph triglyceride transport (Fig. 4). The apparent sigmoidal relationship ($r^2 = 0.9575$) between lymphatic MU and triglyceride transport suggests that a threshold quantity of lipid transport was required to support significant increases in lymphatic drug transport, but that beyond that threshold relatively small changes in lymphatic triglyceride transport resulted in large changes in MU lymphatic transport. At high lipid transport rates, further increases in MU transport were not evident suggesting that lymphatic lipid transport rates were no longer limiting lymphatic drug transport.

**Lymphatic transport of MU in rats**

The lymphatic transport of MU in rats after oral administration in formulations designed to stimulate different rates of triglyceride transport across the enterocyte also increased with increasing lymph triglyceride transport (Fig. 5 and 6). Formulations stimulating low levels of triglyceride transport in lymph (lipid free or 4 mg oleic acid or 4 mg castor oil/lauroglycol FCC)
resulted in only low levels of MU transport in lymph, formulations stimulating intermediate levels of triglyceride transport (containing 40 mg oleic acid or 40 mg castor oil/lauroglycol FCC) resulted in intermediate levels of MU transport, and pre-dosing rats with a 100 mg of lipid prior to administration of MU in 40 mg oleic acid resulted in the greatest extent of MU transport. In the group pre-fed 100 mg of lipid, lymphatic drug transport was notably delayed in comparison to the other groups, presumably reflecting the time required to process the very large quantities of co-administered lipid. The maximum rate of triglyceride transport in lymph (on a mg·h⁻¹ basis) was much lower in rats compared with dogs in both fasted animals and in the presence of lipid or triglyceride-stimulating formulations (Table 3), and this was also reflected in the up to 300-fold difference in total mass of triglyceride transported over the course of the 8-10 h study period. Whilst the differences in lymphatic lipid transport largely reflected the differences in lipid mass administered, the maximum rate of MU transport and total quantity of MU transported in lymph per g of triglyceride were similar in rats and dogs suggesting that the absolute mass of triglyceride transported into lymph was the most important determinant of MU, and hence M, bioavailability.

**Solubility of MU and M**

As poor intestinal solubility is a potential limitation to the oral bioavailability of MU the solubility of MU and M was determined in aqueous media, diluted bile, a representative long chain triglyceride (soybean oil) and micellar solutions representative of both fed and fasted conditions. As expected the aqueous solubility of MU was low when compared with the aqueous solubility of the less lipophilic parent compound (0.42 μg·mL⁻¹ vs. 73 μg·mL⁻¹) but much higher in lipid (437 mg·mL⁻¹ vs. 10 mg·mL⁻¹). The solubility of both compounds in diluted bile, soybean oil and simulated intestinal contents was significantly greater than in water alone (Table 4).
DISCUSSION

The effect of food and in particular lipids on the oral bioavailability of poorly water soluble drugs is well documented. Mechanisms by which increased drug absorption can occur include slowing of gastric emptying, enhancement of gastrointestinal dissolution or solubilisation, protection from intestinal degradation and stimulation of lymphatic drug transport (Charman et al., 1997; Wasan, 2001; Grove et al., 2007; Porter et al., 2007; Dahan et al., 2008). In the current study, the oral bioavailability of MU in fed animals increased 35-fold compared with bioavailability in fasted animals. The bioavailability of M following oral administration of MU also increased substantially post-prandially. The post-prandial exposures to MU and M were consistent with previous data for the structurally similar TU and testosterone (Tauber et al., 1986; Shackleford et al., 2003) and the increase in exposure compared with fasted state bioavailability was consistent with clinical observations for TU (Bagchus et al., 2003). The data are also consistent with observations for MU in humans (data on file Schering Plough, Oss, NL) which suggest a pronounced increase in MU and M exposure when MU is administered with food.

To explore potential mechanisms underpinning the increase in post-prandial exposure, the possible effect of food on intestinal solubilisation of MU and M was estimated in vitro. As expected the aqueous solubility of MU was low but increased dramatically in micellar solutions representative of fasted and fed state intestinal conditions. It is possible that enhanced intestinal solubilisation may therefore contribute to enhanced MU absorption following post-prandial oral administration. Previous studies with testosterone, however, suggest that the primary limitation to M bioavailability is first pass metabolism rather than poor absorption (Tauber et al., 1986), therefore increases in post-prandial absorption alone are unlikely to lead to significant increases in bioavailability without attendant effects on first pass metabolism. In this regard, MU is highly lipophilic (437 mg mL⁻¹ solubility in soybean oil and calculated log P 8.73) and for compounds of this type access to the systemic circulation after oral absorption may occur via the lymphatic system rather than by transport into portal blood (Charman and Stella, 1986; Wasan, 2001; Holm et al., 2002; Hauss, 2007; Dahan et al., 2008). Lymphatic drug transport is typically increased after post-prandial administration since
the lipids in food stimulate the synthesis of lymph lipoproteins, which in turn act as carriers for lymphatically transported drugs (Charman and Stella, 1991; Porter and Charman, 2001a; Wasan, 2001; Holm et al., 2002; Hauss, 2007). This may be particularly important in the case of highly metabolically labile molecules such as M, since lymphatic drug transport circumvents pre-systemic hepatic metabolism and therefore provides an avenue to improved bioavailability.

In the current studies the quantity of MU transported in lymph over a 10 h post-dose period was relatively low but increased more than 50-fold in fed vs fasted animals (0.11 to 5.8% of the administered dose). Importantly, systemic (blood) levels of MU and M were below the limit of quantification in lymph-cannulated animals, presumably reflecting conversion of MU to M and subsequent metabolism of M during first pass through the liver. In this regard, a range of hydroxylated metabolites of M have recently been described (Prasad et al., 2009), but were not quantified here. Intestinal lymphatic transport therefore appears to be essentially the sole mechanism of transport of bioavailable MU and M to the systemic circulation. Given the low blood exposure of M in lymph cannulated animals and the 10-fold higher concentrations of MU in the lymph when compared to M, it is likely that the predominant mechanism of transport of bioavailable M to the systemic circulation is via lymphatic transport of MU followed by systemic liberation of M (rather than via lymphatic transport of M or absorption of MU or M via the blood). That the lymphatic transport of MU is significantly more efficient after co-administration with food provides an explanation for the large increase in post-prandial bioavailability of M following oral administration of MU.

To explore the quantitative impact of co-administered lipid on the lymphatic transport of MU (and therefore the systemic exposure of M) a series of studies were undertaken to map the changes in lymphatic transport after co-administration with differing quantities of lipid. Low levels of MU lymphatic transport were observed under conditions of basal triglyceride transport (i.e. in fasted animals), but administration with increasing quantities of food (and hence lipid), resulted in enhanced triglyceride and MU lymphatic transport. Increases in lymphatic triglyceride transport beyond approximately 6 g over 10 hr, however, resulted in
only minor additional increases in MU transport. Whilst the maximum rate of triglyceride transport in lymph increased with increasing lipid load lymphatic triglyceride transport continued long after lymphatic MU transport was essentially complete (within 2 h post-dosing). The data therefore suggest that high rates of lymphatic triglyceride transport may be an important driver of lymphatic MU transport in the period immediately following oral administration but that the protracted high rates of lymph triglyceride transport observed at higher lipid loads have little further impact on lymphatic MU transport. Indeed the ratio of total MU to total lymph triglyceride transport over the 10 h collection period decreases at higher lipid loads (Table 3). Interestingly, when data obtained in the same model for the structurally unrelated (but lymphatically transported) antimalarial halofantrine (Khoo et al., 2003) are compared to the current MU data, the relationship between lymphatic lipid and drug transport is similar (Fig. 4b). In the case of halofantrine, however, the correlation is even more pronounced, and significant lymphatic drug transport was evident at much lower lymph triglyceride levels than that observed for MU and much higher drug/triglyceride levels were apparent in the lymph. The apparently greater mass of triglyceride required to stimulate lymphatic transport of MU compared with halofantrine is unlikely to reflect differences in lipophilicity since MU is at least as lipophilic as halofantrine when assessed by Log P (8.73 vs 8.5) (Porter et al., 1996) and solubility in long chain triglycerides (437 mg·ml\(^{-1}\) vs 47.3 mg·ml\(^{-1}\)) (Kaukonen et al., 2004). However, maximum concentrations of MU per g of lymphatically transported lymph triglyceride were much lower than that of halofantrine (<1\% vs ~25\% of the equilibrium solubility of drug in triglyceride, (Khoo et al., 2003)) suggesting that factors other than solubility in lymph triglyceride were limiting MU lymph transport. In this regard an important differential between halofantrine and MU, is the potentially lower metabolic stability of MU in the enterocyte. In the current studies the efficiency of enterocyte-based conversion of MU to M was evaluated in a previously described isolated perfused rat intestine model (Johnson et al., 2003). After perfusion of an MU emulsion through an isolated section of jejunum in anesthetised rats, the concentrations of MU in blood draining the perfused intestinal segment were below the limits of quantification at all time points, whereas appearance of M was clearly evident (data not shown). This confirmed that a significant proportion of the pre-systemic conversion of MU to M occurred in the enterocyte, consistent
with previous reports for TU (Coert et al., 1975; Horst et al., 1976). Unpublished investigations have also shown that plasma esterases play a negligible role in the hydrolysis of MU and that hydrolysis by hepatic esterases is considerably more avid (data on file, Schering Plough, Oss, NL). In the latter study conversion by enterocyte-based enzymes was not studied, but collectively these data suggest that after oral administration facile conversion of MU to M will occur on first pass both in the enterocyte and in the liver. Conversely plasma esterases are unlikely to play a significant role in MU to M conversion. Halofantrine is also converted to a less lipophilic and poorly lymphatically transported metabolite desmethylhalofantrine (Khoo, 2002) within the enterocyte (Porter and Charman, 2001b). However, halofantrine is not a high extraction efficiency drug and therefore the extent of enterocyte based conversion of halofantrine to desbutylhalofantrine is considerably lower than that of MU to M.

Previous studies have suggested that increasing the mass of lipid within the enterocyte, may reduce drug exposure to enterocyte-based metabolic enzymes (Van Veld et al., 1987; Trevaskis et al., 2006) and that inhibition of enterocyte based cytochrome P450 metabolism and intestinal P-gp efflux may significantly enhance the extent of lymphatic transport of metabolically labile drugs (Griffin and Driscoll, 2008). In the light of the significant extent of enterocyte based conversion of MU to M the differences in the lipid dependency of lymphatic transport of MU and halofantrine may therefore reflect the requirement for a larger intracellular lipid pool to protect the more metabolically labile MU from conversion to M before substantial lymphatic transport can occur.

To confirm that the lipid dependency of MU lymphatic transport was not species specific, a subsequent series of experiments were conducted in rats. Similarly to the data obtained in dogs, a strong relationship between triglyceride transport and drug transport was evident. Despite differences in the rate and extent of total triglyceride transport, the ratio of MU transported in lymph to total lymph triglyceride were broadly similar (0.16 – 0.52) to those observed in dogs (0.07 – 0.62) and independent of the formulation used further supporting the hypothesis that it is triglyceride transport which supports lymphatic transport of MU rather than the formulation per se.
In conclusion, the oral bioavailability of M was low after oral administration of MU under fasted conditions, but increased dramatically after co-administration with food. Significant increases in lymphatic transport of MU were also demonstrated post-prandially, and data obtained in lymph-cannulated dogs suggests that the increase in M bioavailability was derived almost entirely from lymphatic transport of MU. Subsequent studies further elucidated, in both rats and dogs, that lymphatic transport of MU was highly dependent on the mass of triglyceride transported in the lymph, especially in the period immediately following dosing. The high lymphatic lipid flux required to stimulate substantial MU transport may reflect the need for protection from enterocyte based hydrolysis since conversion of MU to the less lipophilic M is facile and expected to limit lymphatic transport. The latter suggestion is consistent with recent studies that have shown that inhibition of enterocyte-based efflux and metabolism leads to an increase in lymphatic transport of the metabolically labile protease inhibitor saquinavir (Griffin and Driscoll, 2008).
ACKNOWLEDGEMENTS

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REFERENCES


19-nortestosterone implants for possible use as a long-acting contraceptive for men. 

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1  Mean (± S.E.M., n = 3) plasma concentration profiles of MU (triangles) and M (circles) following intravenous administration of MU (closed symbols) and M (open symbols) to non lymph-cannulated greyhound dogs.

Figure 2  Mean (± S.E.M., n = 3) plasma concentration profiles of MU (A) and M (B) following oral administration of MU to non lymph-cannulated greyhound dogs in both the fasted (open symbols) and fed (closed symbols) state.

Figure 3  Mean (± S.E.M., n = 3) lymphatic transport of MU (A) and M (B) in lymph duct cannulated greyhound dogs following oral administration of MU in both the fasted (open symbols) and fed (closed symbols) state. Data represents the total mass of MU and M collected over each time point.

Figure 4  Percentage of the lymphatically transported dose of MU recovered in the lymph of cannulated greyhound dogs following oral administration with different lipid loads. (A) as a function of time. 34 g lipid, mean ± S.E.M, n = 3 (circles), 15 g lipid, n = 1 (upright triangles), 10 g lipid, n = 1 (diamonds), 7.5 g lipid, n = 1 (crosses), 5 g lipid, n = 1 (inverted triangles), fasted, mean ± S.E.M., n = 3 (squares); (B) as a function of total triglyceride (TG) transport. Data is presented as the percentage of the dose recovered following co-administration with different lipid loads relative to the percentage of MU dose transported in lymph following administration of 34 g of lipid (closed circles). Previously reported data for an unrelated highly lipophilic antimalarial (halofantrine) is plotted for comparison (open circles) (Khoo et al., 2003). Three-parameter sigmoidal regression conducted using Sigmaplot, version 10.0, Systat Software, Inc., $r^2 = 0.9575$. 

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Figure 5  Percentage of the lymphatically transported dose of MU recovered in the lymph of lymph duct cannulated rats following oral administration in formulations designed to stimulate varying rates of triglyceride transport. Data is presented as the mean ± SD (n = 3-7). Lipid pre-dose (circles), 40 mg oleic acid + LPC + BS (crosses), 40 mg oleic acid in Tween (inverted triangles), 40 mg castor oil/lauroglycol FCC in Tween (closed squares), 4 mg castor oil/lauroglycol FCC + LPC + BS (diamonds), 4 mg oleic acid + LPC + bile salt (upright triangles), lipid free formulation (open squares). Refer to text for full formulation details.

Figure 6  Cumulative percentage of the administered dose of MU and mass of triglyceride recovered in the lymph of lymph duct cannulated rats following administration of formulations designed to stimulate varying rates of triglyceride transport. Figure 5A depicts data from individual animals whilst Figure 5B depicts mean ± SD (n = 3-7) data for groups of animals according to formulation. Abbreviations: OA (oleic acid), LPC (L-α-phosphatidylcholine), BS (5 mM sodium taurocholate bile salt solution), CO/PGL (castor oil/lauroglycol FCC [60:40 % w/w]), Tween (0.2% (w/v) Tween 80 in normal saline).
Table 1. Summary of pharmacokinetic parameters for MU and M determined in non lymph-cannulated greyhound dogs (mean ± S.E.M. for n = 3 dogs) following intravenous administration of MU and M as an Intralipid emulsion over 5 min.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MU after iv MU</th>
<th>M after iv MU</th>
<th>M after iv M</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC&lt;sub&gt;0→∞&lt;/sub&gt; (nmol⋅min⋅mL&lt;sup&gt;-1&lt;/sup&gt;⋅mmol&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11773 ± 1237</td>
<td>773 ± 28</td>
<td>661 ± 185†</td>
</tr>
<tr>
<td>CL (mL⋅min&lt;sup&gt;-1&lt;/sup&gt;⋅kg&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>2.2 ± 0.3</td>
<td>N/A</td>
<td>54.9 ± 11.8</td>
</tr>
<tr>
<td>V&lt;sub&gt;D&lt;/sub&gt; (mL⋅kg&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>71.8 ± 8.4</td>
<td>N/A</td>
<td>2530 ± 310</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (min)</td>
<td>23.6 ± 4.2</td>
<td>40.2 ± 4.1</td>
<td>34.4 ± 6.5</td>
</tr>
<tr>
<td>% Conversion&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N/A</td>
<td>117</td>
<td>N/A</td>
</tr>
</tbody>
</table>

<sup>a</sup>Dose-normalised AUC. Actual dose of MU administered was 0.06 ± 0.004 mg⋅kg<sup>-1</sup> (0.14 ± 0.004 µmol⋅kg<sup>-1</sup>), actual dose of M administered was 0.11 ± 0.002 mg⋅kg<sup>-1</sup> (0.39 ± 0.004 µmol⋅kg<sup>-1</sup>)

<sup>b</sup>% conversion <em>in vivo</em> calculated as the dose normalized AUC<sub>0→∞</sub> of M following iv administration of MU divided by the dose normalized AUC<sub>0→∞</sub> of M following iv administration of M.
Table 2. Summary of pharmacokinetic parameters for MU and M following oral administration of MU to non lymph-cannulated greyhound dogs in both the fasted and fed states.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fasted</th>
<th>Fed</th>
<th>Mean ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>MU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (nmol·mL$^{-1}$)</td>
<td>0.02 ± 0.004</td>
<td>0.82 ± 0.08*</td>
<td>41</td>
</tr>
<tr>
<td>AUC$^{b,c}$ (nmol·min·mL$^{-1}$·mmol$^{-1}$)</td>
<td>9.58 ± 2.05</td>
<td>334.38 ± 38.07*</td>
<td>35</td>
</tr>
<tr>
<td>$t_{\text{max}}$ (min)</td>
<td>75 (75-90)</td>
<td>240 (210-240)</td>
<td>-</td>
</tr>
<tr>
<td>Oral BA (%)</td>
<td>0.08</td>
<td>2.8</td>
<td>35</td>
</tr>
<tr>
<td>M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (nmol·mL$^{-1}$)</td>
<td>0.01 ± 0.001</td>
<td>0.07 ± 0.01*</td>
<td>7</td>
</tr>
<tr>
<td>AUC$^{b,c}$ (nmol·min·mL$^{-1}$·mmol$^{-1}$)</td>
<td>13.71 ± 0.24</td>
<td>94.57 ± 1.75**</td>
<td>7</td>
</tr>
<tr>
<td>$t_{\text{max}}$ (min)</td>
<td>75 (60-105)</td>
<td>90 (75-120)</td>
<td>-</td>
</tr>
<tr>
<td>Oral BA (%)$^b$</td>
<td>1.7</td>
<td>12.1</td>
<td>7</td>
</tr>
</tbody>
</table>

Data represents the mean ± S.E.M. for n = 3 dogs except for $t_{\text{max}}$ which is the median (range) for n=3 dogs.

$^a$Dose-normalised AUC. Actual dose of MU administered was 2.21 ± 0.05 mg·kg$^{-1}$ (4.83 ± 0.1 µmol·kg$^{-1}$) in fed animals and 1.84 ± 0.12 mg·kg$^{-1}$ (4.02 ± 0.3 µmol·kg$^{-1}$) in fasted animals.

$^b$ refers to the BA of M after oral administration of MU and assumes administration of a molar equivalent dose of M.

*p < 0.01, **p < 0.001
Table 3. Comparison of mean triglyceride (TG) transport and drug transport rates in lymph of dogs and rats following oral co-administration of MU or halofantrine with different lipid loads.

<table>
<thead>
<tr>
<th>Species/drug</th>
<th>Formulation or lipid load</th>
<th>Maximum rate of TG transport in lymph (mg/h)</th>
<th>Total TG transported in lymph over 8/10 h (g)</th>
<th>Maximum rate of drug transport in lymph per g TG (mg drug/g TG)</th>
<th>Total drug transported in lymph per g lymph TG over 8/10 h (mg drug/g TG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MU</td>
<td>0 g lipid (fasted)</td>
<td>461</td>
<td>1.4</td>
<td>0.27</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>5 g lipid</td>
<td>745</td>
<td>4.1</td>
<td>0.94</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>7.5 g lipid</td>
<td>1374</td>
<td>5.2</td>
<td>1.12</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>10 g lipid</td>
<td>2791</td>
<td>7.0</td>
<td>1.51</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>15 g lipid</td>
<td>2101</td>
<td>11.7</td>
<td>0.93</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>34 g lipid</td>
<td>5015</td>
<td>20.45</td>
<td>0.77</td>
<td>0.23</td>
</tr>
<tr>
<td>Halofantrine</td>
<td>ME-LC (fasted)</td>
<td>850</td>
<td>3.4</td>
<td>12.29</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>ME-MC (fasted)</td>
<td>178</td>
<td>0.9</td>
<td>5.14</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>PEG melt (fasted)</td>
<td>NM</td>
<td>0.5</td>
<td>4.81</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>PEG melt (34 g lipid)</td>
<td>NM</td>
<td>32.6</td>
<td>6.21</td>
<td>1.7</td>
</tr>
<tr>
<td>Rat</td>
<td>Lipid-free</td>
<td>2.88</td>
<td>0.017</td>
<td>0.55</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>4 mg OA + LPC + BS</td>
<td>3.18</td>
<td>0.017</td>
<td>0.52</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>4 mg CO/PGL + LPC + BS</td>
<td>3.41</td>
<td>0.020</td>
<td>1.25</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>40 mg CO/PGL in Tween</td>
<td>5.72</td>
<td>0.028</td>
<td>1.03</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>40 mg OA + LPC + BS</td>
<td>9.94</td>
<td>0.034</td>
<td>0.60</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>40 mg OA in Tween</td>
<td>10.22</td>
<td>0.041</td>
<td>0.77</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>100 mg lipid pre-dose</td>
<td>16.97</td>
<td>0.072</td>
<td>0.56</td>
<td>0.32</td>
</tr>
</tbody>
</table>

*a Data reproduced from (Khoo et al., 2003). ME-LC = long-chain triglyceride microemulsion, LC-MC = medium-chain triglyceride microemulsion, PEG melt = lipid-free amorphous PEG 600 solid dispersion

b For abbreviations refer to Figure 6.

NM not measured
Table 4. Solubility of MU and M

<table>
<thead>
<tr>
<th></th>
<th>Solubility (mg·mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MU</td>
</tr>
<tr>
<td>Water</td>
<td>0.0004</td>
</tr>
<tr>
<td>Bile</td>
<td>2.8</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>437</td>
</tr>
<tr>
<td>Fasted state micelles †</td>
<td>0.24</td>
</tr>
<tr>
<td>Fed state micelles ‡</td>
<td>1.2</td>
</tr>
</tbody>
</table>

† 5 mM sodium taurodeoxycholate/1.25 mM purified egg lecithin in buffer (50 mM Tris maleate, 5 mM Ca₂Cl, 150 mM NaCl, pH 7.5)
‡ 20 mM sodium taurodeoxycholate/ 5 mM purified egg lecithin in buffer (50 mM Tris maleate, 5 mM Ca₂Cl, 150 mM NaCl, pH 7.5)

See bile obtained from beagle dogs diluted 1:1 (v/v) in phosphate buffered saline (pH 7.4).
Figure 1

![Graph showing plasma concentration (nM) vs. time (h)]
Figure 5