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# Examination of the impact of a range of Pluronic surfactants on the in-vitro solubilisation behaviour and oral bioavailability of lipidic formulations of atovaquone

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## Abstract

Exogenous surfactants are increasingly used to enhance the dispersion properties of lipid-based formulations of poorly water-soluble drugs, yet their possible effects on formulation digestion and oral bioavailability in-vivo are not well documented. In this study, in-vitro dispersion and digestion experiments were conducted using formulations comprising a blend of long-chain glycerides, ethanol, a model poorly water-soluble drug (atovaquone), and a series of surfactants including Cremophor EL and a range of Pluronic surfactants (Pluronics L121, L61, L72, L43 and F68). Inclusion of Cremophor EL, a surfactant with a high hydrophilic–lipophilic balance (HLB), promoted complete digestion of the formulation and effective dispersion and solubilisation of the lipolytic products and co-administered drug. Surprisingly, formulations containing the Pluronic (L121) with the lowest HLB (0.5) equally effectively promoted digestion and drug solubilisation and a trend towards decreased digestion and drug solubilisation was observed with Pluronics of increasing HLB values. All formulations effectively prevented drug precipitation, suggesting possible utility in-vivo, and no correlation was evident between the ability of the formulations to self-emulsify on dispersion and to promote drug solubilisation on digestion. Subsequent assessment of the oral bioavailability of atovaquone after administration of formulations containing Cremophor EL or Pluronic L121 or a simple solution of atovaquone in long-chain glycerides confirmed the utility of lipid-based formulations for enhancing the oral bioavailability of poorly water-soluble drugs such as atovaquone, but also indicated that in some cases microemulsion preconcentrate formulations may not provide additional bioavailability benefits beyond that achievable using simple lipid solutions.

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## Introduction

The general ability of lipid-based formulations to facilitate enhanced oral bioavailability for poorly water-soluble drugs is well recognized (Pouton 1997). What is less clear, however, is how changes to the choice of lipid, surfactant and co-solvent used to assemble these formulations may affect in-vitro and in-vivo performance. The classical approach to the formulation of lipidic systems suggests that the ability to self-emulsify to form a colloidal dispersion with small particle size (in combination with adequate drug solubility in the formulation) is the key in-vitro indicator of in-vivo performance. More recently, however, a number of studies have suggested that the impact of digestion of the formulation on drug solubilisation patterns must also be considered, since the majority of lipid formulations contain digestible components, and the process of lipid digestion inevitably follows dispersion in the gastrointestinal tract where digestible components are included (Reymond & Sucker 1988; MacGregor et al 1997; Zangenberg et al 2001a; Sek et al 2002; Porter et al 2004a).

These more recent studies have described the development of in-vitro digestion models (Sek et al 2001; Zangenberg et al 2001b) and have examined in broad terms how digestion of lipidic formulations can dictate the extent of drug solubilisation (Zangenberg et al 2001a; Kaukonen et al 2004). More specifically, the relative benefits of inclusion of long-chain ($C_{18}$) and medium-chain ($C_{10\text{-}12}$) lipids in lipid formulations

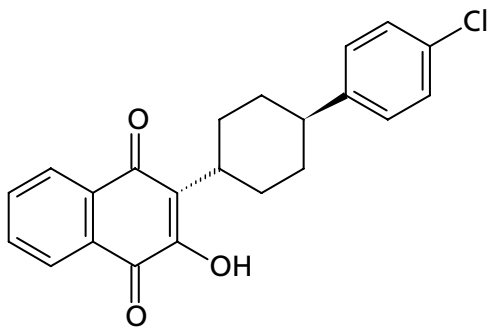


**Figure 1** Chemical structure of atovaquone (MW 366.8).

has been examined and, at least in the case of halofantrine, danazol, and LU 28-179, long-chain lipids appear to prevent drug precipitation on digestion of the formulation more effectively than their medium-chain comparators (Christensen et al 2004; Porter et al 2004a, b). In contrast, however, relatively little attention has been directed towards the differential impact of inclusion of surfactants with varying physicochemical properties on drug solubilisation properties, although nonylphenol ethoxylate surfactants with hydrophilic–lipophilic balance (HLB) values in the range of 12–15 have been suggested to inhibit the in-vitro digestion of medium-chain triglycerides, thereby raising the possibility that surfactants may adversely impact on the bioavailability of a co-administered drug if lipid digestion is a key aspect of bioavailability enhancement (Solomon et al 1996).

This study was designed to investigate the relationship between the dispersion and digestion properties of lipid-based formulations containing exogenous surfactants (Cremophor EL (HLB 12) and a range of Pluronic surfactants (Pluronic L121, L61, L72, L43 and F68 with HLB values of 0.5, 3, 6.5, 12 and 29, respectively) and the in-vivo bioavailability of an incorporated poorly water-soluble compound. Atovaquone (Figure 1) was chosen as a model poorly water-soluble drug as it is highly lipophilic (log $P_{(octanol/water)}$ of 5.31), has low water solubility ($< 0.2\,\mu g\,mL^{-1}$) (data on file, GlaxoSmithKline, Hertfordshire, UK; DeAngelis et al 1994) and its oral bioavailability is enhanced (3 fold) after administration with food (Rolan et al 1994; Dressman & Reppas 2000). The current data suggest that the choice of surfactant should be informed by the pattern of drug solubilisation post digestion, rather than simply the ability to efficiently self-emulsify on dispersion.

## Materials and Methods

### Materials

Atovaquone (*trans*-2-[4-(4-chlorophenyl)-cyclohexyl]-3-hydroxy-1,4-naphthalenedione) was kindly supplied by GlaxoSmithKline (UK). Sodium taurodeoxycholate 99% as bile salt and porcine pancreatin (8 × USP specifications activity) were from Sigma Chemical Co. (St Louis, MO, USA). Soybean oil (Sigma Chemical Co, St Louis, MO, USA) and Maisine 35-1 (Gattefossé, Saint-Priest, France) were used as received. Soybean oil contains long-chain triglycerides with fatty acid composition of 54% w/w linoleic acid ($C_{18:2}$), 22% oleic acid ($C_{18:1}$), 11% palmitic acid ($C_{16:0}$), 9% linolenic acid ($C_{18:3}$) and 4% stearic acid ($C_{18:0}$) (certificate of analysis, lot no 038H0103; Sigma Chemical Co., St Louis, MO, USA). An average molecular mass of 870.5 Da was used for soybean oil. Maisine 35-1 is a blend of long-chain monoglycerides diglycerides and triglycerides (38% monoglycerides, 48% diglycerides, 13% triglycerides, < 1% fatty acids) consisting of 57% (w/w) linoleic acid ($C_{18:2}$), 28% oleic acid ($C_{18:1}$), 11% palmitic acid ($C_{16}$), 2% stearic acid ($C_{18:0}$) and < 1% linolenic acid ($C_{18:3}$), arachidic acid ($C_{20:0}$) and eicosenoic acid ($C_{20:1}$); an average molecular mass of 540 was used for Maisine 35-1 (certificate of analysis, batch no 20300; Gattefossé, Saint-Priest, France). Cremophor EL (HLB 12), and Pluronics L43 and L61 (HLB 12 and 3, respectively) were obtained from BASF (Ludwigshafen am Rhein, Germany). Pluronics F68, L72 and L121 (HLB 29, 6.5, 0.5, respectively) were obtained from BASF (Parsipanny, NJ). Cremophor EL, Pluronic F127 and Pluronic F68 are included in the current FDA list of inactive ingredients. Data regarding the regulatory acceptability of the other Pluronic excipients is not available at this time. Lecithin (approximately 60% pure phosphatidylcholine by HPTLC from egg yolk (Sek et al 2002)) was a gift from Pharmacia LKB (Uppsala, Sweden). Sodium hydroxide 1 M (Titrisol), which was diluted with water to obtain 0.2 M NaOH titration solution, was purchased from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q (Millipore, MA) purification system. All other chemicals and solvents were of analytical purity or HPLC grade, respectively.

### In-vitro dispersion and digestion studies

#### *Preparation of lipid-based formulations for in-vitro studies*

The compositions of the different study formulations and their notations are listed in Table 1 and are based on similar self-emulsifying formulations that have previously been shown to enhance the bioavailability of halofantrine and danazol (Khoo et al 1998; Porter et al 2004a). All formulations were based on the same lipid mixture (1:1 w/w blend of soybean oil and Maisine 35-1), but differed in the nature of the added surfactant. The formulations contained either Cremophor EL or one of a series of Pluronic surfactants of varying HLB. The ratio of surfactant to lipid mixture was kept constant to permit direct assessment of the impact of surfactant on the formulations' behaviour (Table 1). No attempt was made to match the self-emulsifying properties of the different formulations as this was not possible without dramatically changing the relative proportions of the included components. A control formulation (LC-SOLN) with no surfactant was also prepared. The formulations were prepared by weighing atovaquone into a glass vial, followed by the addition of the lipid mixture and surfactant (where

**Table 1** Composition of the formulations containing atovaquone (ATQ) investigated in the in-vitro dispersion and digestion studies

| Formulation | Surfactant | HLB | Formulation components | | | | ATQ solubility in the formulation ($mg\,g^{-1}$) |
|---|---|---|---|---|---|---|---|
| | | | ATQ (mg) | Surfactant (mg) | Lipid mixture[a] (mg) | Ethanol ($\mu$L) | |
| LC-SOLN | Surfactant-free | — | 2.5 | — | 900 | 100 | 5.4 |
| LC-CREL | Cremophor EL | 12 | 2.5 | 300 | 600 | 100 | 9.4 |
| LC-PL121 | Pluronic L121 | 0.5 | 2.5 | 300 | 600 | 100 | 7.9 |
| LC-PL61 | Pluronic L61 | 3 | 2.5 | 300 | 600 | 100 | 8.3 |
| LC-PL72 | Pluronic L72 | 6.5 | 2.5 | 300 | 600 | 100 | 8.8 |
| LC-PL43 | Pluronic L43 | 12 | 2.5 | 300 | 600 | 100 | 8.8 |
| LC-PF68 | Pluronic F68 | 29 | 2.5 | 300 | 600 | 100 | —[b] |

[a]The lipid mixture was a 1:1 w/w blend of long-chain triglyceride (soybean oil, LCT) and Maisine 35-1 (long-chain $C_{18}$ mono-/diglycerides).
[b]Solubility in the LC-PF68 formulation could not be measured as it was a semi-solid paste.

required), and mixed by vortexing until all the atovaquone had dissolved. On cooling to ambient temperature ethanol was added and the mixtures vortexed before equilibrating overnight before use.

*Atovaquone solubility in lipid vehicles*

The equilibrium solubility of atovaquone in each of the lipid vehicles was assessed by addition of excess atovaquone to glass vials containing blank (atovaquone-free) formulations. Vials were incubated at 37°C and vortexed periodically (~ 6 times per day over a period of up to 1 week). At set time points, samples were centrifuged at 1600 *g* for 30 min and a portion removed for analysis of atovaquone by HPLC. Complete separation of atovaquone from the lipid samples was confirmed by viewing under cross-polarised light. Equilibrium solubility was assumed to be attained when consecutive readings were within ± 5%.

*In-vitro dispersion and particle size evaluation*

The dispersion and emulsification characteristics of the formulations were assessed as previously described (Khoo et al 1998). Briefly, 1 mL of formulation was added drop-wise to 200 mL of either water or 0.1 M HCl maintained at 37°C in a USP II dissolution apparatus. Gentle agitation was provided by a stainless-steel dissolution paddle mounted immediately below the solution meniscus and operated at 60 rev $min^{-1}$. The relative dispersibility of the formulations was assessed visually, and the mean particle size of the resulting dispersion was determined by photon correlation spectroscopy (PCS) using a Malvern Zetasizer 3000.

*In-vitro digestion and atovaquone solubility in blank digest aqueous phases*

The in-vitro digestion experiments were performed as previously described (Kaukonen et al 2004) using a simulated fasted-state digestion buffer (50 mM Tris maleate, 150 mM NaCl, 5 mM $CaCl_2 \cdot 2H_2O$, pH 7.5) containing 5 mM bile salts and 1.25 mM phosphatidylcholine (Hay et al 1993; Duane et al 1976). Experiments were run at a pH (7.5) slightly higher than the expected luminal pH to facilitate more effective ionisation (and therefore titration) of liberated fatty acid. In each case 250 mg of formulation was digested at 37°C in a total volume of 10 mL of digestion buffer by addition of 1000 U $mL^{-1}$ of pancreatin extract. The fatty acids produced were titrated to maintain pH 7.5 using a pH stat apparatus (Radiometer Analytical, Lyon, France). Following a 30-min digestion period, 50 $\mu$L of 1 M *p*-bromophenyl boronic acid was added to inhibit further lipolysis. At the end of each experiment two 4-mL samples of the post-digestion mixture were ultracentrifuged for 30 min at 37°C and 334 000 *g* (Optima XL-100K centrifuge, SW-60 rotor; Beckman, Palo Alto, CA) to separate the digests into a floating oily phase (where present), an aqueous dispersed phase and a pellet phase. Samples obtained from each phase were assayed for atovaquone content by HPLC as described below. The equilibrium solubility of atovaquone in blank aqueous phases was also measured. This was achieved by conducting blank digestions as described above (but where drug was omitted from the formulation) and separating samples of the blank aqueous phase into glass vials. Excess solid atovaquone was then added before vortexing for 1 min to mix and wet the powder. Samples were periodically vortexed (~ 6 times per day over a period of up to 1 week) and incubated at 37°C. At set time points, samples were centrifuged at 1600 *g* for 30 min and a portion was removed for analysis by HPLC for atovaquone content. Equilibrium solubility was assumed to be attained when consecutive readings were within ± 5%.

*Assay for lipids and lipid digestion products using high performance thin-layer chromatography (HPTLC)*

At the end of the 30-min digestion period, separated oil, aqueous and pellet phases were also analysed for their lipid content (triglyceride, TG; diglyceride, DG; monoglyceride, MG; and fatty acids, FA) using a previously validated HPTLC method (Sek et al 2001).

*Sample preparation and HPLC analysis of atovaquone-containing digestion samples*

Samples of atovaquone in oil and pellet phases were initially dissolved in chloroform–methanol (2:1, v/v), then diluted as required in methanol–DMF (99:1, v/v)

before analysis by HPLC. Aqueous phase samples were diluted as required in methanol–DMF (99:1, v/v) before HPLC analysis. Calibration standards for atovaquone were prepared by dilution of a stock solution of 100 $\mu$g mL$^{-1}$ atovaquone in methanol–DMF (99:1, v/v) to give concentrations of 0.05, 0.1, 0.25, 2.5 and 5.0 $\mu$g mL$^{-1}$. Unknown concentrations were determined by comparison of the unknown atovaquone peak area with the standard curve.

Atovaquone chromatography was conducted using a Waters 510 pump, a Waters 717 Autosampler, a Waters tuneable absorbance detector (Millipore, Bedford, MA, USA) set at 252 nm, an Ultrasphere $C_8$ bonded reverse-phase column (5 $\mu$m particle size, 4.6 × 250 mm; Beckman Instruments, Palo Alto, CA, USA) and a Newguard RP-8 cartridge guard column (Aquapore 7 $\mu$m, 3.2 × 5 mm; Applied Biosystems, Foster City, CA, USA). Peak data were analysed on a Shimadzu C-R5A Chromatopac integrator (Shimadzu Corp., Kyoto, Japan). The mobile phase comprised acetonitrile–water–trifluoroacetic acid (70:30:0.4, v/v/v). The flow rate was 1.2 mL min$^{-1}$ and all analyses were carried out at room temperature.

The assay was validated by analysis of n = 5 quality control samples made up at three different concentrations (0.1, 2.5, 5 $\mu$g mL$^{-1}$). Inter-assay variability was assessed on five different days. Intra-assay validation revealed accuracy of 97.8%, 98.5% and 119.9% and precision to within ± 0.8%, 0.4% and 1.1% of target at 0.1 $\mu$g mL$^{-1}$, 2.5 $\mu$g mL$^{-1}$ and 5.0 $\mu$g mL$^{-1}$, respectively. Inter-assay validation revealed accuracy of 108.5%, 98.4% and 100.0% and precision to within ± 6.4%, 1.5% and 1.3% of target at 0.1 $\mu$g mL$^{-1}$, 2.5 $\mu$g mL$^{-1}$ and 5.0 $\mu$g mL$^{-1}$ respectively.

## In-vivo atovaquone bioavailability studies

*Formulations for oral administration*

The compositions of the formulations administered in the oral bioavailability study are listed in Table 2. Lipid-based formulations were prepared and individually filled into soft gelatin capsules by syringe as described previously (Khoo et al 1998). The three lipid formulations contained 5 mg atovaquone/1 g of formulation. The ethanol content differed slightly in the LC-CREL formulation to avoid phase separation that was found to occur at higher ethanol concentrations after filling into capsules. The aqueous suspension formulation (AQ-SUSP) was prepared by dilution of the commercially available aqueous suspension of atovaquone (Wellvone, 750 mg g$^{-1}$; GlaxoSmithKline, UK) with 0.5% (w/v) aqueous Xanthan gun (Sigma Chemical Co., St Louis, MO, USA) to a concentration of 15 mg g$^{-1}$. The suspension was equilibrated overnight before encapsulation immediately before dosing.

*Administration and sampling*

All experimental procedures were approved and performed in accordance with the guidelines of the local Institutional Animal Experimentation Ethics Committee. The study was conducted as a four-treatment crossover in three fasted male beagle dogs with a 21-day washout period between treatments. The treatments involved either three capsules of each lipid formulation (LC-SOLN, LC-CREL or LC-PL121), containing 1 g of formulation per capsule, or a single capsule of the aqueous suspension formulation (AQ-SUSP), such that the total dose of atovaquone administered per treatment was 15 mg. Each oral treatment was administered with 50 mL of water. Water was freely available throughout the study period. Food was withheld for a period of 12 h before dosing. Dogs were fed approximately 200 g of standard dry dog food at 10 h and 28 h post dose, then daily thereafter. Blood samples (2.5 mL) were collected pre dose (−10 min) and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 10 h post dosing from the cephalic vein via an indwelling cannula. The cannula was flushed with approximately 2 mL heparinised saline (2 IU mL$^{-1}$) after each blood sample to maintain cannula patency. Later samples were obtained at 24, 28, 50 and 73 h post dosing by individual venipuncture. Samples were collected into sterile tubes containing 4.5 mg of dipotassium edetate. Plasma was separated by centrifugation and stored at −20°C before atovaquone analysis by HPLC.

*Plasma sample preparation*

Calibration standards for atovaquone were prepared by spiking 0.5-mL volumes of blank dog plasma with 100 $\mu$L of a methanol–DMF (99:1, v/v) solution containing 0.10, 0.50, 1.0, 2.5 or 5.0 $\mu$g mL$^{-1}$ atovaquone. This provided plasma standards in the concentration range of 20–1000 ng mL$^{-1}$ atovaquone. A 100-$\mu$L volume of methanol–DMF (99:1, v/v) solution was also added to the plasma samples to compensate for the volume of the spiking solution added to plasma standards. A 100-$\mu$L volume of an internal standard (IS) solution (5.0 $\mu$g mL$^{-1}$ of *trans*-2-hydroxyl-3-(4-phenylcyclohexyl)-1,4-naphthalenedione in

**Table 2** Composition of formulations for oral administration to fasted beagle dogs

| | Formulation components | | | | | Capsules per subject |
|---|---|---|---|---|---|---|
| | Atovaquone (mg) | Surfactant (mg) | Lipid mixture[a] (mg) | Ethanol ($\mu$L) | Aqueous vehicle (mg) | |
| AQ-SUSP | 15 | — | — | — | 985 | 1 |
| LC-SOLN | 5 | — | 900 | 100 | — | 3 |
| LC-CREL | 5 | 310 | 620 | 70 | — | 3 |
| LC-PL121 | 5 | 300 | 600 | 100 | — | 3 |

methanol–DMF (99:1, v/v)) was also added to each plasma sample or standard and the tubes vortexed for 1 min. Aqueous $ZnSO_4.7H_2O$ (10% w/v, 0.5 mL) and NaOH (0.5 M, 0.5 mL) were subsequently added to the samples and the mixture vortexed for 2 min to precipitate plasma proteins. An 8-mL volume of *tert*-butyl methyl ether was then added and each tube was vortexed for 10 min before centrifugation for 15 min at 1600 *g* in a Beckman GS-6R refrigerated centrifuge at 25°C. The organic phase was transferred into new tubes and the contents evaporated to dryness under nitrogen at approximately 30°C using an N-EVAP evaporator (Organomation, USA). The dried extracts were reconstituted with 100 $\mu$L of methanol–DMF (99:1, v/v) and vortexed for a further 5 min, before transfer to limited volume autosampler vials and injection of 25 $\mu$L onto the HPLC.

Unknown concentrations were determined by comparison of the unknown atovaquone:IS peak height ratios with the calibration curve of atovaquone:IS peak height ratio versus concentration constructed using the calibration standards. The plasma assay was validated by analysis of $n = 5$ quality control samples made up at three different concentrations between 20 and 1000 ng mL$^{-1}$. The assay was found to be accurate to 100.6%, 106.4% and 105.4% and precise to within ± 10.8%, 2.9% and 2.8% at 20 ng mL$^{-1}$, 200 ng mL$^{-1}$ and 1000 ng mL$^{-1}$, respectively.

*Atovaquone plasma sample chromatography*

Chromatography was conducted using a Waters 2690 separation module (Alliance solvent delivery system), a Waters tuneable absorbance detector (Millipore, Bedford, MA, USA) set at 252 nm, an Ultrasphere $C_8$ bonded reverse-phase column (5 $\mu$m particle size, 4.6 × 250 mm; Beckman Instruments, Palo Alto, CA, USA) and a Newguard RP-8 cartridge guard column (Aquapore 7 $\mu$m, 3.2 × 5 mm; Applied Biosystems, Foster City, CA, USA). Peak data were analysed on a Shimadzu C-R5A Chromatopac integrator (Shimadzu Corp., Kyoto, Japan). Gradient elution occurred over 30 min using a mobile phase comprising three solvents systems. Solvent A comprised acetonitrile–0.1% (v/v) aqueous trifluoroacetic acid (TFA) (90:10); Solvent B was acetonitrile–0.1% (v/v) aqueous TFA (10:90); and Solvent C was 100% acetonitrile. The initial mobile phase was Solvent A–Solvent B (70:30). The mobile phase composition was changed to Solvent A–Solvent B (75:25) over the first 10 min of the analytical run and then held at that composition for 5 min. The composition was then changed to Solvent C 100% over a period of 1 min, and held at 100% C for a further 8 min. The composition was then returned to Solvent A–Solvent B (70:30) over a period of 1 min and held for 5 min until the end of the run. Total run time was 30 min. The flow rate was 1.2 mL min$^{-1}$ and all analyses were carried out at room temperature.

*Pharmacokinetic data analysis*

Non-compartmental analysis was employed to determine pharmacokinetic parameters using WinNonlin Version 3.2 (Pharsight Corporation, Mountain View, CA, USA). The maximum plasma concentrations ($C_{max}$) and the corresponding time of maximum plasma concentration ($T_{max}$) were determined directly from the atovaquone plasma concentration vs time profiles. The terminal elimination rate constant ($k_e$) was determined using the slope of the terminal portion of the log atovaquone plasma vs time curve over the 23–73 h time period. Area under the atovaquone plasma concentration vs time profiles ($AUC^{0-73}$) were calculated using the linear trapezoidal method, and relative bioavailability comparisons for the lipid formulations were generated using the ratio of $AUC^{0-73}$ obtained for the lipid formulations vs that obtained for the aqueous suspension.

## Results

### Dispersibility and particle size

Stirring of the LC-CREL and LC-PF68 formulations for 30 min in either water or 0.1 M HCl yielded completely or partly-emulsified dispersions, respectively. Particle size determinations by PCS showed that the LC-CREL formulation dispersed to form small particles with mean particle sizes of 196.6 ± 1.9 nm and 149.0 ± 1.5 nm (Z-ave mean ± s.d., $n = 3$) in water and 0.1 M HCl, respectively. Dispersion of the LC-PF68 formulation yielded larger particles with diameters of 439.1 ± 4.0 nm and 424.2 ± 3.0 nm (Z-ave mean ± s.d., $n = 3$) in water and 0.1 M HCl, respectively. The particle size determinations were in agreement with visual observations. The remaining four surfactant-containing formulations did not show improved dispersion properties over the surfactant-free lipid formulation, which phase separated into grossly dispersed lipid droplets. Particle size determinations were not possible for these poorly dispersed systems.

### Influence of surfactants on digestion of long-chain glycerides

The progress of in-vitro lipid digestion of the surfactant-containing lipid formulations and surfactant-free control lipid vehicle was monitored by pH-stat titration and was performed in the presence of 1.25 mM lecithin and 5 mM bile salts. The lecithin/bile salt concentrations were chosen to reflect the likely concentration range encountered in human fasted duodenal contents (Duane et al 1976; Hay et al 1993). Figure 2 shows the rate of fatty acid liberation over the 30-min digestion period and Figure 3 shows the patterns of distribution of the lipid digestion products across the aqueous phase, pellet and undispersed oil phase at the end of the digestion period. In all cases, addition of surfactant increased the rate and extent of fatty acid production, although this effect was marginal for systems containing Pluronic F68. Cremophor EL (HLB 12) provided the largest increase in lipid digestion, and production of fatty acid was increased by approximately 54% above that of control. However, it was also apparent that the ethoxylated glycerides present in Cremophor EL were themselves digested by pancreatic lipase/colipase,

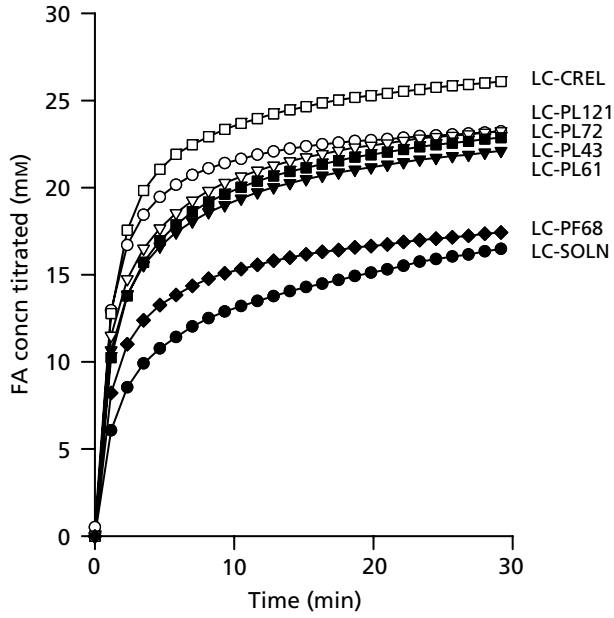


**Figure 2** Profiles of fatty acid (FA) titrated with 0.6 M NaOH during 30 min digestion of LCT/$C_{18}$ mono-/diglycerides blend (1:1 w/w) under low (1.25 mM lecithin:5 mM bile salts) lecithin/bile salts conditions without surfactants (closed circles, control) and with the addition of Cremophor EL (HLB 12; LC-CREL; open squares), Pluronic L121 (HLB 0.5; LC-PL121; open circles), Pluronic L61 (HLB 3.0; LC-PL61; closed triangles), Pluronic L72 (HLB 6.5; LC-PL72; open triangles), Pluronic L43 (HLB 12; LC-Pl43; closed squares), and Pluronic F68 (HLB 29; LC-PF68; closed diamonds). The digestion mixture initially comprised of 250 mg lipid formulation and pancreatic lipase/colipase (1000 TBU $mL^{-1}$) in standard digestion buffer (5 mM $CaCl_2.2H_2O$, 150 mM NaCl and 50 mM Tris-maleate, pH 7.5) at 37 °C. Data are the mean of duplicate determinations. In all cases the range of duplicate measurements was within ± 10% of the mean.

leading to production of fatty acids from the digestion of surfactant, in addition to digestion of the lipid components in the formulation. In a separate control experiment, in-vitro digestion of the same quantity of Cremophor EL as that contained in the lipid formulations (75 mg) led to the liberation of approximately 5 mM of titratable fatty acid in the digestion mixture, suggesting that at least some of the difference between the LC-CREL formulation and the other Pluronic-containing formulations could be explained by the digestibility of the Cremophor surfactant. In contrast, none of the Pluronic surfactants were digested by pancreatin. Closer inspection of Figure 3, however, provides further evidence that the LC-CREL formulation was more completely digested than many of the other formulations, since complete digestion of TG and the lack of a persisting oil phase at the end of the digestion period were evident. Surprisingly, very similar data was obtained on digestion of the LC-L121 formulation containing the extremely lipophilic surfactant Pluronic L121. In contrast, the digestion profile for the LC-PF68 formulation containing Pluronic F68, the most hydrophilic Pluronic, was not markedly different to the surfactant-free lipid solution formulation. The formulations containing the Pluronics of intermediate HLB (LC-PL61, LC-PL72 and LC-PL43), gave similar profiles and the extent of digestion lay between the values obtained for formulations containing Pluronic L121 and F68.

## Solubilisation behaviour of atovaquone during in-vitro digestion of lipid vehicles

The concentrations of atovaquone in the aqueous phase of the digests and the proportion of atovaquone present in each of the digestion phases obtained following digestion of the surfactant-containing lipid formulations and the surfactant-free control lipid formulation are presented in Table 3. For the formulations containing Cremophor EL (LC-CREL) and Pluronic L121 (LC-PL121), digestion was essentially complete after 30 min, and these digests therefore separated into an aqueous solubilised phase and pellet phase only (i.e. no remaining oil phase) after ultracentrifugation. In both cases, a large proportion of the atovaquone was solubilised in the aqueous phase (95–97%) and only a small proportion of the drug was recovered in the pellet (3–5%), after digestion of the formulations.

A relatively high proportion of the atovaquone was also solubilised in the aqueous phase obtained after digestion of the lipid formulations containing the other Pluronic surfactants (i.e. those with HLB values in the range of 3–12), although in this case, digestion was not complete and an undigested oil phase was present after 30 min digestion. However, in all cases, only a small proportion (11–17%) of the atovaquone remained in the undigested oil phase. As expected, a greater quantity of lipid remained undigested and undispersed in the oily phase following digestion of the surfactant-free control formulation and a large proportion of the atovaquone present (67%) remained sequestered in this phase. A similar profile was also evident on digestion of the LC-PF68 formulation. In no case, however, did digestion of the lipid-based formulations result in significant amounts of drug precipitation and > 90% of the atovaquone remained solubilised in either the dispersed aqueous phase or the remaining oil phase.

## Equilibrium solubility of atovaquone in blank aqueous digestion phases

To determine whether the atovaquone concentrations attained in the aqueous phase of the digests were simply a reflection of the solubility of drug in the phase formed, drug-free blank digests were conducted and the equilibrium solubility of atovaquone in the blank aqueous phases assessed. In all cases the equilibrium solubility of atovaquone in the blank aqueous phases was considerably lower than the trafficked concentrations obtained at the end of the digestion period, suggesting that digestion resulted in supersaturation of the aqueous phase. On allowing the aqueous phases obtained post digestion to stand overnight, drug precipitation was visually evident, further supporting this concept. In general the rank order

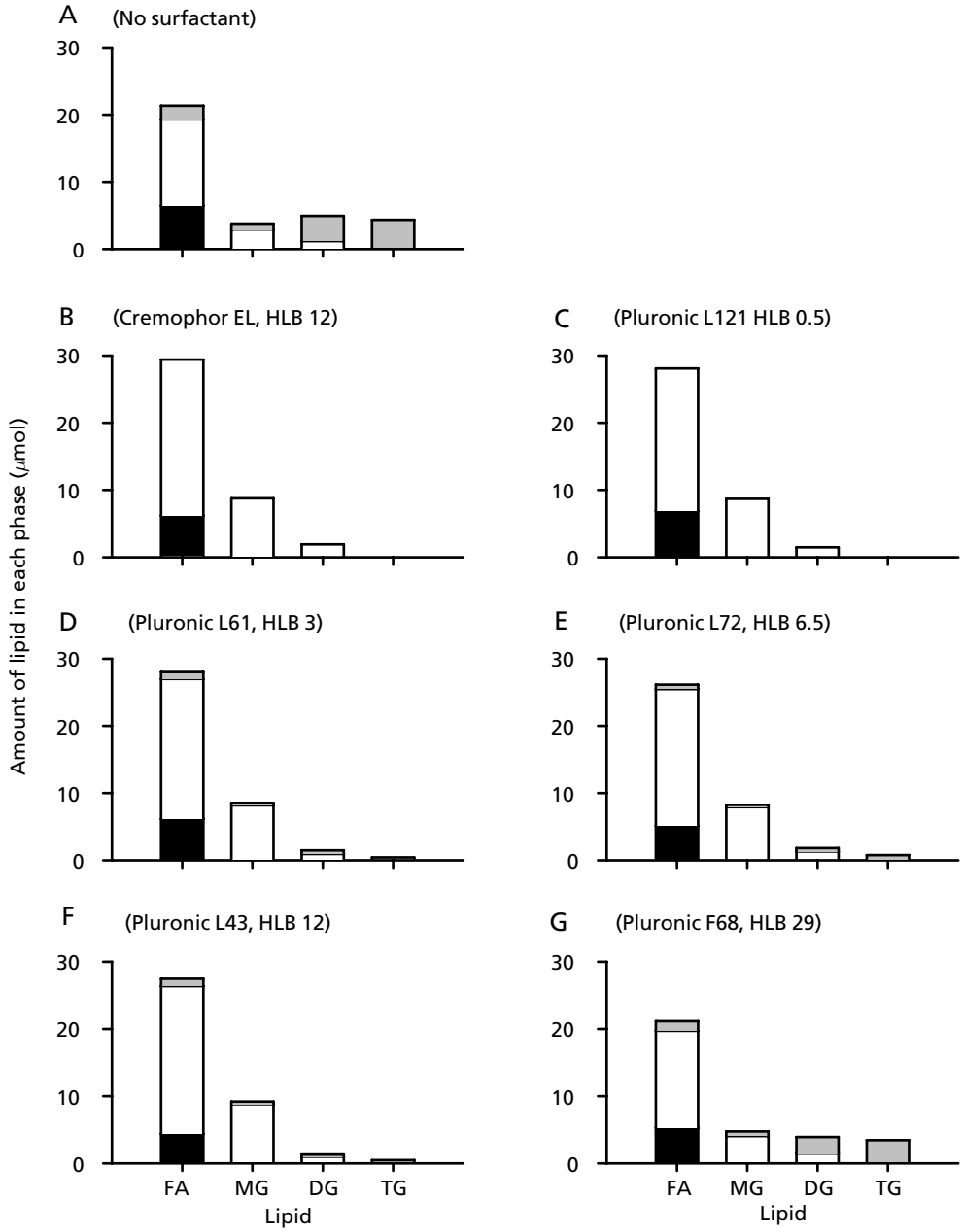


**Figure 3** Distribution of long-chain lipid digestion products across the oily (grey fill), aqueous phase (white fill) and pellet fraction (black fill) formed following 30 min digestion without (A) and with (B–G) surfactants. The digestion mixture initially comprised 250 mg long-chain glyceride lipids and pancreatic lipase/colipase (1000 TBU $mL^{-1}$) in standard digestion buffer (5 mM $CaCl_2.2H_2O$, 150 mM NaCl and 50 mM Tris-maleate, pH 7.5) at 37°C. Data presented are the amount of each particular lipid recovered in each separated phase per 1 mL of digest. Data are the mean of duplicate determinations. In all cases the range of duplicate measurements was within ± 10% of the mean.

of solubility in the phases and the atovaquone concentration obtained at the end of the digestion period were consistent and higher drug concentrations were obtained in the digests obtained from formulations containing the lower HLB Pluronics. The LC-CREL formulation, in contrast, showed markedly different behaviour and the equilibrium solubility of atovaquone in the LC-CREL blank digests was quite low relative to the other formulations, whereas the trafficked atovaquone concentrations were higher than the other formulations. As such, the degree of supersaturation of the aqueous phase obtained post digestion of the LC-CREL formulation was higher than that of the other systems.

### Oral bioavailability studies

The mean plasma atovaquone concentration vs time profiles following oral administration of atovaquone in the

**Table 3** Solubilisation and distribution of atovaquone (ATQ) into the aqueous phase, oil phase and pellet phases formed on digestion of formulations. Equilibrium solubility of ATQ in blank digests at 37 °C is also reported

| | Concn in aqueous phase after digestion ($\mu g\,mL^{-1}$) | Proportion of ATQ distributed to phases after digestion (%) | | | Solubility in blank aqueous phase ($\mu g\,mL^{-1}$) |
|---|---|---|---|---|---|
| | | Aqueous | Oil | Pellet | |
| LC-SOLN | 20.6 | 30.4 | 66.9 | 2.7 | 12.5 |
| LC-CREL | 70.8 | 97.3 | 0 | 2.7 | 27.2 |
| LC-PL121 | 70.6 | 95.2 | 0 | 4.8 | 46.7 |
| LC-PL61 | 64.9 | 76.9 | 17.4 | 5.7 | 37.8 |
| LC-PL72 | 67.4 | 83.9 | 11.2 | 4.9 | 37.4 |
| LC-PL43 | 63.6 | 80.7 | 14.2 | 5.1 | 25.9 |
| LC-PF68 | 24.3 | 33.4 | 61.5 | 5.1 | 12.8 |

Data are the mean of duplicate determinations. In all cases the range of duplicate measurements was within ± 10% of the mean.

four formulations, LC-SOLN, LC-CREL, LC-PL121 and AQ-SUSP, are shown in Figure 4A. To aid comparison of $C_{max}$ and $T_{max}$, truncated data to 10 h are presented in Figure 4B. Mean pharmacokinetic parameters for all dose forms are presented in Table 4.

The terminal elimination half-lives (t½) for atovaquone were consistent across all formulations and of the order of 40 h in each case. Administration of atovaquone in all of the lipid-based formulations resulted in a significant (approx. 3.5-fold) increase in oral bioavailability when compared with atovaquone administration as a simple suspension formulation (Figure 4, Table 4). In contrast, no significant differences were apparent across the different lipid formulations and the increase in atovaquone bioavailability was essentially the same after administration of the poorly dispersed lipid solution formulation (LC-SOLN), the poorly dispersed Pluronic L121-containing formulation (LC-PL121) or the highly dispersed Cremophor EL-containing formulation (LC-CREL).

## Discussion

Enhanced dispersion and a reduction in the particle size of the lipid droplets formed on interaction of lipidic formulations with gastrointestinal fluids is commonly cited as a mechanism by which the absorption, and therefore bioavailability, of incorporated poorly water-soluble drugs may be improved. Surfactants are therefore often added to lipid-based delivery systems in an attempt to improve formulation dispersion characteristics (and in the majority of cases also to improve the drug-loading capacity of the formulation, since most poorly water-soluble drugs are more soluble in surfactant mixtures than pure lipids). This study has evaluated the effect of the addition of a range of surfactants to lipid-based formulations of atovaquone, and has specifically evaluated the effect of surfactant substitution on the ability of the formulation to self-emulsify, on their in-vitro digestion behaviour and on the resulting oral bioavailability of atovaquone after oral administration in formulations

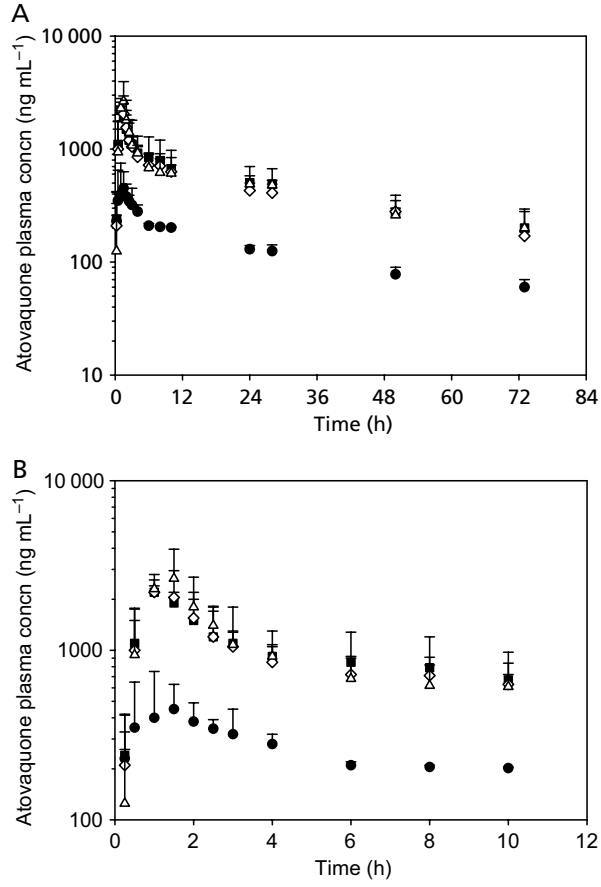


**Figure 4** Mean plasma concentration vs time profiles (mean ± s.e., n = 3) for atovaquone following oral administration to beagle dogs. A total dose of 15 mg atovaquone was administered for each treatment. Three treatments involved administration in the fasted state as lipidic solutions containing atovaquone as a 5 mg $g^{-1}$ solution (3 × soft gelatin capsules) in LC-SOLN (open triangles), LC-CREL (open diamonds), or LC-PL121 (closed squares). The fourth treatment was 15 mg atovaquone in aqueous suspension (closed circles) (1 × soft gel capsule). A. Resulting plasma profiles obtained over the entire sampling period (73 h). B. Data truncated to 10 h to facilitate easier comparison of $C_{max}$ and $T_{max}$. LC-SOLN, long-chain lipids without surfactant; LC-CREL, long-chain lipids formulated with Cremophor EL; LC-PL121; long-chain lipids formulated with Pluronic L121.

**Table 4** Summary pharmacokinetic parameters after oral administration of atovaquone formulations to fasted male beagle dogs

| Formulation | $C_{max}$ ($\mu$g mL$^{-1}$) | $T_{max}$ (h) | Elimination t½ (h) | $AUC_{0-73h}$ ($\mu$g.h mL$^{-1}$) | Rel BA[a] (%) |
|---|---|---|---|---|---|
| LC-SOLN | 2.98 ± 0.98 | 1.2 ± 0.3 | 44.7 ± 18.1 | 31.8 ± 9.3 | 338 ± 99 |
| LC-CREL | 2.37 ± 0.13 | 1.2 ± 0.3 | 33.3 ± 10.1 | 31.8 ± 8.4 | 338 ± 89 |
| LC-PL121 | 2.17 ± 0.34 | 1.0 ± 0.0 | 37.8 ± 3.7 | 33.7 ± 13.0 | 358 ± 138 |
| AQ-SUSP | 0.56 ± 0.09 | 1.8 ± 1.0 | 42.6 ± 6.5 | 9.4 ± 1.0 | 100 |

Data are means ± s.d., n = 3. A total dose of 15 mg atovaquone was administered for each treatment. [a]Rel BA, relative bioavailability in comparison with fasted administration of the atovaquone aqueous suspension (nominally assigned a value of 100%), calculated from the ratios of the $AUC_{0-73h}$ values.

with demonstrably different in-vitro dispersion and digestion properties.

### Influence of exogenous surfactants on lipid-based formulation dispersion and digestion

The digestion profile of the surfactant-free soybean oil/Maisine 35-1 blend (LC-SOLN) was in qualitative agreement with previous data (Sek et al 2002), and showed a rapid rate of lipolysis in the first 5 min of digestion, followed by a marked slowing in digestion rate and incomplete digestion at the end of the 30-min experimental period. Since pancreatic lipase/colipase acts at the oil–water interface, the rate and extent of lipolytic product removal from the interface is a major determinant of the progress of long-chain triglyceride digestion (Patton & Carey 1979). The reduction in the rate of lipolysis at later time points therefore most likely reflects saturation of lecithin/bile salt micelles with lipolytic products and the accumulation of long-chain lipolytic products on the droplet surface preventing pancreatic lipase/colipase binding. The inclusion of Cremophor EL (HLB 12) in the LC-CREL formulation resulted in a formulation that dispersed rapidly and completely on contact with the digestion media, providing a relatively large surface area for attachment of the lipase–co-lipase complex, and for removal of lipolytic digestion products. Cremophor EL was effective in promoting the digestion of the soybean oil/Maisine 35-1 blend and was itself digested, leading to the production of additional long-chain fatty acids, providing further long-chain lipids for incorporation into lecithin/bile salt mixed micelles.

With the exception of the LC-PF68 formulation, substitution of Cremophor EL with any of the series of Pluronic surfactants studied resulted in lipid formulations that dispersed poorly when added to aqueous media but which nevertheless increased the extent of lipid digestion relative to the control lipid solution formulation, regardless of their poor initial dispersion properties. In contrast the LC-PF68 formulation dispersed more effectively, but failed to substantially increase the extent of lipid digestion.

The exact mechanisms by which the different surfactants facilitate the digestion process is still not clear, although Cremophor EL is known to form micelles in aqueous solution, and may promote digestion by allowing intercalation of lipid digestion products into the micellar structures, in a manner analogous to bile salts. The efficiency of lipid digestion, however, is likely to be a complex function of the structures that form on integration of endogenous lecithin and bile salts with exogenous surfactants and the capacity of these structures to solubilise lipid digestion products such as fatty acids and monoglycerides. Whilst the HLB likely plays a role in determining the structures formed, solubilisation behaviour is also likely to be dependent on the specific structures of the incorporated surfactant, since the Pluronic surfactants with equivalent or higher HLB values to that of Cremophor EL were less able to facilitate digestion. For example, Pluronic L43, which like Cremophor EL has an HLB of approximately 12, and the very hydrophilic Pluronic F68, which is also known to form micellar structures in aqueous solution (Constantinides 1995), were particularly poor at improving digestion. In fact, for the Pluronic series, the extent of digestion increased with reducing HLB value of incorporated surfactant, possibly reflecting an increase in available hydrophobic solubilisation volume with reducing HLB. For the very low HLB Pluronics, such as Pluronic L121 which is essentially insoluble in water, the surfactant may rely on the presence of ionised fatty acids, lecithin and bile salts to act as co-surfactants to aid in the stabilization of the colloidal structures formed on digestion. Although the more water-soluble Pluronics, such as Pluronic F68, more readily form micelles in aqueous solution, the proportion of the surfactant mass that makes up the hydrophobic core of the micellar structure is lower and may restrict the solubilisation capacity for digestion products leading to a reduced extent of digestion.

Interestingly, the data obtained here are in contrast to those presented in a previous investigation, which described a substantial inhibition of medium-chain triglyceride (MCT) lipid digestion in the presence of nonylphenol ethoxylate surfactants with HLB values in the range of 12–15 (present in a 1:1 mass ratio with the lipid) (Solomon et al 1996). In the previous study, the inhibitory effect of nonylphenol ethoxylates on MCT digestion by pancreatic lipase/colipase was suggested to be due to the thickness of the hydrated oxyethylene mantle produced by adsorption of the surfactant at the surface of oil–surfactant droplets preventing binding between pancreatic lipase/colipase and the triglyceride substrate. The discrepancies between the earlier study and this investigation may reflect differences in the surfactant series examined or the lipid substrates used (previous studies used MCT as opposed to the long chain soybean oil/Maisine 35-1 blend utilised in this study). Medium-chain digestion products are more hydrophilic than long-chain lipids and are less dependent on micellar solubilisation to promote aqueous

dispersion and digestion (Sek et al 2002). In contrast, the digestion of long-chain glyceride lipids is limited by dispersion of the digestion products into the aqueous phase, a process that is likely to be enhanced by the presence of the included surfactants. The increase in digestion resulting from enhanced solubilisation of digestion products may therefore override any digestion inhibition caused by surfactant adsorption onto the surface of digesting oil droplets.

### Effect of lipids and surfactants on atovaquone solubilisation behaviour during digestion

The solubilisation profiles of atovaquone across the physical phases obtained following digestion of the surfactant-containing formulations typically reflected the extent of digestion and the distribution profile of the liberated digestion products. Thus the LC-CREL and LC-PL121 formulations, where digestion was most effective, also led to attainment of the highest aqueous phase concentrations of atovaquone and the lowest levels of drug precipitation. Similarly for LC-PF68, where digestion and dispersion of digestion products was relatively inefficient and similar to the profile obtained for the surfactant-free lipid solution, a substantial proportion of the atovaquone remained associated with the undigested and undispersed lipid phase at the end of the digestion period, effectively mirroring the atovaquone profiles seen with the control lipid solution formulation. No significant differences in atovaquone solubilisation were evident across the other Pluronic-containing formulations, again reflecting the similar profiles of lipid digestion product solubilisation. Interestingly, the aqueous phase concentrations of atovaquone obtained after in-vitro digestion of all the atovaquone lipid formulations were higher than the respective equilibrium solubilities in identical aqueous phases obtained after ultracentrifugation of drug-free lipid digests. These data suggest that supersaturation of the aqueous phase occurred under digestion conditions. The mechanism supersaturation of this phase is unclear, although previous studies have suggested the existence of a hydrophobic continuum, linking dispersed oil droplets with the liquid crystalline product phases formed on the surface of a lipid droplet during lipid digestion (Patton & Carey 1979). The existence of this direct pathway for the flow of an incorporated molecule from digesting oil droplets to the micellar/vesicular phase (without requiring partition into the aqueous phase) may help to promote the formation of a supersaturated phase immediately following lipid digestion (Kaukonen et al 2004).

These data are in general agreement with the recent studies described by Zangenberg et al (2001b), which examined correlations between increased drug solubilisation and aqueous phase lipolytic product concentrations, using a similar in-vitro lipid digestion model to investigate the solubilisation behaviour of danazol and probucol following digestion of soybean oil-in-water emulsions (emulsified with Tween 80 and Span 80). A good correlation was seen between the solubilisation of danazol (log P 4.5) and the aqueous phase concentration of lipids and bile salts, although for the more lipophilic drug probucol (log P $\sim$ 10), the correlation was less effective due to the preferential partitioning of the drug into an undigested oil phase.

In our studies, where formulation digestion was incomplete, a residual oil phase was present and a significant proportion of the atovaquone remained solubilised within this undigested and poorly dispersed phase (Table 3). However, recent studies examining the solubilisation behaviour of halofantrine on digestion of simple triglyceride lipid solutions suggest that although digestion may be incomplete under some conditions in-vitro, limiting transfer of incorporated drug into the aqueous phase, the key aspect to in-vivo performance is avoidance of drug precipitation (Kaukonen et al 2004). In this regard all the lipid-based formulations examined in the current study effectively prevented drug precipitation during in-vitro digestion and were therefore expected to provide for significant improvements in in-vivo bioavailability when compared with a non-solubilised suspension formulation.

### Effect of exogenous surfactants on the bioavailability of atovaquone from lipid-based formulations

While the in-vitro digestion studies suggested that all of the formulations examined might be expected to resist drug precipitation in-vivo, substantial differences in initial dispersion behaviour across the different systems were evident. Since previous studies have suggested that the particle size of the dispersed systems formed on interaction of lipidic formulations with the gastrointestinal fluids is a key determinant of in-vivo performance (Tarr & Yalkowsky 1989; Gao et al 1998), we subsequently sought to evaluate the relative oral bioavailability of atovaquone after administration in a selection of the formulations examined in-vitro in the first aspect of this study, but which displayed markedly different dispersion characteristics. To this end the LC-SOLN formulation comprising a simple solution of atovaquone in a soybean oil/Maisine 35-1 blend was examined to represent a simple, non-dispersible, surfactant-free, lipidic formulation, but which nonetheless was expected to be readily digested and dispersed in-vivo. The LC-CREL formulation was also evaluated as a representative example of a formulation that disperses extremely readily in-vitro to form emulsion or microemulsion droplets with particle sizes typically below approximately 250 nm (commonly referred to as self-emulsifying or self-microemulsifying drug delivery systems (SEDDS or SMEDDS)). A third formulation (LC-PL121), which displayed poor dispersibility characteristics but excellent solubilisation properties under in-vitro digestion conditions, was also examined. A simple aqueous suspension was administered to provide a reference point for exposure after administration in a non-solubilised, non-lipidic formulation.

Interestingly, while all the lipid-based formulations provided for a significant (3.5-fold) increase in atovaquone bioavailability when compared with the aqueous suspension

formulation, no significant differences in atovaquone exposure were apparent across the different formulations, which in turn had markedly differing initial dispersion properties. These results are consistent with the lack of drug precipitation evident in each case during in-vitro digestion and are also consistent with a previous study that examined the relative oral bioavailability of danazol after administration of long- and medium-chain SMEDDS formulations (Porter et al 2004a). In this previous study, while significant differences in danazol bioavailability were apparent after administration of the long- and medium-chain SMEDDS formulations, no differences in danazol bioavailability were seen after administration of the SMEDDS when compared with a poorly dispersed simple lipid solution formulation (Porter et al 2004a). Together these data provide further evidence that the initial physical form of a lipid-based formulation may be less important than the solubilisation capacity of the colloidal species that are eventually formed in-vivo once digestion of the formulation has occurred and the digestion products produced have become incorporated into lecithin/bile salt mixed micelles.

Although our data are consistent with previous results for danazol (Porter et al 2004a) and are also supported by, for example, studies with ontazolast, a lipophilic anti-inflammatory compound, where a simple soybean oil emulsion provided significantly greater bioavailability than a SMEDDS formulations at the same drug loading (Hauss et al 1998), it is also apparent that specific examples may be seen in the literature where administration of relatively surfactant-rich SMEDDS formulations appears to lead to an increase in drug bioavailability when compared with a less-well-dispersed lipid solution or emulsified formulation (Trull et al 1994, 1995; Julianto et al 2000). Of these examples, perhaps the best known is that of ciclosporin, where oral administration of the second generation Neoral SMEDDS formulation led to a significant increase in ciclosporin bioavailability when compared with the previous, and more poorly dispersed Sandimmun formulation. An unequivocal explanation for these differences is not evident at this time and may simply reflect subtle differences in the physicochemical properties of the drugs involved in the current and previous studies and their relative proclivities with regard to precipitation. However, it is also clear, at least in the case of ciclosporin, that bioavailability may be limited not only by solubility, but also by P-glycoprotein-mediated efflux and enterocyte-based first-pass metabolism (Wu et al 1995; Benet et al 1996). Under these circumstances, and in the light of studies that suggest that surfactants may inhibit efflux pumps such as P-glycoprotein (Nerurkar et al 1996; Solomon et al 1996), it is possible that the improved oral bioavailability of drugs such as ciclosporin when administered in surfactant-rich SMEDDS-like formulations, may reflect the capacity of certain formulation components to inhibit biochemical barriers to bioavailability, such as efflux pumps or enterocyte-based metabolism, rather than an effect on drug solubilisation per se. It is also apparent, however, that significantly more data are required to confirm this hypothesis.

## Conclusion

Examination of the in-vitro digestion and solubilisation profile of a well-dispersed SEDDS formulation of atovaquone (containing long-chain glycerides, Cremophor EL and ethanol) has shown that substantially improved lipid digestion and atovaquone solubilisation profiles may be obtained when compared with a surfactant-free control lipid formulation. Surprisingly, substitution of the Cremophor EL (HLB 12) with a series of Pluronics with HLB values ranging from 0.5 to 29 also improved the extent of atovaquone solubilisation following digestion, even though the replacement of Cremophor EL with the Pluronics resulted in lipid formulations that did not self-emulsify well upon exposure to aqueous media. This was particularly evident with the formulation containing Pluronic L121 (HLB 0.5), where the extent of atovaquone solubilisation was similar to that obtained with the Cremophor EL-containing system, even though Pluronic L121 is practically insoluble in water. Despite the differences in dispersibility of these formulations, the oral bioavailability of the Cremophor EL and Pluronic L121 formulations were indistinguishable from each other and also from a surfactant-free lipid solution formulation. The data highlight the importance of lipid digestion in the efficient solubilisation of lipidic formulations of poorly water-soluble drugs and also suggest that pre-digestion dispersion and particle size measurements are less powerful indicators of in-vivo performance. As such, while the choice of surfactant included in lipid-based formulations should in part reflect its capacity to increase drug solubility in the formulation (and perhaps to alter biochemical processes such as P-glycoprotein efflux or enterocyte-based metabolism), subsequent attention should be more keenly focused on the ability of the surfactant to promote drug solubilisation during lipid digestion, as opposed to simply improving in-vitro dispersion properties.

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