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Research Paper

Lipid-based Formulations for Danazol Containing a Digestible Surfactant, Labrafil M2125CS: *In Vivo* Bioavailability and Dynamic *In Vitro* Lipolysis

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Purpose. To evaluate the use of Labrafil® M2125CS as a lipid vehicle for danazol. Further, the possibility of predicting the *in vivo* behavior with a dynamic *in vitro* lipolysis model was evaluated.
Methods. Danazol (28 mg/kg) was administered orally to rats in four formulations: an aqueous suspension, two suspensions in Labrafil® M2125CS (1 and 2 ml/kg) and a solution in Labrafil® M2125CS (4 ml/kg).
Results. The obtained absolute bioavailabilities of danazol were 1.5±0.8%; 7.1±0.6%; 13.6±1.4% and 13.3±3.4% for the aqueous suspension, 1, 2 and 4 ml Labrafil® M2125CS per kg respectively. Thus administration of danazol with Labrafil® M2125CS resulted in up to a ninefold increase in the bioavailability, and the bioavailability was dependent on the Labrafil® M2125CS dose. *In vitro* lipolysis of the formulations was able to predict the rank order of the bioavailability from the formulations, but not the absorption profile of the *in vivo* study.
Conclusions. The bioavailability of danazol increased when Labrafil® M2125CS was used as a vehicle, both when danazol was suspended and solubilized in the vehicle. The dynamic *in vitro* lipolysis model could be used to rank the bioavailabilities of the *in vivo* data.

KEY WORDS: Labrafil; lipid-based formulations; lipolysis; poorly water soluble drugs; suspensions.

INTRODUCTION

Lipid-based formulations enhance the oral bioavailability of many poorly water-soluble drug molecules, i.e. compounds belonging to the Biopharmaceutics Classification System (BCS) class II and IV (1,2). The mechanisms behind the enhancement have yet not been fully elucidated, however, according to the current understanding, an important part of the mechanism is the increase in the rate and extent of *in vivo* solubilization, which is a prerequisite for absorption of the compound (2). Lipid-based formulations can present the compound in solution or facilitate the formation of solubilizing phases upon the intraluminal processing of the lipids (3,4). The use of lipids and lipid-based formulations to optimize the oral delivery of lipophilic drugs has recently been reviewed by Porter *et al.* (5). A poor solubility in aqueous media is not necessarily equal to a good solubility in pharmaceutically relevant lipids; hence it is not always possible to dissolve the drug dose in a lipid-based dosage form. As an alternative, a suspension of the drug in lipids can be used—a formulation approach that has not been thoroughly investigated and not received much attention as a feasible formulation strategy. A classic study by Carrigan and Bates demonstrates that griseofulvin suspended in corn oil results in a significantly higher bioavailability compared to administration of an aqueous suspension when dosed orally to rats (6). In support of this, a clear trend towards a higher bioavailability of phenytoin was seen when administered orally to rats in a corn oil suspension compared to an aqueous suspension (7).

Macrogolglycerides is a group of non-ionic surfactants, which with increasing popularity have been used as excipients in pharmaceutical formulations. Different types of macrogolglycerides have been used in the preparation of lipid-based drug delivery systems (8–15). Formulations containing macrogolglycerides have been shown to increase the oral absorption of different compounds, e.g. formulations containing Labrasol® (11,14), Gelucire® 44/14 (10,12), and Labrafils (8,15). Labrafil® M2125CS used in the present study is composed of a mixture of mono-, di-, and triglycerides and mono- and di-fatty acid esters of polyethylene glycol 300 (PEG 300), but may also contain some free PEG 300 (16). The fatty acid moieties are mainly linoleic acid (C18:2) and oleic acid (C18:1).

Development of *in vitro* evaluation methods for lipid-based formulations requires a different approach than for *in vitro* evaluation of conventional solid dosage forms. In the former, the drug is often dissolved in the formulation. The formulation excipients are not always miscible with the dissolution media, therefore a two-phase system may be generated. Further, some excipients are hydrolyzed in the gastrointestinal tract by lipases,

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thereby changing the solubilizing environment (17–20). With the aim of distinguishing between the performances of lipid-based formulations *in vivo* and thereby facilitate the formulation development, several *in vitro* digestion models have been developed (1,21–24). Attempts have been made to correlate the solubilization of the drug in the aqueous phase generated during *in vitro* lipolysis with the *in vivo* performance of lipid-based formulations. The *in vitro* lipolysis model has been used successfully to rank the *in vivo* performance of lipid formulations based on different natural triglycerides containing halofantrine (25), danazol (26), progesterone (21), griseofulvin (27) and dexamethasone (27) in solution. However, to our knowledge the possibility of using the *in vitro* lipolysis model to evaluate the *in vivo* performance of lipid-based solutions and suspensions of drugs has not yet been addressed.

The purpose of the present study was to investigate the *in vivo* use of Labrafil® M2125CS (termed Labrafil in the following) as a vehicle for the oral delivery of a poorly soluble model compound, danazol, and to determine if the bioavailability of danazol dosed in suspensions with various levels of Labrafil would be equally effective as a solution. Further, the study aimed at investigating the predictability of the *in vitro* lipolysis model applied to lipid-based suspensions compared to solutions.

MATERIALS AND METHODS

Materials

Danazol USP was purchased from Unikem A/S (Copenhagen, Denmark), and hydroxypropyl-β-cyclodextrin from Roquette (Lestrem, France). The internal standard for the bioanalytical assay, citalopram, was produced by H. Lundbeck A/S (Valby, Denmark). Sodium hydroxide pellets >99% and calcium chloride dihydrate >99% were all purchased from Merck (Darmstadt, Germany), Sodium chloride ≥99.5%, 4-bromobenzeneboronic acid (BBBA) ≥95% and glucose monohydrate Ph. Eur. grade was obtained from Fluka Chemie AG (Buchs, Switzerland). Pancreatin at least 3xUSP, porcine bile extract, Trizma® maleate reagent grade and methylcellulose USP grade were all purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), Epikuron 200, containing at least 92% PC, was purchased from Degussa (Hamburg, Germany), Labrafil® M2125CS was kindly donated by Gattefossé (Saint-Priest, France), rapeseed oil was delivered by Broekelmann food grade (Hamm, Germany), 99.9% ethanol, acetonitrile HPLC grade and methanol HPLC grade were purchased from VWR (Roedovre, Denmark). Purified water was obtained from a Millipore Milli-Q Ultrapure Water purification system (Billeria, MA, USA).

Saturated Solubility of Danazol in Labrafil, Rapeseed Oil or Lipolysis Media

Excess of danazol was added to Labrafil, rapeseed oil or lipolysis media without lipase (composition given in Table I). The suspensions were placed on a magnetic stirrer (IKA, Wilmington, NC, USA) at ambient temperature or in an end-over-end rotating device at 37°C, protected from light. Samples were taken out at specified time points for up to 4 days, centrifuged at 15,000 rpm for 30 min (Biofuge 15, Heraeus Sepatech, Oterode, Germany). The amount of danazol in the supernatant was quantified as described in the analytical section. Equilibrium solubility was assumed attained when the measured concentration in subsequent samples varied by less than 5%. Solubility determinations were performed in triplicate.

Table I. Initial Composition of the *In Vitro* Lipolysis Medium

Substance	Initial concentration
BS (mM)	5
PC (mM)	1.25
Pancreatic lipase (USP units/ml)	800
Trizma-maleate (mM)	2
Na^+ (mM)	150
Ca^{2+} (mmol/min[a])	0.045
Total volume (ml)	300

[a] Dispensing rate (0.09 ml/min of a 0.5 M Ca^{2+} solution)

Preparation of Formulations

Three different formulations containing danazol either dispersed or solubilized in Labrafil were produced; danazol were weighed and added an amount of Labrafil, making the final concentrations 7, 14, or 28 mg danazol per ml vehicle. All orally dosed animals received 28 mg danazol per kg and in order to obtain that dose with the different formulations, 7, 14 and 28 mg/ml, the animals received different amounts of Labrafil, 4, 2 and 1 ml Labrafil per kg respectively. The mixtures were mixed on a magnetic stirrer for 48 h at ambient temperature before use, ensuring that equilibrium was reached. Only the formulation with 4 ml Labrafil per kg was capable of solubilizing the entire danazol dose, the two other formulations were suspensions.

An aqueous suspension, containing 28 mg danazol per ml in 0.5% (*w*/*v*) methylcellulose solution, was prepared by mixing danazol with the methylcellulose solution for 5 min. The particle size in the crude suspension was reduced by means of a Sonifier Cell Disrupter, model B15 equipped with a standard microtip from Branson (Pusan, Korea). The suspension was placed on ice and treated with the power output on 5 three times for 10 min.

The intravenous formulation contained 1.4 mg danazol per ml in a solution containing 10% (*w*/*v*) hydroxypropyl-β-cyclodextrine and 4.4% (*w*/*v*) glucose monohydrate. Danazol was dissolved in the hydroxypropyl-β-cyclodextrine and glucose monohydrate solution by magnetic stirring at ambient temperature. The formulation was filtered through a 0.22 μm filter before use.

All formulations were kept protected from light both during preparation and storage, and the quantitative amount of danazol was determined before use.

Quantitative Analysis of Danazol in Vehicles and Formulations

The danazol content in the formulations was determined by reversed-phase HPLC, a slightly modified version of a validated method previously described by Gadkariem *et al.* (28). A Hitachi system from Merck (Darmstadt, Germany) was

used, equipped with a D-7000 interface, an L-7200 autosampler, an L-7100 pump, an L-7300 column oven (30°C), and an L-7400 UV detector. Data was processed by using HPLC System Manager Version 4.0 from Merck (Darmstadt, Germany). Exact amounts of the oral formulations were weighed (approximately 30–50 mg) and diluted to 10 ml with ethanol. The solutions and the intravenous formulation were subsequently diluted to suitable concentrations with acetonitrile, and 50 μl was injected into the chromatographic system. The analytical column was a Luna C18(2) (5 μm, 150×4.6 mm) column fitted with a C18(2) guard column, both from Phenomenex (Torrance, CA, USA). The mobile phase consisted of acetonitrile and purified water (60:40) (*v*/*v*), and the flow rate was set at 1 ml per min and the UV detector at 285 nm. The method was linear from 80 to 4,000 ng/ml.

In Vivo Study

The protocol was approved by the Animal Welfare Committee, appointed by the Danish Ministry of Justice, and all animal procedures were carried out in compliance with EC Directive 86/609/EEC and with the Danish laws regulating experiments on animals. Male Sprague–Dawley rats weighing 250–296 g at the day of the experiment were purchased from Charles River Laboratories (Wilmington, MA, USA) and maintained on standard food, carrots and water *ad libitum* in the laboratory for at least 1 week prior to entering the experiment. Food was removed 24 h prior to dosing to avoid interactions from lipid components in the food. Water was available *ad libitum* at all times. The animals were allowed access to carrots 4 h after dosing. The animals were randomly assigned to receive one of the five treatments.

Parallel groups were administered with 28 mg/kg of danazol by oral gavage in the oral treatments. Another group was administered with 7 mg/kg of danazol by intravenous injection into the tail vein and flushed with 5 ml/kg of saline. Blood samples of 0.15 ml were collected from the tail vein by individual vein puncture and into plasma collection tubes containing dipotassium EDTA from Sarstedt (Nürnbrecht, Germany). Samples were taken at −5 min (pre-dose) and 0.5, 1, 2, 3, 4, 6, 8, 24, and 28 h after the drug administration for the animals dosed orally and additionally at 10 and 20 min for the animals dosed intravenously. The plasma was harvested immediately by centrifugation for 10 min at 1,000×*g* and stored at −80°C until further analysis. After the experiment, the animals were euthanized.

Quantitative Analysis of Danazol in Plasma

The concentration of danazol in plasma was determined using turboflow chromatography with MS/MS detection. A quantity of 25 μl plasma was mixed with 25 μl 10% methanol containing 200 ng/ml citalopram (internal standard). Calibration curves (1–1,000 ng/ml) were prepared from plasma from non-treated rats. The samples were centrifuged at 6,000×*g* for 20 min at 5°C. A quantity of 10 μl was injected to the chromatographic system by a CTC autosampler from Cohesive Technologies (Franklin, MA, USA).

A turboflow TLX-2 system with a Cyclone turboflow column (0.5×50 mm, 60 μm) from Cohesive Technologies (Franklin, MA, USA) and a C8 XTerra MS analytical column (2.1×20 mm, 3.5 μm) from Waters Corp. were used (Milford, MA, USA). First danazol and the internal standard were retained on the turboflow column where matrix components from plasma were removed. Danazol and the internal standard were then eluted from the turboflow column and thereby transferred to the analytical column from where the compounds where eluted into the MS. The eluents were water with 0.1% NH_3 and methanol with 0.1% NH_3. Different mixtures of these two were used throughout the method with different flow.

The danazol and internal standard content were detected by MS/MS using a Sciex API 3000 mass spectrometer from Applied Biosystem (Foster City, CA, USA). The compounds were detected using positive ionization electrospray with multiple-reaction monitoring set at optimal conditions for each compound. For danazol, the single reaction monitoring (SRM) of m/z 338.2→148.2 was used and for the internal standard m/z 325.0→109.0. The concentration of danazol was determined by standard calibration curve analysis, using linear fitting of a $1/x$-weighted plot of the compound/internal standard peak area ratios *versus* compound concentration.

Pharmacokinetic Data Analysis

Pharmacokinetic parameters were determined using WinNonLin version 4.1 from Pharsight Corp. (Mountain View, CA, USA). The peak plasma concentration (C_{max}) and the time for their occurrence (t_{max}) were obtained directly from the individual plasma concentration *versus* time profiles. Plasma concentrations *versus* time data for danazol in individual rats were analyzed by noncompartmental estimations for the animals dosed orally. After intravenous administration, the plasma concentration data were analyzed using a two-compartment model with first order elimination from the central compartment, and the extrapolated area from the last measured point to infinity was calculated using the linear trapezoidal rule. The absolute bioavailability of danazol was estimated by normalizing the area under the curve (AUC) after oral and intravenous administration with the doses.

In Vitro Dynamic Lipolysis

The procedure for the dynamic *in vitro* lipolysis experiments was based—with minor modifications—on a previously developed lipolysis model (24,29,30). The initial composition of the lipolysis medium in the lipolysis studies is shown in Table I. Bile salts (BS), phosphatidylcholine (PC) and buffer were mixed into a solution in a thermostatically controlled vessel (37±0.5°C). The formulation was added to the BS medium, and the pH was immediately adjusted to 6.5 with 1.00 M NaOH. The amount of formulation used in the model corresponded to the dose that a rat weighing 0.5 kg would receive. After an equilibration time of 3 min, the lipolysis process was initiated by addition of lipase. Simultaneously with adding the lipase, the continuous addition of 0.5 M Ca^{2+} solution was initiated at a rate of 0.09 ml/min. The hydrolysis was followed by potentiostatic titration at pH 6.5 with 1.00 M sodium hydroxide. The titration was carried out on an 842 Titrando titrator fitted with a Unitrode pH electrode with a Pt1000 temperature sensor, two Dosino 10 ml dosing units,

and a rod stirrer with a 96 mm stirrer propeller. The entire system was acquired from Metrohm AG (Herisau, Switzerland). The background lipolysis was determined from a lipolysis experiment without adding formulation nor drug.

At specific times, 3 ml samples were withdrawn, and the lipase was inhibited with a BBBA solution as previously described (24). The samples were centrifuged in thickwall polycarbonate ultracentrifuge tubes 13×56 mm at 37°C at 100,000 rpm for 16 min (5.4×10^5 g at r_{max}) in a Beckman Optima™ MAX benchtop ultracentrifuge with a TLA-110 fixed angle rotor, all obtained from Beckman-Coulter (Fullerton, CA, USA). From the ultracentrifuged samples, 100 µl of the aqueous phase was taken. The samples were diluted with acetonitrile to suitable concentrations and centrifuged at 15,000 rpm for 10 min (2.0×10^4 g at r_{max}) in a Biofuge 15 centrifuge (Heraeus Sepatech, Oterode, Germany) and the supernatant was analyzed for danazol content as described previously in the analytical section.

The total danazol concentration in the lipolysis media was calculated taking the sampling and the dilution occurring due to the addition of the NaOH and Ca^{2+} solutions into account. The percentage of danazol released to the aqueous phase was calculated based on the measurements described above.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (one-way ANOVA) for the obtained AUC and bioavailabilities. One-way ANOVA on ranks was used for t_{max} and C_{max}. Pairwise comparisons between means were performed by the Tukey method. A 5% level of significance was used. All calculations were carried out using SigmaStat for Windows version 3.5 from Systat Software Inc. (Richmond, CA, USA).

RESULTS AND DISCUSSION

The effect of Labrafil® M2125CS used as a lipid vehicle for oral administration of a poorly soluble drug compound was addressed in the present study. Labrafil is a non-ionic surfactant from the group of macrogolglyceride surfactants. Danazol was used as a model drug. Danazol is a good representative of the type of compounds, where both formulation in lipid vehicles and the presence of food can benefit the absorption (26,31,32). The solubility in water is 0.61 µg/ml at 37°C (33) and the log *P* is 4.53 (34). The dose is up to 800 mg per day (35), which makes danazol a class II compound in the biopharmaceutical classification system (36). However, danazol is not very lipophilic; solubilities of 8.0±1.0 mg/ml in Labrafil at 25°C (37) and 4.8 mg/g at 37°C in soybean oil (24) have been reported. Therefore the clinically effective dose cannot be dissolved in a pharmaceutically relevant amount of lipid vehicle. In a study by Charman *et al.* (31), the danazol dose was administered to humans (50–200 mg) in an emulsion, in volumes ranging from 15–60 ml; Porter *et al.* (26) administered 15 mg danazol to dogs in three capsules containing 1 g formulation each (approximately 0.9–1.2 mg danazol per kg). With the aim of obtaining a more pharmaceutically relevant vehicle dose, the possibility of using a suspension of danazol in a lipid vehicle was investigated in the present study. The use of suspensions was also compared with a solution in the lipid vehicle. Furthermore, the possibility of using the *in vitro* lipolysis model to rank the *in vivo* performance of lipid-based suspensions was evaluated.

Characterization of Formulations

The solubility of danazol, at ambient temperature, in Labrafil and rapeseed oil was found to be 8.3±0.2 and 3.2±0.0 mg/ml (mean±SD, n=3) respectively. The obtained solubilities are in accordance with previously published results (24,37) mentioned above. At 37°C the solubility of danazol in Labrafil had increased to 10.3±0.3 mg/ml (mean±SD, n=3). The amount of Labrafil dosed to the animals was based on the solubility at ambient temperature, and the percentage of the danazol dose dissolved in the formulations at ambient temperature is shown in Table II.

The particle size of the suspensions was measured by laser diffraction using the Frauenhofer theory on a Malvern Mastersizer S (Malvern Instruments Ltd., Worcestershire, UK). The aqueous suspension was diluted in purified water, and the two Labrafil suspensions were diluted in rapeseed oil containing danazol at 80% of its saturation solubility. The mean particle size was 2.43±0.03 µm (2.08), 5.36±0.02 µm (1.69), and 5.95±0.02 µm (1.72) (mean±SD, n=3) for the aqueous suspension and the formulations where 2 and 1 ml Labrafil/kg was dosed respectively. The values given in brackets are the span values. These results demonstrate that the particle size in the formulations was in the same particle size range; consequently variations in the *in vivo* results can not be attributed to variations in particle size.

Table II. Formulations Administrated to Fasted Male Sprague–Dawley Rats, Oral Dose 28 mg/kg and Intravenous Dose 7 mg/kg

Formulation	Concentration of danazol (mg/ml)	Route	Labrafil® M2125CS dose	Percent of danazol dose dissolved in formulation[a]
Solution of danazol in Labrafil	7	Oral	4 ml/kg	100
Suspension of danazol in Labrafil	14	Oral	2 ml/kg	59
Suspension of danazol in Labrafil	28	Oral	1 ml/kg	30
Aqueous suspension (suspension of danazol in 0.5% (*w*/*v*) methylcellulose)	28	Oral	0 ml/kg	0
Solution of danazol in 10% hydroxypropyl-β-cyclodextrin	1.4	IV	0 mg/kg	100

[a] Based upon the solubility at ambient temperature

In Vivo Study

The danazol plasma concentration–time profile obtained after intravenous administration is shown in Fig. 1. The following pharmacokinetic parameters were obtained: $AUC_{0-\infty} = 2,832 \pm 293$ h ng/ml , clearance=690±46 ml/h, and a volume of distribution of 1,117±222 ml. The resulting biexponential equation describing the plasma concentration–time profile can be given as:

$$C(t) = 5,882 \times e^{3.71 \times t} + 358 \times e^{0.33 \times t}.$$

The pharmacokinetic results obtained after intravenous administration of danazol in this study were in accordance with results previously published in the literature (33).

Effect of the Dosed Amount of Labrafil® M2125CS

The plasma concentration–time profiles obtained after oral administration of the danazol formulations are presented in Fig. 2, and the pharmacokinetic parameters are listed in Table III. All formulations containing Labrafil had significantly higher bioavailabilities compared to the aqueous suspension, see Table III. There was no difference in the bioavailability of danazol when dosed (28 mg/kg) in 4 ml/kg of Labrafil (solution) or in 2 ml/kg (suspension); both formulations increased the bioavailability ninefold compared to the aqueous suspension. Decreasing the administration of Labrafil to 1 ml/kg (suspension) resulted in a fivefold increase compared to the aqueous suspension. Consequently, administration in a lipid vehicle has a positive effect on the bioavailability of danazol, even though the vehicle is not capable of dissolving the entire drug dose. This is in accordance with results reported in the literature in which lipid suspensions were compared to aqueous suspensions (6,7). Porter *et al*. found a tenfold bioavailability increase in dogs when dosing danazol in a soybean oil solution compared to a micronized powder dosed in a hard gelatin capsule (26). This is in agreement with the present results, even though Labrafil was used as lipid vehicle and the study was performed in rats.

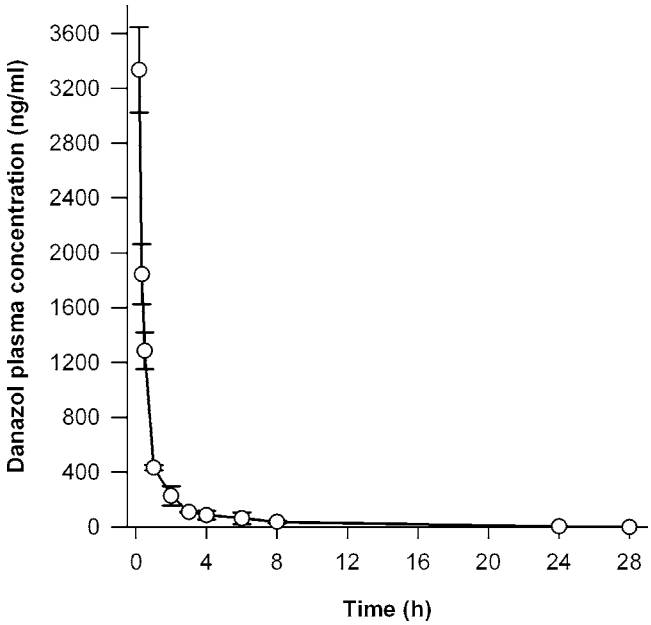

Fig. 1. Plasma concentration–time profile following intravenous administration of 7 mg danazol per kg to male rats (mean±SE, n=7).

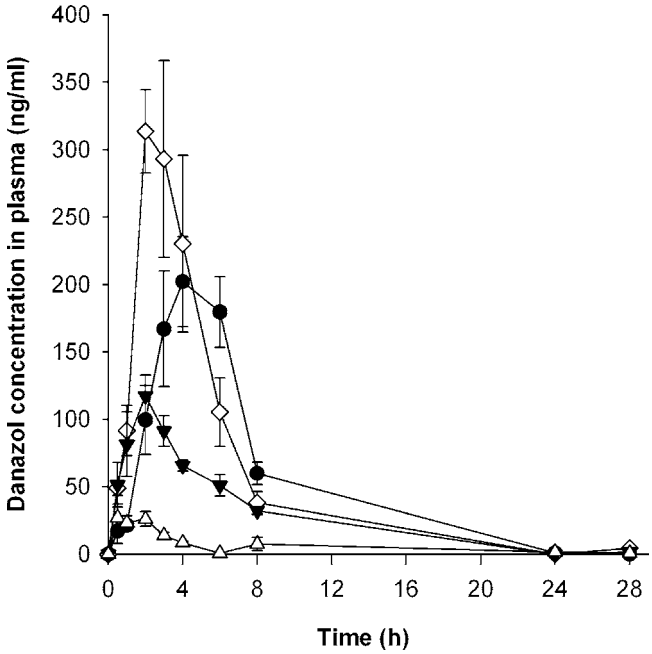

Fig. 2. Plasma concentration–time profiles (mean±SE, n=6) for danazol following oral administration of 28 mg/kg to male rats in either an aqueous suspension with 0.5% (*w/v*) methylcellulose (*open triangles*), suspended in 1 ml Labrafil per kg (*filled inverted triangles*), suspended in 2 ml Labrafil per kg (*open diamonds*), or dissolved in 4 ml Labrafil per kg (*filled circles*).

The bioavailability of danazol increased along with the amount of dosed Labrafil but only until a certain level, see Table III. Administration of danazol (28 mg/kg) with 4 ml/kg Labrafil (solution) resulted in the same bioavailability as administration with 2 ml/kg Labrafil (suspension), 13.3±1.4% (mean±SE, n=6) *versus* 13.6±1.4% (mean±SE, n=6), respectively. Both were significantly higher than for the animals dosed with danazol suspended in the lowest Labrafil amount (1 ml/kg), which gave a bioavailability of 7.1±0.6% (mean±SE, n=6). Hence, a threshold for the amount of Labrafil seems to exist for danazol. Other authors have previously published results with threshold amount of lipid excipients, e.g. Bates and Sequeria found a threshold for the amount of corn oil able to enhance the bioavailability of griseofulvin (38). The higher bioavailability of danazol observed from the 14 mg/ml compared to the 28 mg/ml Labrafil suspension, may be a reflection of the higher amount danazol solubilized at the time of administration. Furthermore, more Labrafil release a higher amount of lipolysis products, important for the solubilization of danazol during the lipolysis of Labrafil.

There were no significant differences between the t_{max} obtained after administration of the three suspensions (the aqueous and the two Labrafil suspensions (1 and 2 ml Labrafil per kg)). Dosing 4 ml Labrafil per kg lead to a significant increase in t_{max} compared to the aqueous suspension. This increase is most likely caused by the presence of lipids in the duodenum, which retards the gastric emptying of remaining stomach content (39).

Table III. Pharmacokinetic Parameters (mean±SE, $n=6$) Following Single Oral Administration of Danazol (28 mg/kg) to Male Rats in Four Different Formulations

	Treatment			
Parameter	7 mg danazol per ml Labrafil A	14 mg danazol per ml Labrafil B	28 mg danazol per ml Labrafil C	28 mg danazol per ml D
t_{max} (h)	$4.5\pm0.5^{(D)}$	2.3 ± 0.2	2.0 ± 0.3	$1.8\pm0.9^{(A)}$
C_{max} (ng/ml)	$249\pm22^{(D)}$	$380\pm53^{(C,D)}$	$136\pm13^{(B)}$	$34\pm7^{(A,B)}$
$AUC_{0\rightarrow28}$ (ng/ml h^{-1})	$1{,}503\pm139^{(C,D)}$	$1{,}590\pm163^{(C,D)}$	$792\pm52^{(A,B,D)}$	$178\pm43^{(A,B,C,D)}$
Bioavailability (%)	$13.3\pm1.4^{(C,D)}$	$13.6\pm1.4^{(C,D)}$	$7.1\pm0.6^{(A,B,D)}$	$1.5\pm0.4^{(A,B,C)}$

The parentheses show from which treatments the specific treatment is significantly different ($p<0.05$)

In Vitro Lipolysis

The dynamic *in vitro* lipolysis model was used to evaluate the formulations *in vitro*. Continuous addition of calcium enables the lipolysis to proceed at a controlled rate, making it possible to obtain information about the distribution of drug between the different digestion phases as the lipolysis progresses (24).

Extent of Lipolysis of the Formulations

The amount of fatty acids released from the oral formulations during the *in vitro* lipolysis is shown in Fig. 3.

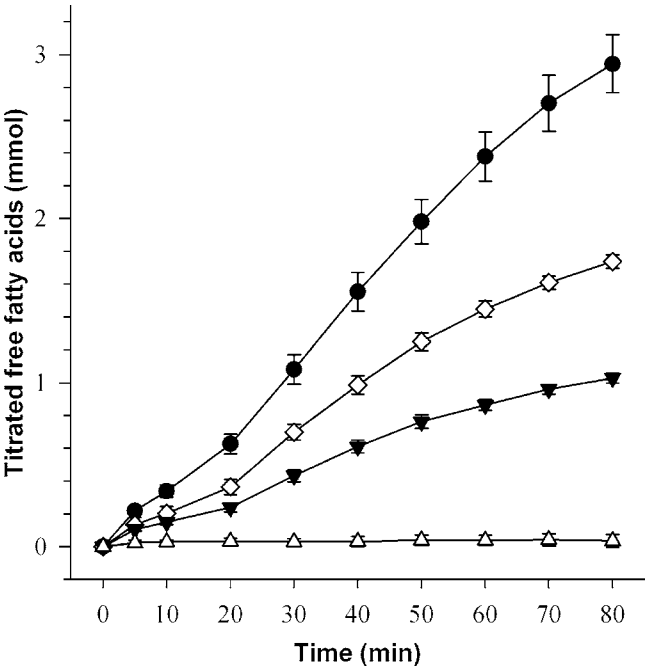

Fig. 3. Amount of fatty acids hydrolyzed from the formulations during *in vitro* lipolysis (mean±SD, $n=3$). The background lipolysis has been subtracted from the obtained results, and results in the figure represent solely lipolysis of the formulations. The amount of formulation added to the model corresponded to the dose that a rat weighing 0.5 kg would receive: 0.5 ml aqueous suspension (28 mg danazol per ml 0.5% (*w*/*v*) methylcellulose) (*open triangles*), 0.5 ml (28 mg danazol suspended per ml Labrafil) (*filled inverted triangles*), 1 ml (14 mg danazol suspended per ml Labrafil) (*open diamonds*), or 2 ml (7 mg dissolved per ml Labrafil) (*filled circles*).

The results represent the fatty acids hydrolyzed from the formulations corrected for background lipolysis. The background lipolysis is mainly the enzymatic conversion of phospholipids to lyso-phospholipids liberating fatty acids and to some extent impurities in pancreatin and the crude bile extract. The amount of released fatty acids increased along with the amounts of Labrafil present in the formulations. This is in agreement with previous data showing that Labrafil is hydrolyzed by enzymes present in pancreatin (A. Larsen, V. Jannin, A. Mülletz. Poster presentation at the 5th World Meeting on Pharmaceutics Biopharmaceutics and Pharmaceutical Technology, Geneva, Switzerland, 2006). The digestion of Labrafil *in vivo* will lead to dynamic changes in the composition of the GI fluids and thereby also the drug solubilization capacity in the GI fluids. Also, other pharmaceutical surfactants have previously been demonstrated to be substrates for lipases, e.g. Labrasol (18,20), Gelucire (20), Cremophor EL (20), and Tween 80 (20,40). As expected, no free fatty acids were released from the aqueous suspension in the dynamic lipolysis model.

Danazol Release During In Vitro Lipolysis

The release of danazol into the aqueous digestion phase during the *in vitro* lipolysis is shown in Fig. 4. The aqueous phase contains mixed bile salt micelles and is suggested to represent the pool of compound available for intestinal absorption. Very fast distribution and dissolution of danazol from the aqueous suspension into the aqueous phase were observed during the equilibration time (3 min) before initiating the lipolysis; at $t=0$ the danazol concentration was 10.4±1.9 μg/ml (22% danazol released). This concentration remained almost unchanged during lipolysis of the aqueous suspension. The saturation solubility of danazol in the lipolysis media at 37°C was measured to be 12.6±0.6 μg/ml (mean±SD, $n=3$). The release of danazol to the aqueous phase from the aqueous suspension thus represented the solubility of danazol in the lipolysis medium when no lipolytic products were released.

While the danazol content in the aqueous phase was relatively stable during the lipolysis of the aqueous suspension, more distinct profiles were obtained when Labrafil was added to the model. The formulation with the lowest Labrafil content lead to a very slow raise of danazol in the aqueous phase until 40 min after the start of the experiment, hereafter the danazol concentration decreased. The initially increased danazol content in the aqueous phase may be a reflection of an

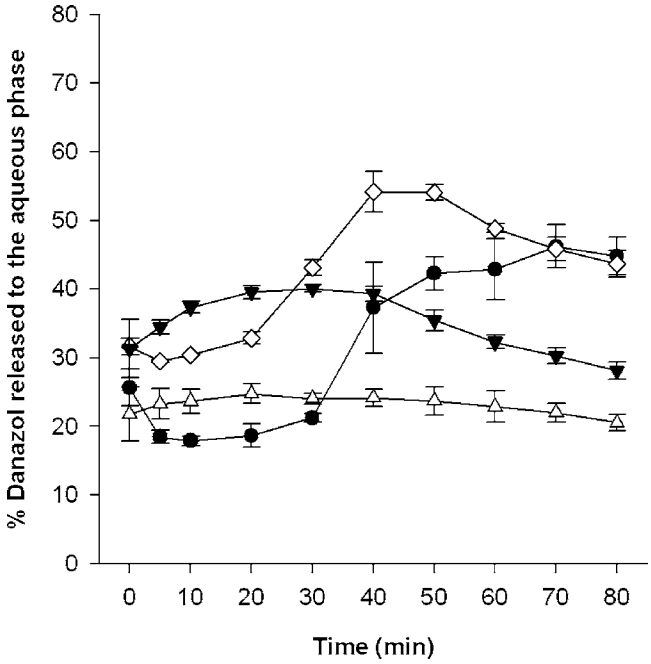

Fig. 4. Percent danazol released from the formulations to the aqueous phase as a function of *in vitro* lipolysis time (mean±SD, n=3). The amount of formulation added to the model corresponded to the dose a rat weighing 0.5 kg would receive: 0.5 ml aqueous suspension (28 mg danazol per ml 0.5% (*w/v*) methylcellulose) (*open triangles*), 0.5 ml (28 mg danazol suspended per ml Labrafil) (*filled inverted triangles*), 1 ml (14 mg danazol suspended per ml Labrafil) (*open diamonds*), or 2 ml (7 mg dissolved per ml Labrafil) (*filled circles*).

increased solubilization power of the mixed micelles, when the digestion products are incorporated. The saturation limit in the aqueous phase occurs at the late stage of the lipolysis process, reflected in the decrease of danazol in the aqueous phase. During the digestion of Labrafil, fatty acids are released and partly removed by precipitation with Ca^{2+} (30) in the lipolysis model. At the later stage of the lipolysis process, the molar amount of Ca^{2+} added may be higher than the amount of free fatty acids released from Labrafil, hence fatty acids incorporated into the mixed micelles may start to precipitate, effectually changing the mixed micelle composition and consequently the solubilizing capacity of these. In addition it has previously been suggested that precipitation of bile acids with Ca^{2+} can occur during *in vitro* lipolysis (24), which generally may change the composition of the micelles leading to the decrease in solubilized danazol.

The two formulations equal to 2 ml Labrafil per kg (danazol suspension) and 4 ml Labrafil per kg (danazol solution) produced very different release profiles in the lipolysis model when compared to the formulation equal to 1 ml Labrafil per kg (danazol suspension). For the 2 ml Labrafil per kg formulation, the danazol concentration raised above the basic solubility in the aqueous phase at the first time point (t=0 min). Zangenberg *et al.* (24) have previously demonstrated that the solubility of danazol in the aqueous phase increase along with the amount of surfactants (bile salts, phospholipids, fatty acids, and monoglycerides). A possible explanation could be that the incorporation of hydrolysis products or possibly Labrafil into the mixed micelles is responsible for the increased partition of danazol into the aqueous phase. After 30 min a pronounced increase in danazol content in the aqueous phase is seen, which is most likely due to the decreased amount of lipid (Labrafil) phase and increased solubilization capacity of the aqueous phase. These changes push the equilibrium towards the aqueous phase. At the end of lipolysis, a decrease in the solubilized amount of danazol is also observed for the 2 ml Labrafil per kg, which could be related to the same phenomenon as described above.

For the 4 ml/kg Labrafil formulations an almost similar profile, compared to 2 ml/kg, is seen, however, initially the danazol content in the aqueous phase is lower than the solubility. This is probably due to danazol partitioning into the lipid phase, which is larger in this case. The initial driving force into the aqueous phase was therefore lower when compared to the other formulations evaluated. After approximately 30 min, danazol starts to participate into the aqueous phase probably for the same reasons as explained above. These findings are in agreement with data previously published by Kaukonen *et al.* (41), in which the release of danazol from a solution in soybean oil after 30 min *in vitro* lipolysis was correlated with the extent of lipolysis. The study by Kaukonen *et al.* is a snapshot of the partitioning of danazol after 30 min of lipolysis, and no information about the trafficking of danazol during digestion is obtained. In the present study the danazol content in the aqueous phase was determined at several time points during lipolysis, producing a more dynamic view of the trafficking that takes place during *in vitro* lipolysis.

Formulation Performance *In Vivo* and *In Vitro*

The use of the dynamic *in vitro* lipolysis model has increased the general understanding of the trafficking of lipophilic compounds during digestion of lipid vehicles. Previously, the lipolysis model have primarily been used in an attempt to correlate the *in vivo* performance of lipid-based formulations containing lipids with different chain lengths fatty acids, e.g. medium chain *versus* long chain. Porter *et al.* recently reported a correlation between the fraction of halofantrine absorbed in dogs when solubilized in medium or long-chain triglycerides and the amount solubilized in the aqueous phase in the lipolysis model. They found that the achievement of the correlation was dependent on the amount of formulation used in the model (25). Another study by Dahan and Hoffman (27) demonstrated a good correlation between released amount of compound during *in vitro* lipolysis and *in vivo* absorption of dexamethasone and griseofulvin when administered in triglycerides with different chain lengths.

In this study, three different formulations, with similar lipid phase composition, were evaluated both *in vivo* and in the dynamic *in vitro* lipolysis model. In the *in vivo* study, the absolute bioavailability of danazol was ranked, 4 ml Labrafil per kg≈2 ml Labrafil per kg>1 ml Labrafil per kg>aqueous suspension. From the *in vitro* lipolysis study the ranking of the formulations differed at different times of lipolysis. After 70 min of lipolysis the ranking of the formulations were similar to the ranking of the bioavailability of danazol from the formulations. The profile of the lipolysis data was affected by the partition coefficient between the aqueous and the lipid

phase. The pharmacokinetic parameters t_{max} and C_{max}, which physiologically was affected by Labrafil, could partly be explained by the *in vitro* lipolysis profiles. The formulation with the longest $t_{max,}$ the 4 ml Labrafil per kg, was also the formulation in which the *in vitro* rise in the danazol content in the aqueous phase was slowest. The C_{max} was ranked in the order 2 ml Labrafil per kg>4 ml Labrafil per kg>1 ml Labrafil per kg>aqueous suspension. This ranking corresponded well with the maximum danazol content in the aqueous phase obtained from the formulations during dynamic *in vitro* lipolysis. Even though *in vitro–in vivo* relations were found in the present study, it was not possible to obtain an *in vitro–in vivo* correlation as defined by the FDA guideline (42).

A standard experimental design for conducting lipolysis of pharmaceutical lipid-based formulations is at present not available. There are many different ways to conduct *in vitro* lipolysis: (1) calcium can be added at the beginning as a bolus or continuous during the experiment, (2) the level of bile salt can be altered, (3) the amount of formulation added to the model can be varied, (4) the amount of lipase added can be varied, and (5) the length of the experiment and the sampling times can be varied. At the present time, it is not defined at which time or to which extent of lipolysis where the amount of drug compound in the aqueous phase correlates with the bioavailability under the various conditions used. This demonstrates the importance of using the dynamic lipolysis model until more knowledge about *in vitro–in vivo* correlations using the *in vitro* lipolysis is achieved. Lipid-based formulations are a very diverse group of formulations, and therefore it seems unlikely that one optimal experimental design for *in vitro* lipolysis can cover this large group of formulations. Systematic studies where the different ways of conducting lipolysis is compared are therefore needed.

CONCLUSION

The present study demonstrates that the use of Labrafil as a vehicle for danazol delivery is able to increase the oral bioavailability to a level corresponding to the level achieved when danazol is solubilized in long-chain triglycerides. Further, the present study showed that a suspension of danazol in Labrafil gave rise to the same oral bioavailability as a solution of danazol in Labrafil. This demonstrates that the use of compounds suspended in lipid vehicles may perform just as well as solutions. The dynamic *in vitro* lipolysis model was used to rank the oral bioavailability from formulations with the same vehicle differing with respect to type, e.g. suspensions *versus* solutions of the drug compound. It was not possible to obtain a level A *in vitro–in vivo* correlation with the use of the dynamic *in vitro* lipolysis model on lipid-based formulations containing danazol in solution or suspended in Labrafil.

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