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Research paper

# Danazol oral absorption modelling in the fasted dog: An example of mechanistic understanding of formulation effects on drug pharmacokinetics

Devendra Pade[a], Masoud Jamei[a], David B. Turner[a], Bipin Mistry[b,c], Marilyn N. Martinez[b,*]

[a] Certara UK Limited, Simcyp Division, 1 Concourse Way, Sheffield S1 2BJ, United Kingdom
[b] US FDA Center for Veterinary Medicine, Rockville, MD 20852, United States
[c] Eisai Inc., Woodcliff Lake, NJ 07677, United States[1]

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ABSTRACT

Oral bioavailability of poorly water soluble (BCS II) drugs like **danazol** can be minimal without the necessary **formulation strategies**. Availability of *in vitro* physicochemical and *in vivo* pharmacokinetic studies can be valuable when designing these strategies but cannot reveal the drug-formulation-gastrointestinal physiology interplay that impact the successful optimization of intestinal solubilization and resulting oral drug absorption. *In silico* mechanistic oral drug absorption models can serve as a tool for providing this important perspective and for integrating information generated across various *in vivo* and *in vitro* studies. In this work, we detail the development and application of the Simcyp canine ADAM model to nine danazol oral formulations and compare the model predictions to **canine** *in vivo* pharmacokinetic data from published literature. The application of this mechanistic approach revealed insights suggesting: (1) complete danazol solubilization *in vitro* may lead to an over-estimation of oral bioavailability when predictions are not corrected for the *in vivo* conditions promoting gut luminal precipitation; (2) some solubilizing excipients can influence intestinal physiology in a manner that may reduce danazol absorption; (3) danazol-formulation-luminal bile salts interplay can result in the formation of mixed micelles that negatively impact danazol intestinal permeability; and (4) the magnitude of danazol bioavailability enhancement associated with the use of solubilizing agents can be affected by the presence of saturable gut metabolism that can lead to concentration-dependent differences in its influence *in vivo* formulation behaviour at high versus low doses.

## 1. Introduction

The low aqueous solubility of many therapeutic compounds presents a significant formulation challenge during oral drug product development. Therefore, it is not surprising that much attention has been given to the topic of improving drug oral bioavailability using solubility enhancers and other formulation mechanisms to improve *in vivo* dissolution. Examples include the development of nano-formulations, use of surfactants and changes in drug crystalline form [1]. However, when our understanding of formulation effects relies solely upon *in vitro* dissolution studies or an assessment of *in vivo* blood level profiles, much information is lost. There still needs to be an appreciation of the multidimensional effects that these variables can have on *in vivo* product behaviour. It is from this framework that *in silico* based mechanistic oral drug absorption models can be highly informative.

Given the wealth of published information available on methods for enhancing its oral bioavailability, danazol was selected as a model compound to explore the insights that can be derived using the Simcyp *in silico* based canine Advanced Dissolution, Absorption, and Metabolism (ADAM) model. Danazol, a low solubility, high permeability (BCS Class II) compound, has become one of the model drugs for exploring methods of solubility enhancement since formulation effects can be examined without the limitations imposed by the presence of an *in vivo* absorption window.

A difficulty encountered when using published data to evaluate formulation effects is the inter-individual and inter-study variability in blood level profiles that often occurs with low solubility drugs such as danazol. The occasional report with disparate outcomes can challenge our ability to generate a comprehensive understanding of the formulation optimization strategies and of the strengths and weakness that each may pose. As discussed later in this paper, another complication is the range of physicochemical properties reported for these compounds,

* Corresponding author.
*E-mail address:* marilyn.martinez@fda.hhs.gov (M.N. Martinez).
[1] Current affiliation.

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adding a layer of uncertainty to our understanding of the drug-excipient-bioavailability relationships.

To predict the *in vivo* effects associated with the various solubilization techniques, it was necessary to develop a basic model that could be applied when oral bioavailability is assessed across investigations. Since many of the published danazol formulation strategies were evaluated using *in vivo* blood profiles in beagle dogs, the Simcyp canine oral absorption module provided an appropriate platform with which we could explore the *in vivo* implications of a range of oral dosing conditions and formulation strategies.

In this manuscript, we demonstrate how *in silico* mechanistic models can be used to facilitate our understanding of formulation effects and the physiological variables that modify the extent to which these formulations will facilitate *in vivo* drug absorption. Formulations were examined from the perspective of their effects on drug solubility and permeability. The latter is particularly critical for BCS class II compounds when there is a critical balance due to the effect of drug micellisation on solubility enhancement versus its negative effects on drug diffusivity. Thus, the method by which a drug is formulated as an "oral solution" can significantly affect the corresponding magnitude of improvement of oral bioavailability [2].

While gaps remain in our understanding of canine gastrointestinal (GI) physiology, sufficient information is available to enable the development of predictive models. *In silico* studies such as this promote an opportunity to test our assumptions, and when the model fails to reflect observed data, allows exploration of potential sources of model bias. With that, we can identify remaining data gaps and expand our insights into the interaction between formulation and GI physiology impacting oral absorption of low solubility compounds.

## 2. Materials & methods

Simcyp Dog (Version 16) is an *in silico* physiologically based pharmacokinetic (PBPK) dog model based on the general anatomy and physiology of a 10 kg beagle dog. The Advanced Dissolution Absorption and Metabolism (ADAM) model is the canine oral drug absorption model within the dog PBPK framework. These models are structurally similar to that of the Simcyp Human Simulator [3,4].

A PBPK model for danazol utilized the default beagle physiology and anatomy (system) parameters incorporated in the Simcyp dog simulator. Drug elimination and distribution attributes were defined on the basis of intravenous (IV) data collated from the publications used in our assessments. Drug effective regional intestinal permeability ($P_{eff,Dog}$) was predicted using the drug physiochemical parameters (logP, pKa, etc.) and the built-in mechanistic permeability model (MechPeff) [2,5]. Combining drug and systems data, the influence of formulation on danazol oral bioavailability was simulated. The predicted plasma concentration time (Cp-t) profiles of danazol were compared to the *in vivo* data published in the literature.

To ascertain the accuracy of the model predictions, all simulations were generated using n = 50 beagle dogs. Inter-individual variability was achieved by Monte-Carlo sampling, assuming a log-normal distribution of the means and coefficients of variation for oral absorption parameters (e.g., gastric emptying time, intestinal transit time, gastrointestinal pH, etc.) based on a 10 kg beagle dog [6]. Predicted mean Cp-t profiles were overlaid with observed data along with their standard deviation (SD). The 5th and 95th percentiles of the predicted mean values were also overlaid to compare with the observed inter-individual variability. If *in vivo* studies reported the standard error of the mean (SEM), this value was converted to a SD using the general equation:

$$SD = SEM \times \sqrt{N}$$

where 'N' is the number of animals included in the study for that observation.

To overlay the observed and the predicted PK profiles, all observed PK profiles were extracted digitally using GetData Graph Digitizer Version 2.22 (http://getdata-graph-digitizer.com). These digitized concentrations served as the basis for our fitting procedures and are reported as the "observed" values in the graphs of the Cp-t profiles or as the "Digitized observed" parameter values in our results tables.

During this work, the following orally administered formulation options are available within the Simcyp dog simulator were used:

**Solution (Soln):** When selecting this formulation option, it is assumed that changes in media composition has no impact on drug absorption and that the oral absorption can be modelled based solely on membrane permeability of the monomer. For the solution option, the drug remains in its fully solubilized form throughout its residence within the GI tract.

**Solution with precipitation (SwP):** Although administered orally as a solution, this option allows for drug precipitation and re-dissolution to occur after administration. The diffusion layer model (DLM) is used to estimate the dissolution of the precipitated drug. When the DLM is active, effects due to bile solubility enhancement, super-saturation effects etc. are considered due to the changing environment of the GI tract during drug transit.

**Suspension:** The formulation is an oral suspension where the user can define the fraction of API dissolved prior to dosing. The remaining dose fraction is undissolved (solid) and its dissolution rate is predicted using the DLM that requires information on drug solubility and formulation critical quality attributes (CQA) such as particle size. This dosage form can be used in situations where the drug is administered as an emulsion or where there is partitioning of the drug into bile micelles. It should be noted that simulation results obtained using SwP are identical to suspension (with 100% API dissolved in formulation) when the same model input parameters are used.

**Immediate release formulation (IR):** This formulation option allows for the dissolution of the drug via input of *in vitro* dissolution profiles or prediction of *in vivo* dissolution rate via the DLM. When *in vitro* dissolution profiles are used as input, the DLM model is bypassed. When the DLM is used to predict the rate of dissolution, information of drug/formulation CQA is required. The *in vivo* dissolution profile can also be estimated using a middle out approach by using best fit Weibull parameter values.

**Controlled/Modified Release (CR/MR) formulation:** While this option enables the user to define a % total mass released over time, such predictions necessitate estimations either based upon *in vitro* dissolution data or via the use of a middle-out approach where the *in vivo* release profile is estimated by the best fit Weibull parameter values. For the formulations modelled in this exercise, the data were not available from the cited studies with which to utilize this option.

Efforts to understand the *in vivo* behaviour of each formulation and to reproduce the reported PK profiles, was based upon a combination of using either of the above formulation options and insights into the pivotal *in vivo* formulation effects available from published information.

### 2.1. In vivo studies used for evaluation

Studies were selected to reflect the range of formulation strategies used to enhance the solubility of danazol. This includes alteration of particle size (e.g., nanoemulsions, alteration of particle radius) or the use of solubilizing excipients such as beta-cyclodextrins, polyvinyl pyrollidone (PVP), polyethylene glycol (PEG), polysorbate 80, Labrafil® and dimethyl sulfoxide (DMSO). Information on the properties of these excipients can be found in Supplemental Table A and in the 2004 review by Strickley [7]. Unless otherwise noted, all *in vivo* studies were conducted in the fasted state.

### 2.2. Model development

#### 2.2.1. Model input parameters

A single set of formulation-independent model input parameters

**Table 1**
Input parameters to the PBPK model for prediction of Cp-t following oral administration of danazol (excipient/formulation effect independent parameters).

| Danazol Parameters | Value | Reference |
|---|---|---|
| *A. Drug Physicochemical Properties* Molecular Weight | 337.45 | [8] |
| Log $P_{Octanol:Water}$ | 4.53 | [48] |
| Compound type | Neutral | [8] |
| Blood:plasma ratio | 1.306 | Predicted[1] |
| Fraction unbound in plasma, $fu_{plasma}$ | 0.0272 | Predicted[2] |
| *B. Drug ADME Properties* Caco-2 cell membrane passive intrinsic transcellular permeability ($P_{trans,0}$ cm/s) | 0.23 | [16] |
| Intestinal Passive Permeability ($P_{eff,dog}$) × $10^{-4}$ cm/s | Duodenum:1.46; Jejunum: 1.4–1.6; Ileum: 0.74 | [2,4] |
| Intrinsic solubility (mg/mL) | 0.00054 | [21,22,27,49,50] |
| Supersaturation ratio (SR) | 28.1 | [20,21] |
| Precipitation Rate Constant (PRC; $h^{-1}$) | Danazol:16.8; Danazol + PVP: 0.65 | [20,21] |
| Vss (L/kg) | 5.35 | Simcyp Predicted |
| Intravenous Clearance, CLIV (mL/min/kg) | 16.84 | [15] |
| *In vitro* $CLint_{DLM}$ (μL/min/mg microsomal protein) | 716* | Simcyp retrograde calculation based on $CL_{IV}$ |
| *In vitro* $CLint_{DIM}$ (μL/min/mg intestine microsomal protein) | Vmax: 10 pmol/min/mg microsomal protein; Km: 0.02 μM | Fitted value (also see Results & Discussion) |
| $fu_{gut}$ | 0.0272 | assume same as $fu_{plasma}$ |
| *C. Simulation Default Conditions* Fluid intake with oral dose (mL) | 50 | Default value unless specified |
| Duration of simulation (h) | 24 | Covering all blood sampling time points for all studies |

*Used as a default value, but adjustments were made as appropriate (where dogs showed high $CL_{IV}$ in individual studies).
[1–2] Predicted beagle values using Prediction Tool Box within the Simulator.

were used across all the datasets. In so doing, reported profiles across a range of oral formulations could be modelled, irrespective of whether the published report contained IV data. When deviations from the basic model parameters were deemed appropriate, the rationale for such adjustments were provided and subsequently discussed in terms of the implications of these changes on the mechanisms associated with a given formulation strategy.

Formulation-independent model input parameters (drug and species-specific) were obtained from literature (as cited within this manuscript) and from online databases such as PubChem (Table 1) [8].

In the absence of literature information, the fraction unbound in plasma ($fu_{plasma}$) for danazol was predicted from $logP_{O:W}$ using the model published by Lobell et al. (also incorporated as a prediction tool within the Simcyp human simulator) [9]. Considering the neutral charge and lipophilicity of danazol, it was assumed that albumin would be the dominant binding plasma protein. The value of blood to plasma ratio (also not available in the literature), was predicted based upon an built-in model which utilizes as input the $logP_{O:W}$, compound charge type, dog plasma pH, dog hematocrit and the predicted $fu_{plasma}$ [10].

The fraction of unbound drug in the intestinal enterocyte ($fu_{gut}$) described the fraction of total drug within the enterocyte available for intracellular metabolism and transport. A value of 1 indicates that the entire amount of drug permeating into the enterocyte remains unbound (free) to the intra-enterocyte components. Being highly lipophilic, it is unlikely that danazol will remain completely unbound within the enterocyte. Since the $fu_{gut}$ value cannot as yet be determined experimentally, it was assumed that the $fu_{plasma}$, reflecting binding to plasma components, is equivalent to the binding that occurs to the enterocyte components.

*2.2.1.1. Organ metabolic clearance determinations.* Danazol *in vivo* Cp-t profiles after bolus IV administration to beagle dogs were taken from Liversidge et al., Anby et al., and Devalapally et al. (Table 2a) [13–15]. The PBPK model enables the input of a whole organ metabolic clearance for the liver [as well as for the intestine] in the form of a steady state intrinsic clearance ($CL_{int}$) or as enzyme-specific saturable Michaelis-Menten $V_{max}$ and $K_m$ values. A scale-up of these values is then based upon an *in vitro-in vivo* extrapolation (IVIVE) of the $CL_{int}$ estimates to that of the whole-body clearance [6].

Given the goal of defining a singular model that could be used irrespective of whether a published investigation contained an IV unit impulse response, it was necessary to ascertain how best to select a value to represent the $CL_{iv}$. In turn, $CL_{iv}$ was used to estimate $CL_{int}$. Upon examining the estimates of $CL_{int}$ derived from the information from Liversidge (Supplemental Table B), Anby and Devalapally, the Liversidge values (16.8 mL/min/kg) were found to be approximately midway between those of Anby (13.5 mL/min/kg) and Devalapally (22.3 mL/min/kg).

Clearance was expressed relative to the dog liver microsomes ($CLint_{DLM}$) [or intestinal microsomes ($CLint_{DIM}$)]. The method for calculating $CLint_{DLM}$ is provided in Supplemental Table B. Several methods were explored for obtaining a single $CLint_{DLM}$ value to be used for all data evaluations. An averaging of the four sets of clearance values derived from published danazol IV studies resulted in a mean value 151 mL/min (% CV = 41). This translates to a $CLint_{DLM}$ of 644 μL/min/mg of microsomal protein (Supplemental Table C). When applying that value versus the one derived from the Liversidge study (which was the median value), it was observed that the Liversidge estimate typically provided the smaller deviation between observed and predicted values (concentration-time profiles). Accordingly, the Liversidge-derived estimate was selected as the value incorporated into our PBPK model. This $CLint_{DLM}$ estimate of 716 μL/min/mg microsomal protein was applied to all datasets, except for those reported by Devalapally (see results for additional information) where a $CLint_{DLM}$ value of 1052 μL/min/mg microsomal protein was applied.

Developing an IV solution of a poorly water soluble drug necessitates the use of drug solubilization strategies. To that end, cyclodextrins have been extensively employed, leading to the need to evaluate their potential effects on drug clearance. Although the possibility of altered PK cannot be completely excluded, we considered cyclodextrin to have at most minimal influence on danazol PK in light of it being a high extraction ratio (ER) drug (the ER in dog appears to be > 0.70) and the negligible renal excretion of the unchanged drug [11].

*2.2.1.2. Volume of distribution.* The built-in Poulin & Thiel model (corrected by Berezhkovskiy, Method 1), in combination with the

**Table 2a**
Danazol IV formulations selected from various literature studies.

| IV Formulation | Dose (mg/kg) | Dosing | N | Body Wt. (Kg) | Reference |
|---|---|---|---|---|---|
| Danazol in HP-β-CD solution (50% w/w) | 3 | Bolus | 5 | 10 | [15] |
| Danazol in HP-β-CD solution (10% w/w) | 1 | Bolus | 3 | 10 | [14] |
| Danazol in SBE-β-CD solution (20% w/v) | 0.85 | Infusion (5 mins) | 4 | 18* (Range 13–23) | [13] |

HP-β-CD: Hydroxypropyl-Beta-Cyclodextrin; SBE-β-CD: Sulpho-butyl ether Beta-Cyclodextrin.
* Danazol profiles from the younger beagle cohort used.

danazol physicochemical parameters, was used to predict the total drug steady state volume of distribution (Vss) and the tissue to plasma partition coefficients (Kp) for the various tissues [12]. The Kp value describes the ratio of the concentration of a compound in the tissue to the concentration of that compound in the plasma at steady state based upon drug physicochemical properties and the composition of the various organs/tissues. When using predicted Kp values, there may be a need for a uniform adjustment scalar to be applied to all tissues in the PBPK model to improve the model estimates of Vss (i.e., improve approximation of observed values). This is accomplished using a $Kp_{scalar}$. The $Kp_{scalar}$ default value is 1 (i.e., no scaling), but can be adjusted to values between $1 \times 10^{-5}$ and $1 \times 10^{5}$. Based upon the results of a sensitivity analysis ranging from a scalar value 1 to 0.01, the $Kp_{scalar}$ value was set as 0.26. This resulted in a Vss estimate of 5.35 L/kg, which is consistent with that reported in the literature (which ranged from 3.9 to 7.9 L/kg) [13–15].

*2.2.1.3. Intestinal permeability ($P_{eff,\ dog}$).* The canine intestinal effective permeability ($P_{eff,\ dog}$) was predicted using the built-in MechPeff model via a 2 step process: (i) Prediction of the Caco-2 cell membrane passive 'intrinsic' transcellular permeability ($P_{trans,0}$); and (ii) calculation of the regional passive effective permeability ($P_{eff,\ dog}$) for the various intestinal segments and for the colon based on morphological surface area differences [2]. The $P_{trans,0}$ was predicted using $logP_{O:W}$ as input based on a correlation published by Sugano et al. ($P_{trans,0} = 2.36\ logP_{O:W}^{1.10}$) [16]. This resulted in a $P_{trans,0}$ of 0.23 cm/s (higher than that published by Sugano et al.) due to the higher (experimental) $logP_{O:W}$ value used in this study (4.53) [17]. The MechPeff model then estimates the effective regional passive permeability based upon the beagle anatomical and physiological environment of the small intestinal segments (e.g., luminal pH, villi morphology, bile salt concentrations, and free fraction of drug available for permeation) [2]. The resulting $P_{eff,dog}$ for the different regions of the small intestine and colon are provided in Table 1, and the rationale for the values used in those estimates are provided in Appendix A. Only passive absorption processes were considered since there is no evidence of active transport of danazol across the intestinal membrane [18].

When estimating drug absorption for cyclodextrin formulations or micellar bound drugs, the assumption is that the danazol high $P_{trans,0}$ results in very rapid absorption of free monomer. Of note here is that only the unbound drug is considered available for transcellular absorption processes. Within the framework of this model, we assume that there is instantaneous equilibration between the micelle bound and unbound. Therefore, any constraints to drug absorption must be attributable to the slow diffusivity of the bound drug through the mucous layer [leading to a lower concentration gradient of the monomer for passive permeability] or to drug solubilization. Accordingly, because of danazol's physicochemical properties, the primary challenge is that of solubilization and the secondary challenge is the diffusion of the drug through the unstirred layer surrounding the intestine.

*2.2.1.4. Intestinal first pass drug loss.* It was also necessary to include a component for saturable intestinal metabolism when simulating all oral dose administrations. It was observed that when modelled in the absence of gut metabolism, there was an over prediction of the danazol Cp-t profile (2 mg/kg solution formulation). Including saturable gut metabolism did not make a qualitative difference to the bioavailability predicted at the higher dose (20 mg/kg solution) but did enable the bioavailability of the lower dose (2 mg/kg solution) to be accurately recovered.

The presence of gut metabolism was identified based upon the work of Lee et al. [19] who provided *in vitro* evidence demonstrating gut metabolism of danazol by intestinal CYP3A4 (canine CYP3A12) and CYP2J2 enzymes. Although danazol itself was not directly tested for saturation kinetics, these authors showed that CYP2J2 exhibits saturable metabolism in the gut wall. Using the *in vivo* Cp-t profile for Takano 2 mg/kg solution formulation, the Km value was set to saturation occurring at 0.02 μM. The $V_{max}$ was then optimized at 10 pmol/min/mg of microsomal protein using parameter estimation and automated sensitivity analysis. This single set of $V_{max}$ and Km values was used across all oral dose administration simulations. In accordance with the reported Cp-t profiles, these values of $V_{max}$ and Km resulted in substantial presystemic drug loss at low enterocyte drug concentrations (consistent with a lower-than expected systemic drug concentration when dogs received relatively low oral doses or with formulations associated with a small amount of drug crossing into the enterocytes), but had minimal impact when relatively large amounts of drug crossed into the enterocytes (leading to enzyme saturation). The assumption of a gut metabolism component was further validated when simulating an even lower dose of 0.2 mg/kg oral solution published by Takano et al. where the resulting predicted versus the observed AUC value was 0.035 and 0.047 μg × h/mL, respectively (for details please see results and discussion section).

The datasets used in the danazol modelling and simulation study are provided in Tables 2a and 2b.

*2.2.2. Formulation-dependent model parameters*

The ADAM model [3,4], in conjunction with the Simcyp Dog PBPK simulator, was used to examine the *in vivo* behaviour of the various danazol formulations, predicting the fraction of drug dissolved (solubilized), the fraction of drug crossing the enterocyte apical membrane (fa), and the fraction of drug escaping gut metabolism (Fg). These components led to the ultimate prediction of product Cp-t profiles and systemic bioavailability (F).

Like the Simcyp human ADAM model, the Simcyp dog gastrointestinal (GI) tract is divided into nine anatomical segments (stomach, duodenum, jejunum segments I-V, ileum and colon). Drug absorption from each segment is described as the solubilized drug available to interact with the intestinal membrane, the rate and extent of drug dissolution (and any subsequent precipitation), luminal degradation, membrane transcellular and/or paracellular permeability (the latter not relevant to danazol), intestinal drug metabolism, the activity of influx or efflux transporter (again, not relevant to danazol), and the transit of the dissolved drug and/or undissolved drug product from one segment to another [4]. The beagle *in vivo* PK studies providing the Cp-t profiles associated with oral danazol formulations evaluated in this cross-study comparison are listed in Table 2b.

A wide range of formulation strategies were included to promote our understanding of the challenges encountered during efforts to optimize danazol bioavailability. With the ADAM model, it is possible to

**Table 2b**
Danazol oral formulations selected from various literature studies.

| Formulation Code | Formulation Type | Formulation Specifics | Dose | N (dogs) | Reference |
|---|---|---|---|---|---|
| Liversidge Soln | Solution | Danazol-HP-β-CD complex solution (50% w/w) | 20 mg/kg | 5 | [15] |
| Takano Soln | Solution | Danazol Aqueous solution (with 10% DMSO & 20% VETPGS) | 2 mg/kg | 5 | [32] |
| Labrafil Soln | Solution | Danazol 4 mg/mL in Labrafil | 5 mg/kg | 3 | [14] |
| Liversidge PVP Susp | Aqueous Suspension | Conventional danazol (5% w/w) + PVP (1.5% w/w) in water | 200 mg | 5 | [15] |
| Erlich PVP | Non aqueous solution in capsules | Danazol (4%) + PVP (23%) in PEG | 100 mg | 6 | [51] |
| Takano IR | IR Capsule | Danazol:Lactose (1:9) | 2 mg/kg | 5 | [32] |
| Erlich DCR 100 | Commercial IR capsule | Danocrine® 100 mg | 100 mg | 6 | [51] |
| Erlich DCR 200 | Commercial IR capsule | Danocrine® 200 mg | 200 mg | 6 | [51] |
| Deva DCR 200 | Commercial IR capsule | Danocrine® 200 mg | 200 mg | 3 | [14] |

HP-β-CD: Hydroxypropyl-Beta-Cyclodextrin; DMSO: Dimethyl sulfoxide; VETPGS: Vitamin E Tocopherol Polyethylene Glycerol Succinate; PVP: Polyvinylpyrrolidone; PEG: Polyethylene Glycol.

account for drug-formulation properties such as intrinsic solubility (IS), degree of supersaturation, precipitation rate constant (PRC) of the solubilized form of drug within the GI environment, and the free fraction of drug available for permeation in the intestinal lumen. The physicochemical properties of the solubility enhancing excipients used in these investigations and a description of their specific effects on danazol oral bioavailability are provided in Supplemental Table A.

The supersaturation ratio (SR) for danazol was calculated as the ratio of kinetic (maximum) solubility to the aqueous equilibrium solubility of danazol published by Higashino et al., (danazol alone, 28.1); Jackson et al. (danazol alone, 21.4, and danazol + PVP, 21); and Larsen et al. (danazol in Labrafil, 31.4) [20–22]. A sensitivity analysis on the SR values in the range of 20–40 for each specific formulation also indicated no major difference on the fa value. Hence a SR value of 28.1 was used for all formulations.

The PRC, which is a first order rate constant, was determined by calculating the slope of the same danazol concentration-time profiles published as was used for estimating the SR (Higashino et al. and Jackson et al). The PRC (1/h) for danazol alone was 16.83, and for danazol + PVP was 0.65. Being lipophilic, danazol exhibits enhanced aqueous solubility in the presence of bile micelles. However, as the concentration of bile micelles and the intraluminal fluid volumes decrease when going from the proximal to the distal parts of the intestine (in ileum and colon), some proportion of solubilized danazol in the aqueous phase may precipitate.

In the case of commercial danazol solid oral dosage forms (Danocrine® capsules), the FDA product label information as well as a report by Porter et al. indicated that the danazol formulation filled within the capsules did not contain any solubility enhancing excipients [23,24]. Hence when simulating oral administration of danazol in capsules, the danazol IS was set to 0.00054 (mg/mL), the SR set to 28.1 and the PRC set to 16.83 (Table 1). While optimally, the SR and the PRC would be obtained by evaluating the relationship between the ability of an active pharmaceutical ingredient (API) to be solubilized within the solvent of interest (canine fasted simulated small intestinal fluid, FASSIFc) across a range of API concentrations, this was not feasible with danazol due to its very low aqueous solubility. As demonstrated by Higashino et al., in the absence of a solubilizing agent such as DMSO (used for generating a danazol-DMSO stock solution), information on SR and PRC could not be obtained. However, since the concentration of DMSO diluted from the stock was low in the final (human) FASSIF-danazol working solution (~1.5%; Bevernage et al.), and because it was reported that this DMSO concentration was not found to significantly influence the thermodynamic solubility of danazol [25,26], we considered this value to provide a reasonable estimate of SR and PRC that could be incorporated into our mechanistic model. These SR and PRC values were subsequently used for formulations where excipients were not added to enhance danazol solubility or where (hypothetically) aqueous danazol was released from a solubility enhancing solution (such as the Labrafil solution formulation).

The free fraction of drug available for permeation was determined by the bile salt micelle to water partitioning coefficient of the drug (logKm:w), where Km:w is the proportion of drug bound to bile micelles versus that proportion remaining free [2,27]. The default model danazol logKm:w value was set as 5.6 based on a dataset of 14 neutral compounds as studied by Glomme et al. [28]. The predicted total solubility of danazol in the jejunum due to bile micelle mediated solubility enhancement was 0.02 mg/mL (Supplemental Table H, Erlich DCR and Deva DCR formulations), which agrees with the experimentally determined value of 0.018 mg/mL as reported by Okazaki et al. However, in the presence of solubility enhancing excipients such as PVP, jejunal total danazol solubility (using the bile micelle mediated solubilization enhancement model) exceeded 0.02 mg/mL due to the formulation-associated increase in danazol intrinsic solubility (Supplemental Table H).

The Labrafil formulation was modelled as a solution with precipitation, thereby requiring an estimate of the PRC (see results for additional discussion). Simultaneously, because of its partitioning into the mixed micelles (during Labrafil lipolysis within the dog GI tract), an increased $\log K_{m:w}$ value different from the default value characterizing its partitioning into bile micelles needed to be evaluated. Further details of the lipolysis process and the estimation of $\log K_{m:w}$ can be found in the discussion pertaining to the Labrafil formulation. Hypothetically, lipolysis could either lead to the danazol molecule being released into the aqueous fluids, which could subsequently precipitate, or stay in solution due to bile micellisation [22,29].

The mean particle size radius for danazol in the commercial Danocrine® capsules was not reported in the *in vivo* studies and hence was set at 10 μm based on literature information [30,31]. For the Labrafil solution, its formulation settings were used to conduct a sensitivity analysis of the relationship between particle radius of precipitating danazol and fraction absorbed (Supplemental figure G). This analysis revealed no differences on the predicted fa values for particle radii within the range of 0.1–30 μm. This implies the presence of a solubility limited absorption of danazol after precipitation from solution during lipolysis of Labrafil [32]. The particle size of precipitating danazol (as API) from the Labrafil solution was assumed to be 5 μm.

A sensitivity analysis for the relationship between fa versus particle radius of precipitated danazol was also conducted for the Erlich PVP formulation. For this formulation, it has been reported that the precipitated danazol (in presence of PVP alone) can have a mean particle size diameter of approximately 350–400 nm, with a very broad distribution [21]. Hence, simulations were conducted using a particle radius of 0.25 μm, 10 μm, 20 μm and 30 μm. This enabled us to span a range of particle sizes from that of nanoscale (250 nm) up to 30 μm. The sensitivity analysis of particle size radius (0.1–30 μm) on the predicted fa (Supplemental figure G), using the Erlich PVP simulation settings, revealed a minor sensitivity of ~10% with the predicted fa decreasing with the increase in particle radius (fa = 0.43 for particle radius = 0.1 μm and fa = 0.3 for particle radius = 30 μm). This low level

**Table 3**
Formulation specific input parameters to the ADAM model of the dog PBPK simulator (formulation dependent parameters).

| Formulation Code | ADAM model Formulation Input | IS (mg/mL) | SR | PRC (1/h) | $logK_{m:w}$ | Mean particle size radius (μm) |
|---|---|---|---|---|---|---|
| Liversidge Soln | Solution | NR | NR | NR | 5.6 | NR |
| Takano Soln | Solution | NR | NR | NR | 5.6 | NR |
| Labrafil Soln | SwP* | 0.00054 | 28.1 | 16.8 | 8.8 | 5 |
| Liversidge PVP Susp | Suspension (API dissolved: 10%) | 0.0013 | 28.1 | 0.65 | 5.6 | 5 |
| Erlich PVP | SwP* | 0.0013 | 28.1 | 0.65 | 5.6 | 0.25, 10, 20, 30 |
| Takano IR | IR^ | 0.00054 | 28.1 | 16.8 | 5.6 | 2.5 |
| Erlich DCR 100 | IR^ | 0.00054 | 28.1 | 16.8 | 5.6 | 10 |
| Erlich DCR 200 | IR^ | 0.00054 | 28.1 | 16.8 | 5.6 | 10 |
| Deva DCR 200 | IR^ | 0.00054 | 28.1 | 16.8 | 5.6 | 10 |

NR: Not required.
* SwP: Solution with precipitation
^ IR: Immediate Release.

of sensitivity was largely attributable to the increase of danazol solubility occurring in the presence of PVP. The solubility of danazol at 25 °C is reported as 0.0013, 0.0011 and 0.0012 mg/mL in presence of PVP concentrations of 10, 100 and 1000 μg/mL, respectively [21]. Based upon the data from Jackson et al. we see that PVP not only enhances danazol solubility but also decreases the PRC. Since the solubility of danazol was effectively constant across PVP concentrations of 10 – 1000 μg/mL, the PRC of danazol in the presence of PVP was not expected to be different from 0.65 h to 1. Furthermore, the model accounts for the solubilization of danazol in PVP as a true solution because of the amphiphilic qualities of PVP. We further assume that due to danazol's very high membrane permeability, it has a higher affinity for the membrane than it does for the PVP thereby enabling rapid permeability without the constraints of solubility limiting its transport to the epithelial membrane.

A summary of formulation-specific input parameters to the ADAM model (formulation dependent parameters) is provided in Table 3. The model predicted enhanced drug solubility in the different GI segments due to presence of bile is shown in Supplemental Table H.

#### 2.2.3. Optimization function

Parameter values were estimated using the Nelder-Mead optimization algorithm. The least squares method weighted by the reciprocal of the predicted squared values ($1/Y^2$) was used as the minimisation method. Parameter estimation employed a termination criterion based upon attainment of a minimum constant objective function value (OFV).

### 2.3. Model predictions

The PBPK model and formulation-specific model parameters were used to simulate a population of 50 healthy beagle dogs. The accuracy of the modelled parameter values and the description of the impact of formulation on the absorption and dissolution of danazol in dogs was assessed by comparing the observed versus predicted values.

## 3. Results

Each of the formulations that were evaluated in this study presented their own unique challenges impacting the corresponding model predictions of the segmental Fa values. The resulting estimates obtained when varying the formulation-associated pivotal considerations are provided in Fig. 1A–H. Details of the rationale for model selections are provided for each set of formulations below.

### 3.1. Danazol bolus intravenous plasma concentration time profiles

The predicted versus observed Cp-t profiles are provided in Fig. 2A–C. Parameter estimates are provided in Table 4.

Liversidge (Fig. 2A): All predicted parameter values were within 2-fold of the reported averages. The reported dose normalized $AUC_{0\text{-}t}$ in the study (19.8 μg·h/mL), when converted to the administered dose (2.97 μg·h/mL), was comparable to the $AUC_{0\text{-}t}$ (2.80 μg·h/mL) calculated manually from the digitally extracted values of the mean IV profile published in the Liversidge study (see Supplemental Table D) [15].

Anby (Fig. 2B and Supplemental Table E): The simulations over-predicted the observed Cp-t profiles, despite the 5% error in mean observed vs predicted $AUC_{0\text{-}t}$ and clearance estimates. The difference between the modelled versus Anby reported estimates of Vss may have contributed to this over-prediction. Despite the prediction error in the latter portion of the profile, the ability to adequately model the initial phases of the Cp-t profile provided the necessary confidence that this misspecification would not negatively impact our assessment of formulation effects.

Devalapally (Fig. 2C and Supplemental Table F): Initial simulations using the model default estimates of $CLint_{DLM}$ = 716 μL/min/mg of microsomal protein markedly over-predicted the Cp-t profiles, as shown by the dark line in Fig. 2C. Subsequent refinement via retrograde calculation of $CLint_{DLM}$ of 1052 μL/min/mg of microsomal protein (~30% higher based upon Devalapally reported $CL_{IV}$ estimates of 22.33 mL/min/kg) resulted in a much closer approximation of the digitized observed Cp-t profiles. Accordingly, all simulations for the Devalapally data were generated with this higher $CLint_{DLM}$, which was derived based on the Devalapally $CL_{IV}$ estimate.

It is important to note that the $CL_{IV}$ for Anby is less than that of Liversidge. Based upon the work of Devalapally et al. [14], the danazol hepatic extraction ratio in dogs was estimated as 0.72, which qualifies as a high ER drug. If danazol is a high ER drug, the $CL_{IV}$ is approximately equal to hepatic blood flow. Within the Simcyp Animal Model, the cardiac output (CO) is kept at a constant value of 2049 mL/min. This value reflects the CO of a 10-kg dog, which is similar to the average body weight of dogs enrolled in the Liversidge study. However, for the Anby study, the dogs had an average body weight of 18 kg. Accordingly, since the CO for a 10 kg dog is the value used in the canine mechanistic model, irrespective of dog body weight, the dogs in the Anby study would have parameter estimates generated based on an assumption of a lower L/kg CO. With that, the CL would be expected to be lower if in fact the $CL_{IV}$ was primarily a function of hepatic blood flow.

### 3.2. Danazol oral drug absorption

#### 3.2.1. Formulation dependent model input parameters

The information on excipient physicochemical properties and their corresponding *in vivo* effects on danazol bioavailability (Supplement Table A) were considered and when necessary, were used to revise the model values for the IS, PRC and the SR ratios.

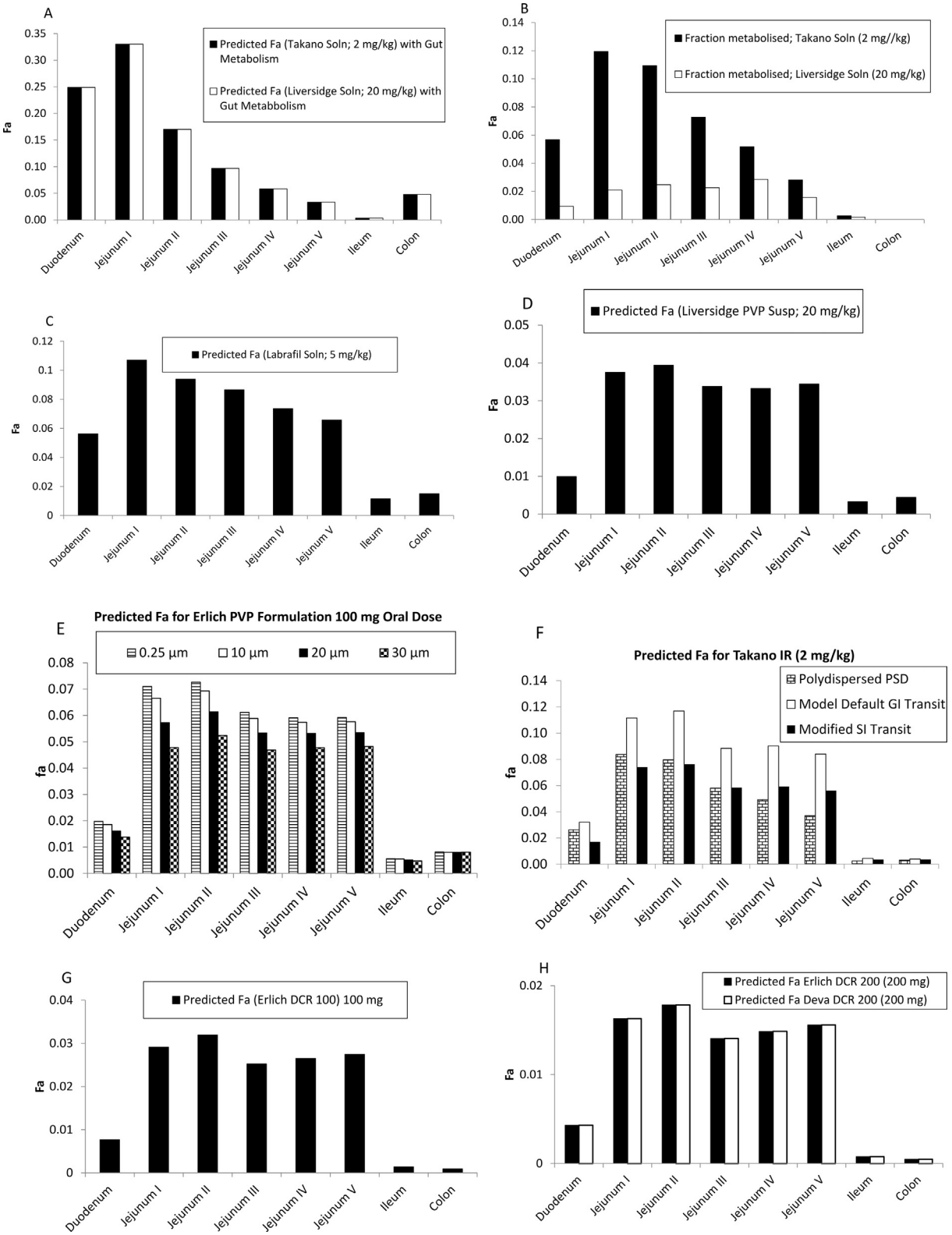


**Fig. 1.** Predicted values of fraction of administered dose absorbed (Fa) or fraction of dose metabolised in different segments of the beagle GI tract for Danazol after administration of various oral formulations: ( A) Predicted Fa for Liversidge Soln (20 mg/kg) and Takano Soln (2 mg/kg); (B) Fraction of dose metabolised -Liversidge Soln (20 mg/kg) and Takano Soln (2 mg/kg) using saturable gut metabolism (Vmax: 10 pmol/min/mg; Km: 0.02 µM); (C) Predicted Fa for Labrafil Soln (5 mg/kg) oral solution formulation; (D) Predicted Fa for Liversidge PVP Susp (20 mg/kg) oral formulation; (E) Predicted Fa for Erlich PVP formulation (100 mg dose) as a function of particle size; (F) Predicted Fa for Takano IR (2 mg/kg) oral formulation using modified or default SI transit times; (G): Predicted Fa for Erlich DCR 100 (100 mg) oral formulation (Danazol particle radius (µm): 10, 20, 30); (H) Predicted Fa for Erlich DCR 200 (200 mg) and Deva DCR 200 (200 mg) oral formulation.

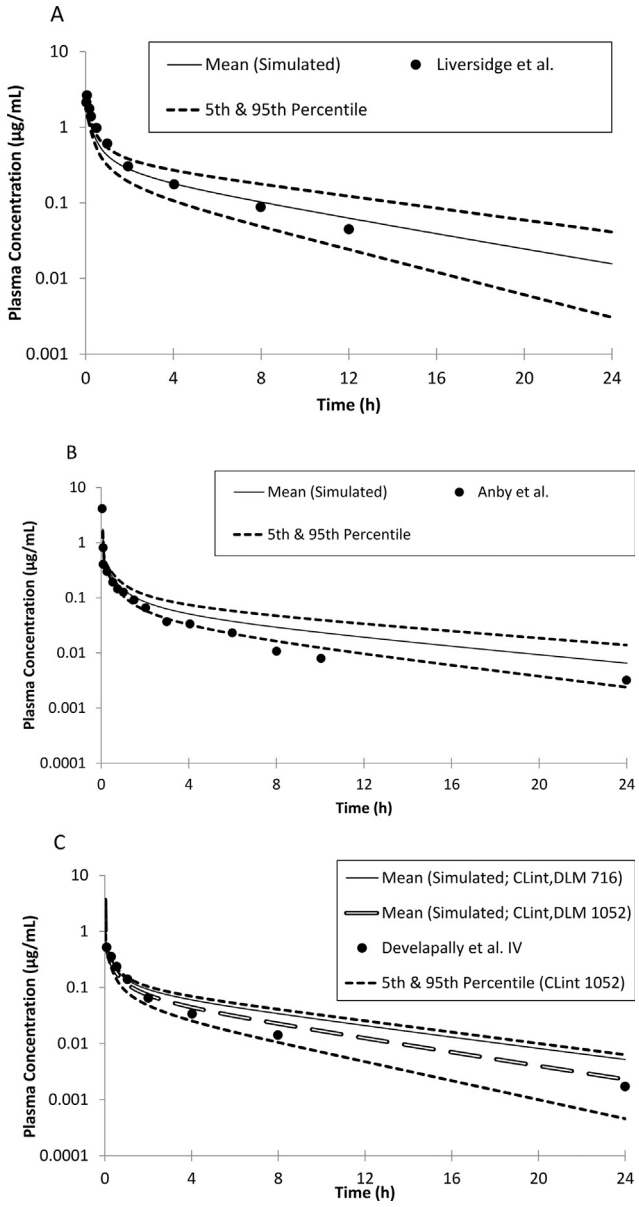


**Fig. 2.** Predicted and observed Cp-t profiles of danazol after intravenous administration; (A) 3 mg/kg bolus (Liversidge et al.); (B) 0.85 mg/kg infusion (Anby et al.); (C) 1 mg/kg bolus (Devalapally et al.). Filled circles reflect the mean observed data.

### 3.2.2. Oral solution (Soln)

The Liversidge Soln (hydroxypropyl β-cyclodextrin) and Takano Soln (10% DMSO; 20% Vit. E TPGS) formulations were modelled as oral solutions. Under the conditions of this model, oral solutions do not precipitate, rendering neither IS, SR nor PRC as relevant parameters.

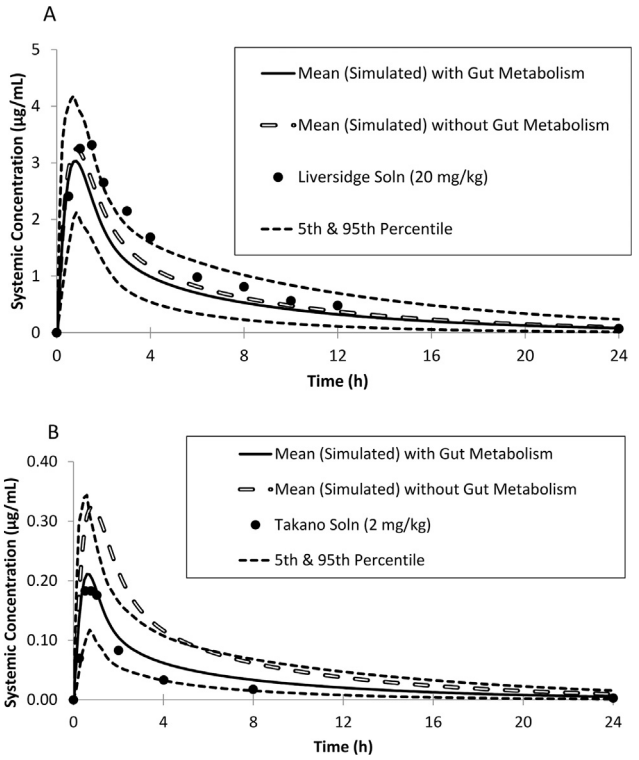


**Fig. 3.** The Liversidge (hydroxypropyl β-cyclodextrin) and Takano Soln (10% DMSO; 20% Vit. E TPGS) formulations modelled as oral solutions with or without the inclusion of gut metabolism . Filled circles reflect the mean observed data. Error bars about the observed means values represent standard deviations. (A) Liversidge 20 mg/kg Soln; (B) Takano 2 mg/kg Soln.

Predicted and digitized observed Cp-t profiles for the Liversidge Soln and Takano Soln formulations are shown in Fig. 3A & B and Supplemental Tables I1, 2. For the two oral solution formulations, the Fa and the fraction of dose metabolised in enterocyte ($E_G$) for each intestinal segment are shown in Fig. 1A & B respectively.

The dose-associated impact of saturable gut metabolism in the danazol ADAM model can be seen from the sensitivity analysis of increasing doses on Fg – fraction of dose escaping gut metabolism (Supplemental Figure A). The observed bioavailability for the Liversidge Soln, when including the optimized gut metabolism component ($V_{max}$: 10 pmol/min/mg; Km: 0.02 µM), changed the overall F for danazol by approximately 10% (Supplemental Table I1). In contrast, in the absence of the inclusion of a saturable gut metabolism, the bioavailability for Takano Soln formulation (Supplemental Table I2) was grossly over-predicted ($C_{max}$: ~2 fold; AUC: ~3 fold; F: ~6 fold). Although there was still some over-prediction of the Takano data

**Table 4**
Observed (reported) and predicted AUC and $CL_{IV}$ parameters for profiles in studies by Liversidge, Anby and Devalapally.

| | Liversidge et al. (3 mg/kg) | | | Anby et al. (0.85 mg/kg) | | | Devalapally et al. (1 mg/kg) | | |
|---|---|---|---|---|---|---|---|---|---|
| | $AUC_{0\text{-}12h}$ | $CL_{IV}$ | Vss | $AUC_{0\text{-}\infty}$ | $CL_{IV}$ | Vss | $AUC_{0\text{-}24h}$ | $CL_{IV}$ | Vss |
| Reported Average Values | 2.80 | 16.84 | 3.91 | 1.046 | 13.54 | 7.9 | 0.737 | 22.33 | 4.89 |
| Predicted | 2.36 | 21.22 | 5.36 | 1.058 | 14.19 | 5.35 | 0.789* | 22.50* | 5.35 |
| Ratio | 0.84 | 1.26 | 1.37 | 1.01 | 1.05 | 0.69 | 1.07 | 1.01 | 1.09 |
| **$CL_{int,DLM}$** | 716 | | | 716 | | | 1052 | | |

$AUC_{0\text{-}t}$, $AUC_{0\text{-}\infty}$ = µg·h/mL.
$CL_{IV}$ = mL/min/kg.
Vss = L/kg.
* Predicted AUC and CL obtained using $CLint_{DLM}$ 1052.

following this model modification (the $C_{max}$ and AUC ratio (Pred/Obsd) 1.2 and 1.4 fold, respectively), the inclusion of gut metabolism substantially improved the model predictions. Increasing the gut metabolism component ($V_{max}$), in an attempt to improve our recovery of AUC, F, and the tailing part of the Cp-t profile, resulted in a gross underprediction of the $C_{max}$.

*3.2.2.1. Labrafil.* When modelling this formulation in the solution mode (without supersaturation or precipitation), the predicted Cp-t profile exceeded the digitized observed profile concentrations (Supplemental Figure B). Thus, although considered a non-aqueous solution by Devalapally et al., the Labrafil formulation did not provide the magnitude of enhanced oral bioavailability observed with the other "danazol oral solutions". If danazol precipitation from the saturated aqueous phase was not a possibility and if the lower absorption of danazol (compared to ideal solution) was attributed to the increased partitioning and gradual release of danazol from the mixed micelle phase, the *in vivo* observed plasma profiles would indicate a delayed Tmax (see sensitivity analysis of $logK_{m:w}$ on Tmax - Supplemental Figure C). Therefore, based on the excipient information in Supplemental Table A, the simulation results were interpreted to indicate that either: (1) a greater than predicted ($logK_{m:w} > 5.6$) proportion of the solubilized drug partitioned into the mixed micelles (thus lowering the free fraction) because of increased micelle concentration due to Labrafil lipolysis, leading to a lower fraction of drug absorbed; or (2) possible precipitation of the drug post digestion of Labrafil lipolysis after the drug is released into the aqueous phase and transits into the distal intestine (ileum and colon) or (3) a sequential occurrence of both processes mentioned above.

Any of these three scenarios would provide an Fa that is lower than anticipated for a true solution. Consequently, in contrast to the Liversidge and Takano oral solutions, we considered applying the alternative formulation classification of SwP. This option allowed for the drug to partition into the mixed micelles (during lipolysis and digestion) and to possibly precipitate following the release of the drug into the aqueous phase.

A substantial over-prediction (using the solution formulation mode) and under-prediction (using the SwP mode) of drug plasma concentrations occurred when the $logK_{m:w}$ was retained at the value of 5.6. Considering the multifaceted nature of the Labrafil-danazol relationship and to mimic the increased partitioning of danazol into bile micelles, a sensitivity analysis was conducted to optimize the $logK_{m:w}$. Any change implemented in the $logK_{m:w}$ would also affect the estimate of $P_{eff}$ as predicted by the MechPeff model, which factors the effect of free fraction on passive membrane permeability [2]. Accordingly, to appreciate the formulation effects of Labrafil on danazol oral bioavailability, it was necessary to account not only for the impact of Labrafil on drug solubilization but also on permeation. With an increase in the concentration of the mixed micellar systems associated with Labrafil lipolysis, the free monomer available for permeation is expected to decrease. To accommodate an increased partitioning of danazol into the mixed micelles, the optimized $logK_{m:w}$ (using the SwP mode) was found to be 8.8. A sensitivity analysis of the PRC (between values 0.1–100 $h^{-1}$ using $logK_{m:w}$ 8.8) revealed no impact on PK parameters Cmax, Tmax, AUC and Fa, indicating that although danazol was allowed to precipitate, it did not affect danazol absorption.

As can be seen from the predicted and observed Cp-t profiles and PK parameters, the revised model conditions resulted in very good predictions of Cmax, with most absorption occurring in the proximal small intestine (duodenum and jejunum). The Cp reduces to some minimal concentration at approximately 4 h postdose (Fig. 1C and Supplemental Figure D). The slight over-prediction of Fa in the mid and/or distal jejunum resulted in a 2-fold over-prediction of AUC and F. Considering the predicted inter-individual variability for AUC (SD = 0.44), this magnitude of error was determined not to impact our conclusions regarding excipient effects on oral drug solubility and permeability.

The appropriateness of using SwP as the modelled formulation was further reinforced by evaluating the Fa in each intestinal segment. At $logK_{m:w} = 8.8$, there was negligible absorption in the ileum using either SwP or solution modes, but there was a tremendous rise (up to ~30%) in Fa in the colon when modelled as a solution. To ascertain if we could rectify this over-prediction by eliminating colonic absorption when modelling as an oral solution, the effective permeability of the colon was set to zero (Supplemental Figures C and D). Although prohibiting colonic drug absorption reduced the magnitude of the overprediction, there remained a markedly greater prediction error as compared to that seen with SwP. Thus, although the extent of aqueous danazol precipitation may have been relatively small (based upon the lack of sensitivity observed to the PRC), the results imply that precipitation did in fact influence the danazol absorption characteristics following its oral administration as a Labrafil non-aqueous solution.

For additional support of our contention that the impact of Labrafil was primarily grounded in its effects on danazol partitioning into the bile micelles, we repeated assessments under three sets of conditions. To that end, nearly identical results were achieved when the Labrafil formulation was modelled as an oral solution with precipitation ($logK_{m:w} = 8.8$, PRC = 16.7 1/h), as an oral solution with precipitation but where precipitation was minimized by setting the PRC to 0.01 h ($logK_{m:w} = 8.8$), or as an oral suspension where 100% of the drug was solubilized upon administration ($logK_{m:w} = 8.8$, PRC = 0.01 or PRC = 16.7 1/h). Based upon these results, the critical factor in danazol absorption was the ability of the drug to partition into the bile salts. While a PRC of 16.7 or 0.01 had no impact on prediction error, the lower $logK_{m:w}$ values (5.6) markedly under-predicted the observed Cp-t profiles (Supplemental Figure H). These outcomes are consistent with the negligible danazol precipitation occurring after administration of the Labrafil formulation. Conversely, movement of the danazol from the Labrafil into the mixed micelles (upon Labrafil lipolysis) controlled the extent of danazol oral bioavailability. Please refer to the discussion section for additional information on this topic.

We further explored this assumption by examining the available literature. Although Larsen et al. [22] did not report evidence of danazol precipitation during the Labrafil *in vitro* lipolysis study, they indicated that danazol approached its saturation limit toward the end of lipolysis process. Our model, while not able to simulate the lipolysis process, resulted in simulations that were reflective of the findings of Larson et al. in that we observed the danazol concentration approaching the saturation limit in the aqueous phase leading to minor precipitation in the distal SI. The possibility of drug precipitation *in vitro* was also reiterated by Sassene et al. in their introduction where they report the possibility of danazol and fenofibrate precipitation from lipid based drug delivery systems during *in vitro* lipolysis (during drug transit) [29]. However, Sassene et al. indicated that the possibility of danazol precipitation during *in vivo* lipolysis was minimal, which coincides with our prediction that the amount of drug precipitated did not have an effect on the Fa. This implies that although precipitation does occur, there could still be redissolution or partitioning of the drug into the mixed micelles. Precipitation of danazol upon digestion of a lipid formulation was also shown by Porter et al. for medium chain (MC) and long chain (LC) self micro-emulsifying drug delivery systems (SMEDDS) [24]. Their observations were not related to Labrafil. Nevertheless, considering their combination of *in vivo* and *in vitro* data, we viewed their result to be supportive of our assessments (their study showed that nearly 70% (for MC-SMEDDS) and 6% (LC-SMEDDS) of danazol precipitated out of the digestion phase [24]).

We note that for a low solubility/high permeability compound such as danazol, the enhanced solubility in the fed state should increase its oral bioavailability. This is precisely what was reported when the oral capsules were administered in fasted and fed human subjects [33]. However, as discussed by Sugano et al. [16], an increase in mixed micelle concentrations within the intestine results in several counteracting forces. This includes enhanced solubility versus a reduction in

the free drug fraction at the epithelial membrane due to a reduction of the drug diffusion coefficient through the mucus layer. So long as drug solubility enhancement due to bile is the rate-controlling effect, any negative influences on $P_{eff}$ will be negligible. However, under the conditions of the Devalapally investigation where the drug was administered in solution, the negative influences on $P_{eff}$ appear to have had the predominant influence, rendering the observed drug concentrations markedly lower than would be predicted if the Labrafil formulation behaved as a true (non-aqueous) solution. Thus, the interesting take home message is that when predicting whether there will be a positive or negative food effect, both drug physicochemical properties and formulation need to be considered.

To account for the decrease in the danazol free fraction available to drive the passive permeability concentration gradient at the epithelial membrane, the 'MechPeff' permeability model reduces the overall $P_{eff}$ from a model default value of $\sim 1.5 \times 10^{-4}$ cm/s to $0.45 \times 10^{-4}$ cm/s (Supplemental Figure D). This change implies that an increase in the danazol fraction partitioning into micelles leads to a decrease in the fraction of danazol monomer which drives the passive permeability concentration gradient at the epithelial membrane. The consequence of these changes is the reduction in the overall $P_{eff}$. In this case, a separate equilibrium prevails between the free drug monomer and the micelle bound drug until all micelle bound drug dissociates to the monomer. Within our model, this lower fraction of danazol monomer became the rate limiting factor determining the overall intestinal permeability (and hence the decrease in the overall $P_{eff}$ value). This decrease in monomer fraction is consistent with the observed difference between the free versus micelle bound danazol when tested in FaSSIF and FeSSIF [16].

As a final test of our rationale for the observed effects of Labrafil, we explored an additional scenario whereby the availability of the free monomer was not considered to be the rate limiting step towards $P_{eff}$ (and Fa) but rather all the solubilized drug concentration (free + micelle bound) functioned as the driving force for passive permeability (concentration gradient). Based upon that assumption, the relationship between total drug concentration and enterocyte permeability was tested via a sensitivity analysis (Supplemental Figure F). As seen from Supplemental Figure F, a single $\log K_{m:w}$ value could not effectively recover the Cmax, Tmax and AUC of the *in vivo* profiles. For example, the $\log K_{m:w}$ of 6.1 that would closely approximate the reported Cmax value (observed = 0.13 µg/mL) would result in an over-prediction of AUC of approximately 0.58 µg * h/mL (observed = 0.41 µg * h/mL) and an over-prediction of Tmax of 2.1 h (observed = 0.5–1 h). At the previously defined optimized $\log K_{m:w}$ of 8.8, all parameter values are grossly over-predicted. This observation served to confirm the importance of the free fraction as the variable determining danazol permeability.

The predicted and observed Cp-t profiles of danazol and the resultant PK parameters, after oral administration of Labrafil Soln formulation are shown in Fig. 4 and Supplemental Table I3.

*3.2.2.2. Liversidge PVP and Erlich PVP formulations*. Based upon the excipient information defined in Supplemental Table A, both simulated datasets were generated using the modified IS of 0.0013 and PRC of 0.65 ($h^{-1}$).

Liversidge: The Liversidge PVP suspension is an aqueous suspension of danazol (5% w/w) and PVP (1.5% w/w). To determine the best fit estimate of the fraction of drug dissolved in the suspension prior to oral administration, a sensitivity analysis was performed. Varying the percent of API dissolved in the suspension showed no effect on the predicted Fa, Fg, Cmax, Tmax or AUC. Hence, the model condition for the percent of API dissolved was set at 10%. The particle size radius for Liversidge PVP was input as 5 µm in accordance with that specified in the study publication [15]. The predicted and digitized observed PK parameters, Cp-t profiles and the predicted Fa values after administration of the Liversidge PVP Susp formulation are shown in Supplemental Table J1 and Figs. 5 and 1D, respectively.

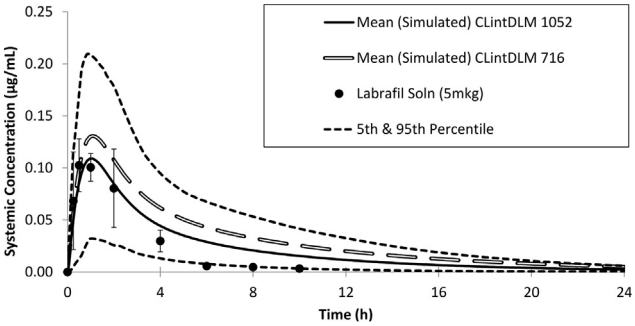


**Fig. 4.** Predicted and observed Cp-t profiles of danazol after administration of Labrafil Soln (5 mg/kg) oral solution formulation. Filled circles reflect the mean observed data. Error bars about the observed means values represent standard deviations.

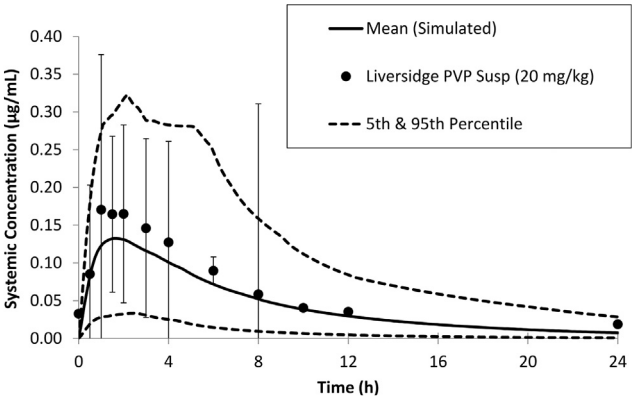


**Fig. 5.** Predicted and observed Cp-t profiles of danazol after administration of Liversidge PVP Susp (20 mg/kg) oral formulation. Filled circles reflect the mean observed data. Error bars about the observed means values represent standard deviations.

Of note is the very wide variability associated with the digitized observed Cp-t profiles and the wide window of $T_{max}$ values. Accordingly, while the simulations were associated with a 0.27 fold under-prediction of the mean $C_{max}$ value, the modelled $C_{max}$ was within the range of the observed $C_{max}$ variability (CV = 65%). The predicted values of AUC and F closely approximated the observed values, and the variability associated with these predictions correlated well with the observed data. With respect to the intestinal segmental involvement in the absorption process, unlike that seen with the oral solution (where the absorption occurred primarily in the duodenum and proximal jejunum, Fig. 1A), the danazol absorption from the oral suspension occurred throughout the jejunum. Minimal to no absorption occurred in segments distal to the jejunum (Fig. 1D).

Erlich: In contrast to the Liversidge PVP oral suspension which was aqueous in nature (PVP in water), the Erlich PVP formulation contained a much higher concentration of PVP (23%) in combination with polyethylene glycol (PEG 400). The danazol:PVP ratio was 1:6 respectively and was prepared by dissolving danazol in a mixture of pre-dissolved PVP in PEG 400. This non-aqueous solution was then filled into gelatine capsules prior to being administered to the beagle dogs (n = 4 dogs; 100 mg danazol/dog). The authors estimated the solubility of danazol in that formulation to be about 48 mg/mL, implying that 2 mL of solution was administered.

We considered various model options to explore possible reasons for the observed profile of the Erlich 100 mg PVP formulation. Although it was administered as a "solution" in a gelatine capsule, the use of the solution formulation option within Simcyp markedly over-estimated the plasma concentrations. Therefore, we applied the option of solution with precipitation, using the intrinsic solubility and PRC estimate

previously applied to the Liversidge PVP data. In this case, the predicted and observed profiles were in close agreement. The simulation output showed negligible absorption in the colon or ileum (Fig. 1E). Given that observation and to ascertain if colonic absorption contributed to the overestimation of the Erlich et al. PVP profiles seen when modelled as a solution, we repeated the simulations using the solution model but without colonic drug absorption. This exploration failed to reduce the magnitude of model misspecification, leading us to consider the solution with precipitation as the best choice for these data.

Tracking the magnitude of precipitation across the various GI segments, we observed that while negligible in the duodenum, the magnitude of danazol precipitation increased as the drug moved down the GI tract (see Supplemental Figure I). This raises the question of if precipitation was occurring, what could be the potential reasons. Although no straightforward explanations are available in the literature, there were reported observations that provided clues as to events that may have occurred:

**I:** PEG: At any given temperature, the presence of water can lead to the aggregation of the PEG [34]. These authors estimated that at 37 °C, the PEG 400 critical aggregation concentration (CAC) is approximately 0.05 mol/L (Fig. 2 in Derkaoui 2007). Considering the amount of water within the dog GI tract after the administration of a 60 mL water flush (as reported by Erlich et al.), our model predicts that the highest water volumes will range from 0.006 (Ileum) to 0.08 (stomach) liter. Given these predictions, the CAC for PEG 400 would be exceeded as soon as it is diluted within the stomach (0.0625 mol/L). Due to the formation of aggregates, the solubilization capacity of PEG would be reduced, leading to the potential precipitation of danazol. However, we speculate that the presence of 23% PVP in the Erlich et al. formulation allowed for the danazol precipitation rate to be reduced from 17 $h^{-1}$ to 0.65 $h^{-1}$ (as was applied to the Liversidge dataset). Thus, most drug remained in solution until it reached the mid to distal segments of the intestine.

**II:** PVP: This postulated scenario was further supported by a BASF study where the investigators examined the magnitude of the "parachute" effect associated with the inclusion of a crystallization inhibitor to a 500 µL soft gel containing danazol solubilized in PEG 400 as PEG 400 LA [35]. The capsules were injected into the Pion InForm$^{R}$ in a 50 mL aqueous buffered solution (which is a lower fluid volume than what was anticipated within the dog stomach at the time of capsule administration), and the concentration of dissolved API was evaluated over time. In either PEG 400 alone or PEG 400 + Kollidon 12PF (a form of PVP), the maximum concentration of solubilized danazol was 50 µg/mL. Despite differences in the form of PVP used by Erlich et al. (PVP K-30 = Kollidon® 30) vs Romanski and Hoffman, we considered the precipitation results occurring in the presence of Kollidon 12 PF or even the most potent effect of Kollidon VA 64 (which unlike Kollidon 30, is a vinylpyrrolidone-vinyl acetate copolymer) to support our model selection of solution with precipitation. To that end, Romanski and Hoffman showed that precipitation began to occur within about 2 min with Kollidon 12PF and within about 8 min for Kollidon VA64 (the duration of the parachute effect was about 5 min with Kollidon 12PF and about 25 min with Kollidon VA 64).

Regardless of the exact mechanism, it is clear from our model that although administered as a "solution", when solubilized in PEG 400 + PVP, danazol does not behave as a true solution and that solubilization-associated changes will continue to compromise its oral bioavailability. Given their limited systemic absorption (see Supplemental Table A), it was assumed that formulation effects for the Erlich treatment would be primarily a function of its effects on drug solubility.

The model did not account for the time for capsule disintegration but rather treated the dosage form (in this case, 'solvation' of the gelatine capsule and 'release' of its contents into the gastric environment) as "instantaneous". Despite this oversimplification, any error associated with this assumption should be minimal so long as the rate of drug release exceeds the gastric residence time (mean 0.37 h; CV 69%). The emptying of the gastric contents is modelled as a first order process for solutions and fine particles.

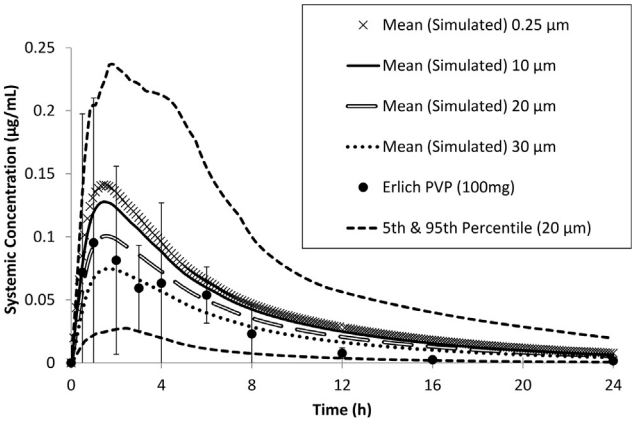


**Fig. 6.** Predicted and observed Cp-t profiles of danazol after administration of Erlich PVP (100 mg) oral formulation. Filled circles reflect the mean observed data. Error bars about the observed means values represent standard deviations.

The applicability of the modelled conditions was demonstrated by the lack of difference between the Tmax associated with the oral solution versus that observed when the drug was administered in the gelatine capsule. Since the "precipitated" danazol would also be available for 're-solubilization' and absorption, simulations were conducted considering particle size of the emulsion globules with a radius of 0.25, 10, 20 and 30 µm. Observed and simulated (0.25, 10, 20 and 30 µm) Cp-t profiles and PK parameters for the Erlich PVP danazol formulation are shown in Fig. 6 and Supplemental Table J2, respectively. Of these, the 20 µm radius resulted in simulated profiles most closely aligned with observed values. Predicted segmental Fa values were greatest in the mid and distal jejunum (Fig. 1E).

Given the shape of the predicted versus observed profiles using the 20 µm size particles where the over-prediction was particularly evident at the latter timepoints, we believe that the over prediction of the Erlich AUC values was attributed to the use of the Liversidge $CLint_{DLM}$ rather than to an over-estimation of the absorption component of the profile. This conclusion is supported by the ratio of AUC values obtained with the PVP formulation versus the Danocrine 100 mg tablets (DCR 100) and by comparing the corresponding ratios generated based on observed versus simulated profiles (1.35 for the PVP formulation and 1.44 for the Erlich 100 mg Danocrine tablets).

### *3.2.3. Solid formulations: hard gelatine capsules and Danocrine® 100 mg or 200 mg strength tablets*

*3.2.3.1. Takano gelatine capsules (Takano IR):.* The lactose included in the Takano IR capsule formulation (danazol and lactose in a ratio of 1:9 respectively) enhanced drug wettability without altering its solubility (Supplemental Table A). Accordingly, the danazol IS was maintained as the default value of 0.00054 mg/mL, with a corresponding SR = 28.1 and PRC = 16.8. The particle size radius, as specified in the study report, was 2.5 µm and the dose was 2 mg/kg.

When using these parameters as model input values, the simulations over-predicted the observed Cp-t profiles of the Takano IR capsules. There was a 2-fold over-prediction in AUC and a 4-fold over prediction in Fa and F. In exploring the possible factors influencing this over-prediction, we considered: (1) a local effect of lactose on the small intestinal transit time (lactose was the only excipient included in the capsule formulation); or (2) bias in the values used for defining the drug formulation's critical quality attributes (CQA).

*3.2.3.1.1. Intestinal transit time.* The initial effort to identify the reasons for this over-estimation focused on the possibility that the

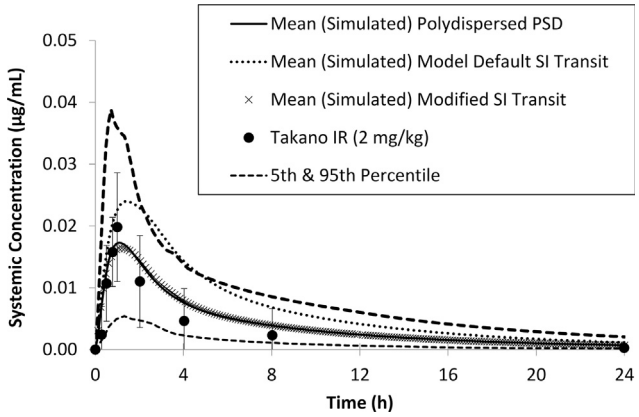


**Fig. 7.** Predicted and observed Cp-t profiles of danazol after administration of Takano IR (2 mg/kg) oral formulation.

lactose included in the soft gelatine capsule caused a change in the small intestinal transit times [36] (Supplemental Table A). Upon conducting a sensitivity analysis, it was observed that a misspecification of the intestinal transit time could lead to our model-generated over-predictions. Accordingly, based upon a sensitivity analysis to recover the observed *in vivo* profiles, we adjusted the small intestinal transit time from the default value of 2.39–1.34 h (Supplemental Figure E). This change succeeded in improving the overall predictions as compared to that obtained when using the default model settings. However, Fa and F remained over-predicted by approximately 3-fold (Fig. 6 and Supplemental Table K). Predictions of AUC were within 1.33-fold of the observed values (Figs. 7 and 1F). Although the model using the modified small intestinal transit time still generated predictions that exceeded the *in vivo* estimate of Fa (Supplemental Table K; Fig. 1F), the very large variability in the simulation output allowed for the decrease in GI transit time to improve our ability to capture the observed mean profile data.

Since the estimation of Fa in Simcyp is via a summation of the segmental Fa values, this outcome indicates that a change in GI transit time alone was unable to adequately compensate for what was occurring with the Takano IR formulation. Moreover, it could be argued that the amount of lactose provided to these dogs (180 mg) would not be expected to cause the formulation-induced local osmotic effect, and corresponding water efflux into the intestine, that would cause the magnitude of physiological effect necessary to explain the difference between observed and predicted values. Therefore, an alternative explanation needed to be explored.

*3.2.3.1.2. In vivo dissolution*. Considering the specifics of the formulation, which contained only two ingredients, drug and lactose, it was suspected that API - CQAs such as particle size, PRC or SR, could be responsible for the *in vivo* profile over prediction. The PRC of danazol was shown to be a robust model input parameter when simulating the other formulations, resulting in a satisfactory recovery of the observed plasma profiles. The SR was not expected to be a sensitive parameter because danazol is a neutral drug and the formulation did not contain any solubility enhancing excipients which would allow the drug to enter a supersaturated state. Upon review of the particle size, it was noted that Takano et al. reported the volumetric mean diameter (VMD) calculated as the Feret's diameter. Hence, when 5 µm was the VMD used in the simulations, we assumed it to follow a monodispersed distribution (i.e., all particles of the same size). This assumption led to profile predictions that were much greater than that of the observed plasma profiles. A sensitivity analysis of the monodispersed particle size distribution (using standard model input parameters) revealed that the particle size was the primary CQA that determined the Fa. At particle size radii values of 5 µm and lower (predicted Fa = 0.7) or 150 µm and higher (predicted Fa ≤ 0.02), the Fa was insensitive to changes in particle size (Supplemental Figure J). Between the region of 5–150 µm, Fa was found to be highly sensitive to particle size.

Based upon the *in vitro* dissolution data generated using $FASSIF_{dog}$, Takano et al. reported an approximately two-fold under-prediction of Fa for the 229 µm particle diameter despite their correlation to the observed Fa for the 5-µm size particles. For the larger particles, they concluded that *in vivo* dissolution was occurring under sink conditions (rendering the formulation dissolution-rate limited) but concluded that absorption was solubility-limited for the formulations containing the 5-µm size particles. That conclusion is consistent with the result of our sensitivity analysis.

We did, however, note a possible challenge introduced by the method used by Takano et al. when generating their particle size evaluation. Firstly, they reported a mean particle parameter, suggesting that they were dealing with a polydispersed rather than a monodispersed system. However, they did not provide any specifics about the particle size distribution, such as the Fmin or the Fmax (minimum or maximum diameters). Secondly, they reported particle size as the Feret's mean diameter, suggesting the presence of irregularly shaped particles. This differs from the Simcyp ADAM diffusion layer model that assumes particle sphericity for prediction of dissolution rate. This difference in shape assumption could have contributed error to our simulation output.

Based on the results of the sensitivity analysis, we explored the impact of varying the particle size distribution on profile predictions. We observed that if, for example, we had a polydispersed particle size input consisting of 40% 2.5 µm and 60% 125 µm, we were able to closely proximate the observed Cmax and Tmax. In fact, the resulting profile was similar to that achieved by reducing the intestinal transit time (Figs. 7 & 1F). Nevertheless, in both cases, we were unable to accurately reproduce the observed Fa (Supplemental Table K).

As a further method for exploring the likelihood that bias in our estimates of *in vivo* dissolution rate (such as that which would occur due to bias in the particle size estimations) was the primary cause of our prediction error, we fitted the *in vivo* dissolution profile using a Weibull function (GI transit time was set to default values). Weibull optimization resulted in parameter values of Fmax = 26.1%, $\alpha = 0.416$ and $\beta = 0.775$. Although over-prediction in AUC and Fa was still observed, the improvement in fit (particularly regarding Cmax and Tmax) was consistent with particle size being the primary source of error leading to the differences between the observed and predicted profiles (Supplemental Table K).

*3.2.3.2. Erlich DCR 100, Erlich DCR 200 and Deva DCR 200*. These three sets of data reflect the *in vivo* performance of the commercially available danazol IR capsules. None of the excipients in this commercial product are known to influence danazol physicochemical properties. Accordingly, the default danazol parameters (with no excipient effect) were used in these simulations (Table 1). An inter-study comparison of the observed profiles identified a divergence whereby the Deva DCR 200 formulation was associated with a lower AUC and similar $C_{max}$ compared to the Erlich DCR 100 despite a doubling of dose. However, there was no corresponding saturable absorption kinetics in the observed data for the Erlich DCR 100 and 200 mg strength IR capsules. The latter raises uncertainties as to why the simulated Erlich DCR 100 mg vs 200 mg product profiles failed to show a corresponding dose-associated increase in danazol concentrations.

*3.2.3.2.1. Erlich DCR 100*. Observed and predicted Cp-t profiles, predicted Fa and the related PK parameters for Erlich DCR 100 are shown in Figs. 8 and 1G and Supplemental Table L1, respectively.

The study did not provide information on the particle size of danazol in the formulation. Therefore, the particle size radius was set at 10 µm, based on literature information [30,31]. The $C_{max}$ and $T_{max}$ were reasonably well predicted with fold ratios of 1.1 and 0.74, respectively.

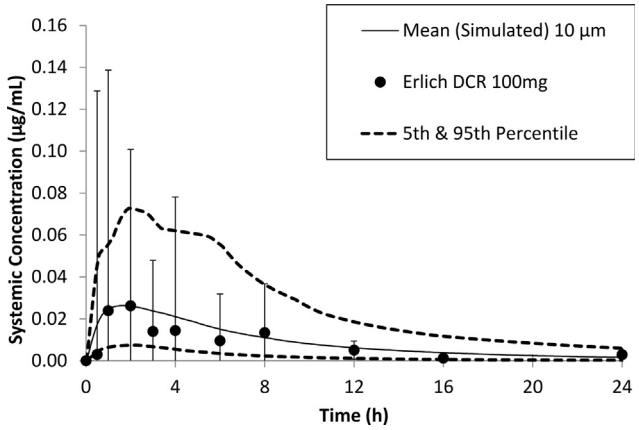


**Fig. 8.** Predicted and observed Cp-t profiles of danazol after administration of Erlich DCR 100 (100 mg) oral formulation.

The AUC was slightly over predicted with a fold ratio of 1.44 when compared to observed data. The observed Cp-t profile exhibited an initial small delay in the onset of absorption, followed by rapid absorption, a decline to a plateau which continued roughly up to 8 h, and lastly a terminal phase of concentration decline. The predicted mean Cp-t profile did not show this trend of an initial small delay in absorption, but when the profile for the 5th percentile was examined, a delay in onset of absorption was observed, indicating that the inter individual variability captured this delay in the simulated population of n = 50. The predicted Fa values for the different GI tract segments corresponded well with a slower rate of *in vivo* dissolution (Fig. 1G).

*3.2.3.2.2. Erlich DCR 200.* The observed and predicted Cp-t profiles and the predicted Fa values are provided in Figs. 9 and 1H and Supplemental Table L2. The simulated profiles under-predicted $C_{max}$ (0.47 fold) and AUC (0.74 fold), but over-predicted $T_{max}$ (1.34 fold). Comparing the observed values of the Erlich DCR 100 and 200 formulations, there was a proportional increase in the AUC (0.0.16 µg·h/mL vs 0.34 µg·h/mL for the 100 and 200 mg capsules, respectively) but the increase in $C_{max}$ was greater than proportional (0.026 µg/mL vs 0.07 µg/mL for the 100 and 200 mg capsules, respectively). Along with a shift in $T_{max}$ (3 h vs 1.88 h for the 100 and 200 mg capsules, respectively), the high inter-individual variability in the reported data from the four study dogs (e.g., the CV for the AUC being 0.34 and 0.65 for the 100 and 200 mg capsules, respectively), confounded our ability to accurately predict the Cp-t profiles. Unfortunately, without additional information about the *in vivo* study, it is difficult to resolve reasons for the errors obtained in the model predictions.

*3.2.3.2.3. Deva DCR 200.* Apart from using a $CLint_{DLM}$ of 1052 to model the Devalapally data, inter-study differences in observed Cp-t

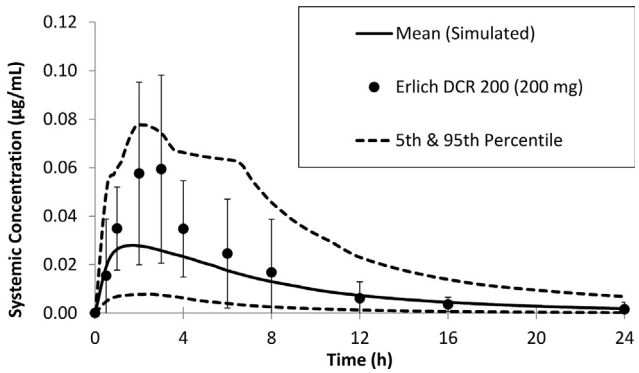


**Fig. 9.** Predicted and observed Cp-t profiles of danazol after administration of Erlich DCR 200 (200 mg) oral formulation.

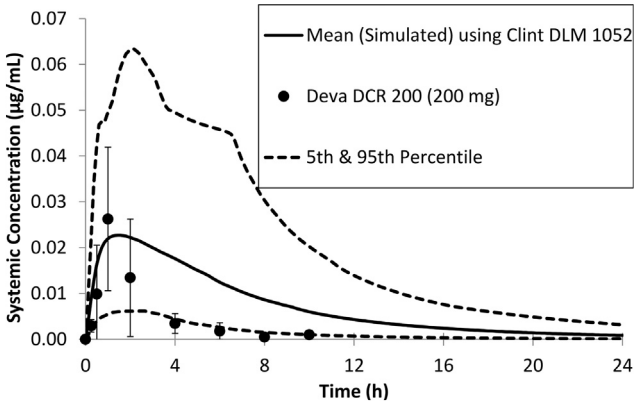


**Fig. 10.** Predicted and observed Cp-t profiles of danazol after administration of Deva DCR 200 oral formulation.

profiles reported by Devalapally and Erlich for the same DCR 200 mg strength tablets were difficult to reconcile. In this regard, it appeared that a smaller amount of drug was absorbed in the Deva DCR 200 study as compared to that of either the Erlich DCR 100 or the 200 mg strength tablets. Furthermore, the Devalapally profiles exhibited a very rapid and sharp peak. The rate of drop corresponded to a $t_{1/2}$ of about 1 h, which was far more rapid than that seen in their IV profiles generated using the same dogs.

The early peaks suggested that absorption was limited to the duodenum and proximal jejunum. Potentially, the early $T_{max}$ values could reflect enhanced drug solubilization as a function of the "nonfasted" condition of the dogs used in this Devalapally study. However, given the expected rate of danazol elimination, this alone could not explain the reason for a very rapid drop in concentrations once the peaks were achieved.

The predicted and observed Cp-t profiles, predicted Fa and relevant PK parameters are shown in Figs. 10 and 1H and Supplemental Table M, respectively.

## 4. Discussion

This work showcases the utility of mechanistic models to identify factors influencing the varied effects that formulations can have on the oral absorption of a highly lipophilic compound. By modelling *in vivo* profiles across a range of formulations, we identified likely causes for differences between observed and predicted formulation effects. For example, it is unlikely that the presence of saturable gut metabolism would have been recognized without the use of a mechanistic model. This saturable metabolism resulted in a dose-dependent impact of formulation-induced solubility enhancement which was particularly impactful at very low doses of danazol.

Prior to identification of pre-systemic drug loss, we were unable to explain the reason for inconsistencies between systemic exposure and dose proportionality, influence of formulation, particle size and predicted danazol oral bioavailability. In particular, the lower the dose or the smaller the danazol particle size, the more extreme became our over-prediction of oral bioavailability. This outcome presented a challenge since based upon drug physicochemical properties alone, the lower danazol oral dose or smaller particle size should have increased (not decreased) the fraction of solubilized dose, thereby enhancing the fraction of dose absorbed. As seen in other cases, the use of formulations that prevent Oswald's ripening typically enhances the *in vitro* dissolution and oral drug bioavailability as particle size decreases [30,31]. However, through a careful assessment of these inter-study inconsistencies, we could identify a plausible reason for this unanticipated outcome. For those studies examining formulation effects at lower doses of danazol (or formulations with low Fa), it is easy to see how failure to identify this pre-systemic metabolism could lead to a

misinterpretation of *in vivo* study results and *in vivo/in vitro* disparities. For example, it may have been attractive to assume that the lower-than-anticipated Fa seen with the Takano 2 mg/kg Vit E-TPGS solution was attributable to drug precipitation. Nonetheless, this possibility was ruled out based on the reports by Childs et al. [37] where it was shown that danazol supersaturates or is maintained in a solubilized form at concentrations of 0.45–0.2 mg/mL when in the presence of just 1% Vit E TPGS for a duration of 2 h. Since the percentage of TPGS in the Takano 2 mg/kg solution was 20%, precipitation of danazol after mixing with the GI milieu was considered to be negligible. Ultimately, with the inclusion of saturable gut metabolism, excellent agreement between observed and predicted parameter values was observed.

Further explanation of the rationale supporting the designation of the Takano formulation as an oral solution is warranted. On the one hand, it can be argued that TPGS is associated with the formation of an emulsion-like dosage form, which would be modelled as an oral suspension) [38,39] (the inhibition of P-gp associated with this excipient can be ignored for danazol because of its excellent passive permeability and absence of P-gp substrate activity). However, the inclusion of DMSO, a highly effective solubilizing agent, can alter the relationship between a low-solubility drug and the emulsifying agents. For example, the presence of DMSO as a co-solvent in an emulsion substantially increases the release of poorly soluble compounds (eg. Nystatin) when formulated in a variety of vehicles intended to form a self-nanoemulsifying delivery system [40]. To that end, the solubility of danazol in DMSO is 30 mg/mL, which means that it would be expected to have markedly enhanced the drug solubility in the Takano TPGS formulation (https://www.caymanchem.com/pdfs/16471.pdf). Nevertheless, confirmation was needed that the Takano formulation did not behave as an *in vivo* emulsion rather than as an oral solution.

As shown in Table I2, when concentrations were predicted as an oral solution, an excellent correlation was achieved between observed and predicted drug concentrations. In contrast, the population simulations poorly predicted the observed profiles when modelled as an oral suspension (with 100% of the suspension delivered in solution and $\log K_{m:w}$ = 8.8 or 5.6). The ratio of predicted (mean of 50 simulated profiles) to reported observed values were 0.90, 1.24 and 1.42 for Tmax, Cmax and AUC when simulated as an oral solution, 2.45, 0.28, and 0.58 when simulated as an oral suspension (100% dissolved, $\log K_{m:w}$ = 8.8) for Tmax, Cmax and AUC, and 1.25, 0.33, and 0.41 for these same parameters when the $\log K_{m:w}$ values were maintained as originally predicted (5.6). Thus, it would appear that the Takano TPGS:DMSO formulation behaviour is more closely aligned with that of an oral solution than that of a suspension. A more detailed evaluation of the *in vivo* formulation effects is not appropriate without additional *in vivo* and *in vitro* data that characterize the multifaceted *in vivo* behaviour of this formulation.

As a side note, the impact of changing $\log K_{m:w}$ underscores the contradictory effects of the bile salts on solubility versus permeability of poorly soluble but highly permeable drugs. On the one hand, it enhances drug solubility, which is expected to increase the oral bioavailability of drugs such as danazol. Conversely, due to its negative effect on drug diffusion across the unstirred mucous layer (monomer versus bound drug), it can decrease the oral absorption of the dissolved drug.

### 4.1. Challenge of cross-study model development

One of the challenges encountered when utilizing data obtained across several published investigations was an absence of the information needed for building study-specific model input parameter values. This necessitated the development of a unified set of input parameter values that could be applied across all formulations. This absence of individualized model input values appeared to contribute to the imprecision in Cp-t predictions and the need to carefully assess if the prediction error was attributable to model misspecification or to differences in danazol PK across the various beagle study populations. At least in part, this problem was addressed through the inclusion of population variability about the estimates of the Cp-t profiles. However, such adjustments were not always adequate. For example, an increase in values of $CLint_{DLM}$ from 716 to 1052 μL/min/mg of microsomal protein was needed for the Devalapally IV dataset. The difference in $CLint_{DLM}$ values for the Devalapally dataset could be a consequence of phenotypic variability across the beagle dog populations such that the dogs used in the Devalapally studies may reflect a subpopulation of extensive metabolizers. However, when considering the danazol metabolic pathway, this possibility does not appear to be a likely primary cause of the observed disparity. Danazol is metabolized primarily by CYP3A4 in humans [41] (canine ortholog is CYP3A12 or CYP3A26), or CYP2D6 (canine orthologue is CYP2D15) [19]. While polymorphic forms of CYP3A12 have been reported in dogs, they do not appear to be associated with marked differences in dog hepatic metabolizing capacity [42]. Moreover, while PK consequences may be associated with polymorphic forms of CYP2D15 [43], that enzyme appears to have a minor role in danazol metabolic clearance. Therefore, an alternative possibility is to consider that since danazol is a high ER drug, a potential increase in hepatic blood flow associated with the "partially fed state" of dogs in the Devalapally study could have contributed to a greater clearance estimate.

We also needed to consider the possibility that an inappropriate assumption of total drug solubilization biased our evaluation of Fa in the presence of solubilizing agents such as the cyclodextrins. In that regard, the Holm study [44] evaluated the effect of cyclodextrin on the oral danazol bioavailability and indicated that an increase in the amount of cyclodextrin can potentially reduce the fraction of danazol absorbed. However, this was also not the case for the Liversidge et al. study (danazol with 50% w/w HP-β-CD) as the absolute bioavailability reported by the authors was 100% for the orally administered danazol-cyclodextrin solution [15]. Therefore, although our model does not provide an option for adjustment based upon the association constant of danazol with cyclodextrin, we did not consider this to bias our conclusions because we were able to recover danazol oral solution observed values at the higher dose.

The predicted volume of distribution at steady state (Vss, L/kg) using the adjusted Kp values (adjusted via use of a $Kp_{scalar}$) was 5.35 L/kg. Since the PBPK dog simulator does not account for inter-individual variability in the distribution model, the predicted Vss was constant across the three simulated IV studies. The predicted Vss value was within a 2-fold error of the observed values reported in the three publications containing IV data. The variability across observed Vss values may be attributed to the lipophilic nature of danazol and the range of body compositions (such as the amount of adipose and muscle tissue) across the three investigations (body weights ranging between 10 and 23 kg for publications including IV administration). While this point most certainly could have led to an increase risk of error when estimating drug concentrations, it should not influence our predictions of segmental drug absorption, Fa and AUC.

### 4.2. Investigating the interplay between formulation, drug, and in vivo physiology

Efforts to explore potential causes of model misspecification led to an identification of potential 'excipient-*in vivo* interactions' that may have influenced the *in vivo* translation of 'excipient *in vitro* solubilization effects'. This includes intestinal digestion of Labrafil, proposed alterations in small intestinal transit time in the presence of lactose-associated formulations, and bile salt-excipient interactions that can restrict drug permeability across the enterocyte. The apparent non-linear effect of enhanced bile micellization on the fraction of oral danazol absorbed indicates that the effort to increase danazol solubility by using non-ionic lipophilic surfactants such as Labrafil may lead to *in vitro* danazol solubilization without a corresponding magnitude in the

increase of Fa.

#### 4.2.1. Lactose

As described in the results, possibilities for the differences between the observed and predicted profiles included error in the estimation of particle diameter and a decrease in GI transit time due to a transient effect of the lactose administered in the capsule formulation.

Our justification for proposing an altered small intestinal transit time was the unusually high amount of lactose (lactose:danazol = 90:10) contained within the Takano IR capsules. With a dose of 2 mg/kg, the amount of lactose administered was 18 mg/kg. The lactase activity in the duodenum, jejunum, and ileum of normal healthy dogs is estimated as 254, 309, and 40 nmol/mg/min (based upon disaccharidases activity in purified brush border membrane vesicles isolated from regions of the canine small intestine) [15]. Translating this to the amount of lactose that would be digested, we estimate 86.8 μg/kg/min, 105.68 μg/kg/min, and 13.68 μg/kg/min. Thus, although the amount of lactose administered may not be overwhelming to a point of inducing a diarrheal effect, we could not exclude the possibility of local transient osmotic effect causing an increase in intestinal water efflux and a reduced residence time in the initial intestinal segment, just enough to lower the Fa.

However, we preferred an alternative consideration: that of model error introduced by the values used to represent the danazol particle size diameter. As previously mentioned, from the perspective of potential drug CQAs (PRC, SR and particle size), only particle size was found to influence the *in vivo* dissolution rate within the framework of our model conditions. Using the Feret's mean diameter reported by Takano (5 μm) either as monodispersed or polydispersed distribution resulted in an over-estimation of the observed drug plasma concentration profiles.

Takano et al. suggested that their mis-match in predicted vs observed *in vitro* danazol dissolution could reflect the presence of a polydispersed rather than a monodispersed system. Taking this into consideration and based on the results of the sensitivity analysis (Supplemental Figure J), we used a polydispersed PSD consisting of 40% 2.5 μm and 60% 125 μm particles, which improved our recovery of the observed drug plasma concentration. Although this was not intended to imply that the 40/60 ratio was an appropriate representation of the true particle size distribution, these results were highly informative. It supported the belief that the reported particle size of 5 μm was misleading. Ultimately, having detailed information on the true particle size distribution would have helped in the recovery of the observed plasma drug concentration profiles and in our efforts to define the *in vivo* behaviour of the Takano 2 mg/kg capsule formulation.

#### 4.2.2. Labrafil

The assessment of the effect of Labrafil was highly complex. There were several aspects of its *in vivo* effects that needed to be carefully explored. Therefore, we will spend some time describing the thought processes that were included in our analysis.

Labrafil undergoes lipolysis in the small intestine, leading to increased mixed micelle formation and increased danazol partitioning into these micelles [22,24]. Larsen et al. examined the consequences of this, both from an *in vivo* (rat) and *in vitro* (lipolysis model) perspective, concluding that lipolysis of lipophilic vehicles such as Labrafil is important in the *in vivo* trafficking of very low solubility compounds. These investigators showed that the Labrafil release of lipolysis products, including mixed micelles, was important for danazol solubilization. In that regard, using an *in vitro* lipolysis model (which contained a combination of bile salts and lipolytic enzymes), the amount of Labrafil dosed with the formulation governed the rate and extent of danazol released into the aqueous phase during lipolysis as compared to that of a 'no-Labrafil' formulation (where the aqueous danazol = 22%). With the lowest content of Labrafil (1 mL/kg), the percent of danazol released into the aqueous phase initially increased from a baseline

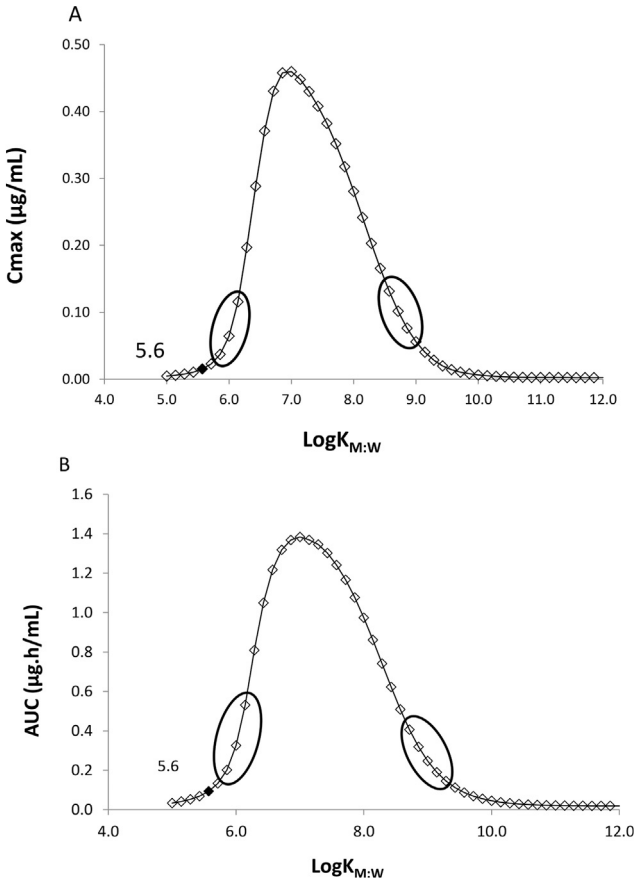


**Fig. 11.** (A & B) Influence of increasing logKm:w on danazol Cmax and AUC values (For detailed sensitivity analysis please see Supplemental Figures C & D).

30–40%, followed by a decrease back to 30% after 40 min. For the 2 mL/kg formulation, danazol was steadily released into the aqueous phase for the first 20 min (30%), then increased to 55% at 40 min and subsequently decreased to 40% at 80 min [22]. We believe that these findings are consistent with our assumption of Labrafil digestion and subsequent partitioning into the bile salts (and mixed micelles) of the dogs used in the Devalapally investigation. The Labrafil formulation dosed by Devalapally et al. contained 1.2 mL/kg of Labrafil with a danazol concentration of 4 mg/mL [14]. Hence the behaviour of danazol during lipolysis is expected to be similar to the 1 mL/kg observation by Larsen et al. [22] where the percent of danazol released into the aqueous phase initially increased from a baseline 30% to 40%, followed by a decrease back to 30% after 40 min.

Using a sensitivity analysis, the effect of increasing $\log K_{m:w}$ on Fa, $C_{max}$, $T_{max}$ and AUC was evaluated. The sensitivity analysis identified two optimal $\log K_{m:w}$ values (6.1 and 8.8) that would recover the *in vivo* Labrafil Fa, $C_{max}$ and AUC values (Fig. 11). Based upon the corresponding effects on $T_{max}$, it was concluded that the optimal $\log K_{m:w}$ value is 8.8 This facilitated our understanding of the relationship between free (micelle unbound) danazol, its transcellular permeability, and the conflicting effects (due to micellisation) of enhanced drug solubility versus decreased permeability associated with the use of lipophilic formulations (such as Labrafil). This has relevance to potential mechanisms underlying a conflict between enhanced solubility but decreased permeability of lipophilic compounds in the presence of several solubility-enhancing conditions [45,46].

With this in mind, as compared to what would be predicted for a true solution, the lower than expected danazol bioavailability may have been attributable to a trapping of drug within the micelle (proportional decrease in monomer in the aqueous phase) which in turn can decrease the diffusivity of the micelle bound drug through the unstirred mucus

layer. As a result of a decrease in monomer proportion at the epithelial membrane, the decrease in concentration gradient for passive permeability results in a decrease in the net effective permeability of the drug. This interpretation is consistent with the finding of Ingels et al. who compared the permeability of danazol across colon rectal cancer (Caco-2) monolayers using transport medium (TM) and FaSSIF (which contains sodium taurocholate and lecithin). In that study, it was observed that FaSSIF reduced the apical to basal and basal to apical movement of danazol across the monolayers [18]. Additional discussion on this topic is provided in Appendix B.

For clarity, the diffusion of drug through the mucous layer was estimated as follows:

$$D_{eff} = f_{unbound} \times D_{mono} + (1 - f_{unbound}) \times D_{micelle}$$

where $D_{eff}$ is the effective diffusion of drug through the mucous layer, $f_{unbound}$ is the fraction of monomer (i.e., not bound to the bile micelles), $D_{mono}$ is the effective diffusivity of the monomer, and $D_{micelle}$ is the effective diffusivity of bile micelles, which is a fixed value of 0.78 within the MechPeff [2].

Based upon several methods of data analysis, it was concluded that there was an increased partitioning of danazol into bile micelles during the process of lipolysis and a subsequent decrease in drug permeability because of the lower diffusivity of micelle-bound drug through the unstirred mucous layer as compared to that of the free monomer.

The other important take-home message was that because changing the PRC had little impact on the Fa of the Labrafil simulations (when modelled as an oral solution with precipitation or oral suspension with 100% of drug solubilized upon administration), we concluded that danazol precipitation was not a result of vehicle lipolysis. This conclusion is consistent with that of Larsen et al. [22], Sassene et al. [29] and Zangenberg et al. [47] who showed that following lipolysis of lipophilic vehicles, danazol solubilization is dependent on the partitioning of danazol into the mixed micelles and that precipitation of danazol in the proximal small intestine is unlikely due to high luminal bile salt concentrations [22,29,47]. However, precipitation could occur in the distal small intestine where bile salt concentrations markedly decrease.

In terms of the precipitation, we need to think of this as a luminal event. Although the $LogK_{m:w}$ for danazol in the Labrafil formulation is high, the amount of bile salts remaining in the lower small intestine is negligible. In the canine intestine, the conjugated bile salt concentration rises from the duodenum until the upper limit of the distal quarter of the small bowel but drops to near zero near the end of the ileum. Thus, we would expect a decrease in the solubilization capability in the distal as compared to the proximal small bowel and that any additional drug released from the Labrafil formulation would risk precipitating before it can cross the unstirred boundary layer and reach the enterocyte membrane.

### 4.3. Model misspecification: The Devalapally data

We noted that the reported exposure of the Devalapally DCR 200 mg strength tablets was lower than that of the Erlich DCR 100 mg and 200 mg strength tablets. However, as seen in Fig. 12, the observed exposure associated with Erlich DCR 100 and 200 mg tablets were effectively dose-proportional. Clearly, it is difficult to reconcile this inconsistency using a single PK model.

Even upon eyeballing the Devalapally data, these dogs clearly exhibited danazol PK attributes that differed from what was observed in the other investigation. Profile differences appeared to be attributable to a more rapid $CL_{IV}$ in the dogs enrolled in the Devalapally study. This observation could not be attributed to model misspecifications since it

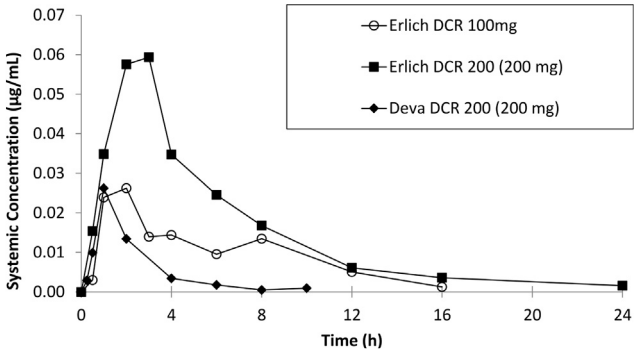


**Fig. 12.** Comparison of observed in vivo Cp-t profiles of danazol after administration of Erlich DCR 100 (100 mg), Erlich DCR 200 (200 mg) and Deva DCR 200 (200 mg) capsule formulations.

was based upon a noncompartmental analysis of the published IV datasets. Despite the absence of any discernable reason for this interstudy difference in CL (such as breed, age, diet, body weight, etc), the $CL_{IV}$ was about 32% higher than that associated with the Liversidge IV dataset. The estimated $CL_{IV}$ was then used to back-calculate a $CL_{intDLM}$, resulting in an estimate of 1052 µL/min/mg of microsomal protein (as opposed to 716 µL/min/mg of microsomal protein as was used for modelling the data generated in the other studies). This is included in the supplemental material as well (Supplemental Table C).

The reality is that this kind of intersubject variability can and does exist and can negatively affect efforts to utilize a single set of physiological parameter estimates (even if averaged across several investigations) to support cross-study extrapolations.

## 5. Conclusions

Experimental determination of drug parameters such as *in vivo* dissolution, supersaturation, precipitation and alteration of bile salt concentrations due to the presence of excipients and their components or to their synergistic effects on overall Fa or (even more complicated) regional intestinal Fa, can require a large number of animals, complex regional intestinal access techniques and lengthy and highly specific experimental procedures. The use of *in silico* mechanistic modelling approaches provide an opportunity to explore the multifaceted ramifications of formulations that may be used to enhance the solubility of highly lipophilic compounds such as danazol. In this study, we showcased the strengths and weaknesses encountered when trying to predict such complex parameters via an *in silico* bottom up modelling approach. Despite those situations, when we encountered a less than optimal reproduction of the observed Cp-t profiles, it was clear that mechanistic models can provide a unique opportunity to explore these complex drug-formulation-host interactions. In so doing, these models provide investigators with insights into conditions when *in vitro* predictions may not accurately reflect *in vivo* formulation effects and help to target specific questions that need to be explored to insure formulation optimization of low solubility compounds such as danazol.

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## Appendix A. Estimation of Peff

Regarding the estimate of $P_{trans,0}$, Avdeef provided an estimate of the $LogP_{PAMPA,intrinsic}$ value (the observed permeability of the unionized,

unbound drug, measured by the cosolvent PAMPA method), which could be converted from a Log (-1.79) to a value representing the $P_{trans,0}$ (expressed as $16218 \times 10^{-6}$ cm/s). When this value was considered as the input $P_{trans,0}$ (16,218) to estimate the $P_{eff,Dog}$, we obtain a jejunal $P_{eff,Dog}$ value of $1.198 \times 10^{-4}$ cm/s. However, if we considered this to be the Log $P_{pampa,0}$ (intrinsic PAMPA) value, we obtain a predicted $P_{trans,0}$ value of $781 \times 10^{-6}$ cm/s. The resulting estimate of jejunal $P_{eff,Dog}$ was then reduced to $0.7164 \times 10^{-4}$ cm/s. This left us with the issue of which of these methods of estimation is the most appropriate.

Insights into likely reasons for this disparity were obtained from the study by Sugano [16] where he explored the importance of bile micellar penetration across the mucous unstirred water layer (UWL), a component not considered when estimating $P_{trans,0}$ from either Log $K_{mw}$ or $P_{pampa,0}$ based upon regression equations that included both ionisable and unionized compounds.

Initially, Sugano used $LogP_{oct}$ to estimate $P_{ep}$ (the epithelial cellular membrane permeability of the free monomer via the equation:

$$P_{ep} = A \times P_{OCT}{}^{B}$$

where A and B were determined via regression of Log P and experimental data generated on Caco-2 intrinsic membrane permeability across a range of ionisable and unionizable compounds.

However, he observed that the resulting $P_{ep}$ under-estimated the $P_{eff}$ of danazol and concluded that for unionized compounds such as danazol, basing Peff solely upon the regression equation for the intrinsic Caco-2 permeability vs $K_{mw}$, when generated across a library of weak acids, weak bases, and neutral compounds, will under-estimate the *in vivo* Peff.

Rather, he observed that for danazol, the effective permeability was estimated with far greater accuracy when including the permeability through the UWL. This was described via his equation:

$$\frac{1}{P_{eff}} = \left(\frac{1}{P_{ep,eff}} + \frac{1}{P_{UWL,eff}}\right) \times \frac{1}{FE}$$

where $P_{ep,eff}$ = effective epithelial cellular membrane permeability of the free monomer
$P_{UWL,eff}$ = the effective USL permeability (which includes considerations for the monomer, the micelles)
FE = the surface expansion by fold factor.

These observations influenced the equation used when we converted $P_{trans,0}$ to $P_{eff}$. In brief, the LogP_int, PAMPA value (the observed permeability of the unionized, unbound drug, measured by the cosolvent PAMPA method), was converted from a Log P to a value of the magnitude of $10^{-6}$ obtained from Avdeef 2009 [46]. This value was then used by the Simcyp software as the membrane passive intrinsic transcellular permeability ($10^{-6}$/s), which in turn was used to predict $P_{eff}$ and the corresponding permeability of the various intestinal segments. We note that Sugano observed that when including permeability of the free monomer and micelle by water convection in their estimates of $P_{UWL,eff}$, they, obtained an estimate of Peff = 1.1 (predicted %fraction absorbed = 17%, observed faction absorbed ranged between 8 and 25% under fasted conditions). Therefore, we concluded that the Avdeef reported danazol $P_{trans,o}$ value of 16218.1 should be used to predict Peff,dog (where our estimate of $1.198 \times 10^{-4}$ was comparable to that reported by Sugano that included considerations for diffusion through the UWL and permeability by water convection.

## Appendix B. Influence of bile micelles on movement through the unstirred boundary layer

Although it is the free (unbound) drug that diffused into the enterocyte, an important component of *in vivo* permeability is the ability of the free drug and bile micelle to diffuse across the unstirred boundary layer [16]. The ability of the free drug to be solubilized in aqueous fluids is impacted by the enhanced diffusivity of the drug when incorporated into a bile salt-danazol complex [17]. Thus, unless this enhanced movement through the mucus UWL is incorporated into our estimates of Peff, we will markedly under-estimate the *in vivo* Peff, which occurs in the presence of bile salts. In fact, unlike steroids such as hydrocortisone, tricinolone, dexamethasone, and beta methasone, the diffusivity of danazol in 0.1 N NaCL and a 30 mM sodium taurocholate solution resulted in a large decrease in the diffusion coefficient ($Cm^2$/sec) of danazol [17]. These authors estimate that the majority of danazol is associated with micelles. This has important implications with regard to the ability of danazol to diffuse through the unstirred boundary layer.

Considering the findings of Bakatselou et al. [17] and the equation by Sugano [16], we see that any decrease in the fraction of drug remaining in the monomer (e.g., due to food or, in our proposal, lipophilic excipients such as Labrafil), there will be a decrease in the $P_{UW,\ eff}$. This is seen in the equation:

$$P_{UW,eff} = \frac{1}{h_{eff}} \times (f_{mono} \times D_{UWL,mono} + f_{mic} \times D_{UWL,mic})$$

Where $P_{UWL,eff}$ = the effective unstirred boundary layer permeability
$D_{UWL,mono}$ = the diffusion coefficient of the free monomer
$D_{UWL,mic}$ = the diffusion coefficient of the micelle bound drug
$F_{mono}$ = the fraction of drug existing as the monomer
$F_{mic}$ = the fraction of drug existing as the micelle bound drug
$h_{eff}$ = the effective thickness of the unstirred boundary layer.

Sukano notes that this equation can be further refined by adding the permeability of the free monomer and micelle bound molecules by water convection, leading to an even greater estimate of $P_{UW,eff}$.

$$P_{UW,eff} = \frac{1}{h_{eff}} \times (f_{mono} \times D_{UWL,mono} + f_{mic} \times D_{UWL,mic}) + (f_{mono} \times P_{WC,mono} + f_{mic} \times P_{WC,mic})$$

where $P_{WC,\ mono}$ and $P_{WC,mic}$ is the permeability of the free monomer and micelle bound danazol by water convection, respectively.

As previously discussed, it is this equation that provided the basis for our decisions regarding the value to use for converting $P_{Pampa,0}$ to $P_{trans,0}$ and therefore the estimate of Peff.

Since we have already accounted for the enhancement of the Peff due to micellar movement through the UBL, the further association with the digested Labrafil (handled by increasing our estimate of in $\log K_{m:w}$) will have a negative effect via its decrease in $F_{mono}$, thereby decreasing $P_{UW,eff}$.

## Appendix C. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.05.024.

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