Computational study on interindividual variability in composition of dog intestinal fluids and its effect on the solubilization pattern for poorly soluble drugs

Dushime Blaise

Master’s Programme in Pharmaceutical Modelling
Department of Pharmacy
Pharmaceutics and Biopharmaceutics
Uppsala University
Master thesis 45 credits

Supervisors: Per Larsson, Aleksei Kабедев, Albin Parrow

Examiner: Per Artursson

Spring, 2021
Table of Contents

Abstract ......................................................................................................................... 3

Introduction .................................................................................................................... 3

  Aim of the project ........................................................................................................ 4

Materials and Methods ................................................................................................. 5

  Molecular Dynamics Simulations section ................................................................. 5

    Composition of DIF samples ..................................................................................... 5

    Setup of the Molecular Dynamics Simulations ..................................................... 5

    Micelle determination and Shape Factor Analysis (Micelle analysis) .................... 6

  Radius of gyration ....................................................................................................... 7

  Free energy calculations ............................................................................................. 7

Experimental Section .................................................................................................... 8

  Solubility study in DIF ............................................................................................... 8

  Phospholipids quantification in DIF ......................................................................... 9

Statistical analysis ......................................................................................................... 10

Results ............................................................................................................................ 10

  Molecular Dynamics Simulations section ................................................................. 10

    Trajectories and micelle visualization .................................................................... 10

    Radial distribution function (RDF) ...................................................................... 11

    Radius of Gyration ................................................................................................. 13

    Prediction of solubility computationally .................................................................. 13

Experimental section .................................................................................................. 14

  Drug solubility in DIF samples ............................................................................... 14

  Phospholipid quantification ..................................................................................... 14

Discussion ...................................................................................................................... 14

Conclusion ...................................................................................................................... 16

References .................................................................................................................... 17

Appendix
Abstract

The complex interplay between intestinal fluid and drugs at molecular level is not well known. Therefore, as dogs are used to study solubility of drugs during formulation development, a model of Dog Intestinal Fluid (DIF) that considers molecular interactions is needed for in vitro in vivo correlation (IVIVC), hence, reducing the need and extent of animal studies. Molecular dynamics (MD) can assist in interpreting biological experiments via in-depth study of structural changes at molecular level during drug dissolution. This project was undertaken to predict and evaluate the solubility of three poorly soluble drugs in DIF samples and model solubilization of these drugs in DIF via coarse-grained MD simulations. Computational part was based on MD simulations (on both published and experimentally observed data) and experimental part studied solubility of three drugs in three DIF samples using shake flask method (thermodynamic solubility). Developed model and experimental data showed qualitative agreement and will be further used for improvement of in silico models of DIF.

Introduction

Oral dosage is the most used and patient convenient form of drug administration despite risk of lower bioavailability. Specifically, drugs with lower solubility which make up around half of new developed drugs (O’Shea et al., 2019). Various strategies to overcome low solubility have been developed (Williams et al., 2013). Lipid-based formulations (LBF) is a strategy that has been used for decades to increase solubility of poorly water-soluble drugs (Feeney et al., 2016).

There is a growing body of literature that recognises the importance of LBF strategies, however, poor prediction of in vivo performance has limited the widespread adoption of LBF strategy (Porter, Trevaskis and Charman, 2007). One of the reasons contributing to poor prediction is that molecular interactions between LBF, intestinal fluid and drugs are not well known, thus, extensive studies at molecular level are needed for reliable prediction of formulation efficiency. For instance, Alskär et al. inferred that fatty acids are an essential component for vitro solubilization of a weak base carvedilol while the in vivo is moderated by both fatty acids and bile salts (Alskär et al., 2019), therefore, in vitro model that can mimic well in vivo medium (intestinal fluid) and its interactions with LBF can improve in vitro in vivo correlation (IVIVC).

Dogs have been used successfully in preclinical studies to assess bioavailability of drugs during formulation development despite differences in human and dog’s intestinal fluid components (Walsh et al., 2017). Dog gastrointestinal(GI) physiology and anatomy differ to that of human in many aspects affecting absorption such as length and size of GI, mobility and transit time,
microflora, intestinal fluid components, etc. that were compared in different studies (Kararli, 1995; Hatton, 2015; Walsh et al., 2017). In previous study comparing solubility of two weak bases in human and dog intestinal fluids, it was found that these drugs solubilized twice as much in DIF as in human intestinal fluid (Arndt et al., 2013). A good DIF model is needed to successfully predict outcome of clinical studies when drugs are tested on humans. In this research project, we used samples collected as part of another study (Alskär et al., 2019) to investigate the effect of interindividual variability of DIF composition on solubilization.

Molecular dynamics (MD) simulations can explain and predict the outcome of biological experiments by understanding molecular movements (trajectories) and interactions as a function of time. MD models of lipids and LBF have shed some light on behavior of lipids. In their study, Larsson et al, found that when the system contains 75% of water, lipids behavior, in particular, diffusion, density of picking, neighboring like/unlike atoms and order of lipid tails changes significantly as compared to 0% of water (Larsson, Alskär and Bergström, 2017). As lipids tend to self-assemble when dissolved in water, Birru et al. showed that phospholipids tend to form micelle phases after digestion rather than lamellar phases (Birru et al., 2017). In addition, the size and shape of lipids aggregations depend on amount (concentration) of lipids in the mixture (Benson and Pleiss, 2014) and the tails of lipid chains (Larsson, Alskär and Bergström, 2017). Not many computational models have been developed to understand complex milieu of drugs, intestinal fluid and enabling formulations. This study is an extension to the study done by (Alskär et al., 2019) in a sense that it uses previously collected DIF samples and adds more drugs to study solubility in DIF. Computational MD simulations of already published DIF compositions were performed in order to understand structural changes and interactions at the molecular level during drug disposition.

**Aim of the project**
The main purpose of the project was to model DIF with three poorly soluble drugs (carvedilol, felodipine and probucol) via coarse-grained MD simulations, study structural organization of the colloids and the energy of drug-colloid interactions, and then compare the latter with experimental solubility data. The model was expected to explain how different drugs behave in DIF by studying distribution of compounds within colloids and drug-DIF interactions. These distribution and interactions can contribute to the overall understanding of how a given drug solubilizes. Additionally, we analyzed the phospholipids composition in DIF samples. An interindividual variability study on DIF samples was done both computationally and experimentally.
Materials and Methods

Molecular Dynamics Simulations section

Composition of DIF samples
The simulations in this study were based on the data from Alskär et al (Alskär et al., 2019). In that study, DIF samples were collected after dogs were administered 75 ml of water. Concentration of bile salts (BS) and free fatty acids (FFA) in DIF for the three dogs (G, P, and V) were analyzed at three different sample time points after administration (early 0–5 min, mid around 5–20 min, and late around 20–90 min). Phospholipid (PL) concentration was not quantified in that study, therefore, as various literatures on DIF compositions have determined (Tuvia Gilat, 1996; Kalantzi et al., 2006; Ikeda, Morita and Terada, 2017; Walsh et al., 2017; Dog/Canine FaSSIF/FaSSGF Biorelevant Dissolution Media, 16:10:43.687025), we estimated the concentration in PL to be a quarter of BS and the concentrations for each dog at mid-point are summarized in Table 1.

Table 1. DIF compositions in BS, PL and FFA collected 5-20 min after administration of 75 ml water to three dogs (G, P, V).

<table>
<thead>
<tr>
<th>dog</th>
<th>BS (mM)</th>
<th>Estimated PL (mM)</th>
<th>FFA (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>7.37</td>
<td>1.83</td>
<td>2.39</td>
</tr>
<tr>
<td>P</td>
<td>6.80</td>
<td>1.7</td>
<td>1.24</td>
</tr>
<tr>
<td>V</td>
<td>0.62</td>
<td>0.15</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Different studies have determined the compositions of DIF where taurocholate and taurodeoxycholate were found to be the most important bile salts and oleate observed to be the major FFA. The proportion of taurodeoxycholate in DIF was found to be a quarter or half of all BS (Kalantzi et al., 2006; Walsh et al., 2017). This study used the two BS (taurocholate and taurodeoxycholate) in equal proportion, oleate for FFA and phosphatidylcholine which makes up to 95% of all phospholipids in the DIF (Wüstner, Herrmann and Müller, 2000) represented PL.

Setup of the Molecular Dynamics Simulations
MD simulations were executed with the GROMACS software version 2018 at coarse-grained (CG) level via Martini force field. The representation in Martini differs from the all-atom one in a way that Martini groups approximately 3–4 heavy atoms into one CG bead. Four water molecules represent one CG bead. This has demonstrated to use less computational resources (Hu et al., 2013; May et al., 2014). We used three local servers (48 CPUs each with GPU for fast water simulations) and Uppsala university supercomputer (UPPMAX) for simulations. The simulation boxes representing each dog components are shown in Table 2, with taurocholate (TAUR),
taurodeoxycholate (TAUD), phosphatidylcholine (DIPC), oleate (FOLE) and water (W) proportionally with the data. BS were negatively charged, and carvedilol positively charged.

Table 2. DIF simulation box representing each dog (number of molecules). Water is the number of beads and dog V values were proportionally multiplied by 10 because the data were low for micelles formation.

<table>
<thead>
<tr>
<th>Components</th>
<th>Dog G</th>
<th>Dog P</th>
<th>Dog V</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAUR</td>
<td>11</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>TAUD</td>
<td>11</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>DIPC</td>
<td>6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>FOLE</td>
<td>7</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>W</td>
<td>41,050</td>
<td>41,050</td>
<td>410,570</td>
</tr>
</tbody>
</table>

Topologies and coordinate files of all the components (TAUR, TAUD, DIPC, FOLE and probucol) were already available at the department and have been used in another study (Parrow et al., 2020), except carvedilol and felodipine that were developed for this study (Figure A1).

For each dog, the drug (API) loading was simulated with 0 (without API) 1, 10, 20, 50, and 100 molecules in triplicate, leading to 48 simulations per dog except for V, done in duplicate because the box contained 10 times molecules than others to keep the correct ratio, requiring more computation time we could not meet. A total 128 simulations were done, each taking 2 to 3 days to complete.

The simulations started by loading randomly molecules representing the dogs in the systems using Packmol (Martínez et al., 2009) setting the a cubic box size to 10nm. The interactions in Gromacs used electrostatic and van der Waals interactions. The temperature and pressure were set to 310 K and 1 bar. The system was minimized before and after charge neutralizations. The positively charged ions were added to neutralize the system from negatively charged bile salts or negatively charged ions were added in case of excess from carvedilol that was positively charged. This was followed by equilibration with an increasing time step. The duration of the production stage of each simulation was 1500 ns.

**Micelle determination and Shape Factor Analysis (Micelle analysis)**

After the simulation run completed, visualization of the trajectories was done with visual molecular dynamics VMD and analysis of the colloid structures and statistics were done using combination of built-in Gromacs tools. The trajectories were visualized for the last 20-50 ns and system snapshots were taken from the last frame.
The micelle was selected in each simulation to analyze the radial distribution function (RDF), a Gromacs function that calculates the positions drug and DIF components vis a vis of each other or the micelle. This analyzed the distributions for the last 1000 ns. Micelle-components distribution helps to understand how far from micelle’s (colloid) center of mass are each DIF component located and can be the starting point to estimate the radius. Additionally, the analysis of components-components distribution provides information on how the molecules interact with each other. The RDF use the density distribution as shown in this equation (1) also known as pair correlation function g_{AB}(r) between particles of type A and B (Lindahl et al., 2021a, 2021b; Radial distribution functions, GROMACS 2021, no date), of which each component is compared to the selected micelle and/or elected components.

\[
g_{AB}(r) = \frac{\langle \rho_B(r) \rangle}{\langle \rho_B \rangle_{local}} = \frac{1}{\langle \rho_B \rangle_{local}} \frac{1}{N_A} \sum_{i \in A} \sum_{j \in B} \frac{\delta(r_{ij} - r)}{4\pi r^2} \]

“with \( \langle \rho_B(r) \rangle \) the particle density of type B at a distance r around particles A, and \( \langle \rho_B \rangle_{local} \) the particle density of type B averaged over all spheres around particles A” (Figure A2). A high-density distribution at a specific radius indicates the particle(s) is more available at the given distance from the center (in that study – of the colloid) or affinity in case of two components.

**Radius of gyration**

The radius of gyration (Rg) was used to predict in addition to visualization, the shape and size of the micelles as a function of time (Lindahl et al., 2021a). Equal average radii values of around x, y and z axes indicate the size (radius), and the shape of the micelle is sphere while equal values around two axes imply the micelle is ellipsoidal and is calculated from equation (2) below.

\[
R_g(x) = \sqrt{\frac{\sum_i m_i (R_i(y)^2 + R_i(z)^2)}{\sum_i m_i}}
\]

**Free energy calculations**

We ran free energy calculations in a separate simulation series to study the strength of the interactions between the drug molecules and colloidal structures. The final configurations of the main runs used input files for 20 subsequent simulations for each colloid-drug combination, where van Der Waals and electrostatic interactions were gradually removed between the API and the rest of the system. Thermodynamic integration was then used to track the energy changes (dG) associated with such molecule removal. Stronger interactions between the solute and colloid would be reflected by higher dG values. In our case, as the API molecules always tend to attach to the micelles, the removal of the molecule would require extra energy of dG. We compared it for all three dogs and for the studied drugs.
**Experimental Section**

**Solubility study in DIF**

**Materials**

Material used for solubility study are: APIs (carvedilol and felodipine), DIF samples (three), DMSO, MilliQ, Acetonitrile (ACN), Mobile Phase (MP)(1:1 MilliQ : ACN), incubator, Shaker, PH meter (Metrohm Biotrode), Vials ≥5 ml, Eppendorf tubes, 1ul to 1000ul pipettes and analysis carried out with HPLC-UV (Agilent Technologies 1290 Infinity) with a Zorbax Eclipse XDB-C18 column (4.6×100 mm) (Agilent Technologies, US).

**Model drugs and DIF samples**

Carvedilol, MW, 406.47 g/mol, T (11 – 116) °C, pKa 8.20, logP 4.14 was acquired from Molekula (CAS72956-09-3) and felodipine MW: 384,256 g/mol, T, 139.1 °C, logP 5.58 from H. Lundbeck A/S (Lu AE39700). Dog samples were collected in the previous study by Alskär et al (Alskär et al., 2019) and stored at -80°C. Samples for mid-point were used except for dog G of which we used early point. (Mid-point sample was not available for dog G, however, the concentrations from both sampling points were close, 7.13 mM early against 7.32 mM for mid-point). Probucol experiment could not be performed as the methods and available equipments at the department was not suited for it within time limit of the thesis project.

**Shake flask method**

HPLC methods were developed according to previous studies, carvedilol (Alskär et al., 2019; Dunn et al., 2019), felodipine (Alvebratt et al., 2020; Henze et al., 2020) and adapted to our study. DIF samples were first thawed, and pH measured before API loading. 300 µL of sample was add to an Eppendorf tube with excess API (4-5 mg). Triplicates of 300 µL per DIF-sample were made. A 300 µL blank sample was made from each DIF-sample. Samples and blanks were gently mixed before they were incubated on shaker board for 24 hours at 37°C and ~200 rpm. After incubation, the samples pH was measured again before they were placed in centrifuge at (37°C, 2300 g, 10 min). The supernatant was removed and diluted 10-fold in cold ACN after which a second centrifugation at (4 °C, 2300 g, 10 min) was done. After the second centrifugation, supernatant was removed and diluted again 10-fold in mobile phase before quantification using HPLC-UV. The solubility values were determined as the mean value of the sample triplicates.

For carvedilol and felodipine, a calibration curve (CC) with a range of 1.594 - 102 µg/ml, with QCs in mobile phase at a high, middle, and low concentration, more details can be seen in the Appendix (Table A3-A8, Figure A3, A4). For dog-QCs, blank DIF-samples were treated as the other DIF samples mentioned, but spiked after the first centrifugation, to bring a final concentration of dog-QCs to 5, 20 and 61 µg/ml. The HPLC method for carvedilol had solvent A:
NaAc (buffer) 25 mM, 0.1% trimethylamine, pH 5 and solvent B: ACN. Gradient started at 50:50 A:B for 2.5 min, then linearly increasing B up to 20:80 with a runtime of 5 min. For felodipine, solvents A: MilliQ water and B: ACN was used with a gradient starting at A:B 50:50 fixed for 1 min, then, linearly increasing the non-polar phase to 20:80 and back to 50:50 at a runtime of 6 min.

**Phospholipids’ quantification in DIF**

**Materials**

Phospholipid quantification assay kit (CS0001) was acquired from sigma Aldrich (USA). 96 well flat-bottom plates (clear), horizontal shaker, MilliQ, pipettes and spectrophotometric (570 nm) multiwell plate reader were used to carry out the experiment.

**Quantification**

The method uses hydrolysis enzymes that oxidize the phospholipids in the sample in different steps (Grignard *et al.*, 2017). First, choline is released from phospholipids by phospholipase D oxidation, which in turn is oxidized to release hydrogen peroxide that will react with peroxidase and appropriate substrates to form end products. Colorimetric method (suitable to detect concentration 1–5 nmol) was used to detect the end products and quantify the concentrations. The kit contains 5 components and was stored at -20 °C. After defrosting the kit, Assay buffer, Probe and Standard were equilibrated at room temperature while Hydrolysis enzyme and Development mix were kept on ice while in use to maintain low temperature.

**Standard Curve**

The standard (50 mM) was diluted 100-fold in MilliQ in duplicate generating 0.5mM. Each duplicate was diluted again with MilliQ in a 96 well clear plate to generate 0 (blank), 1, 2, 3, 4, and 5 nmol/well standard for the total volume of 50 µL par well.

**Samples**

Samples were defrosted, shaken before adding 100 µL of each sample in an Eppendorf in triplicates. Samples were thereafter centrifuged at 37 °C, 2300 g for 10 min before diluting the supernatant 100-fold with MilliQ. 50 µL of each triplicate of 100-fold diluted samples were pipetted in a 96 well plate. One blank sample was made from each dog and added 50 µL of each in the well plate as well.

**Reaction and Control mixes**

Reaction mix (Rx) and control mix (Cx) were developed according to kit manufacturer protocol (Grignard *et al.*, 2017). The difference between reaction and control mixes is that no hydrolysis enzyme is added into control mix. 50 µL of Reaction mix was added to standard and sample wells
while 50µL of control mix was added to blank samples. After, wells were mixed using a horizontal shaker and incubated for 30 minutes at 37°C. The plate was protected from light during the incubation, thereafter, a spectrophotometric multiwell reader measured the absorbances at 570nm.

**Statistical analysis**

Data sorting and manipulation was done mainly with Microsoft excel and on some occasions R. The graph visualization and statistics were performed with GraphPad Prism 9.1.1 (Graphpad Software Inc. USA). The values were reported as the mean ± standard deviation from the tri (duplicates). Analysis of variances for RDF, contact (affinity) and Radius of gyration was done.

**Results**

**Molecular Dynamics Simulations section**

**Trajectories and micelle visualization**

The trajectories were analyzed after reaching equilibrium. The micelle structures were presented after reviewing each triplicate per drug loading and per dog’s simulated sample. Only the molecules that were within or in the vicinity of the micelles were used for the analysis routines, such as radial distribution function and radius of gyration. Therefore, micelle structures presented in this study represent the triplicate with the close average of free monomers in the system. The snapshots of the systems in this study do not show water molecules for the clarity of micelles.

The visualization of the micelles of individual dogs without API loaded showed similar trend for G and P dogs as expected from their compositions (Figure 1). Nevertheless, aggregate observed for the dog V appeared to be different from others. There were no single or large micelles, rather 4- 5 small aggregates and many free monomers exclusively taurocholates (TAUR).

![Figure 1: Systems representation without API. From left to right dog G, dog P and dog V.](image)

Simulations for the dog V, with or without drug, only yielded small aggregates and API self-aggregations in case of high API loading (Figure A5). The shape similarity between dogs G and P remained regardless the API and the amount loaded. However, there was a slight difference among both dogs on available free monomers in the system, with dog P having 1-3 monomers more than dog G at lower API loading but not difference at high loading (Figure A6). The slight variability was not observed at 50 API molecules and over.
Comparing how different APIs behave for the same dog, we found that the trends were similar, except a slight difference between felodipine and the other two APIs. Interestingly, free monomers were observed only at a low drug loading for probucol (1 molecules) and carvedilol (1 and 10 molecules), but at all APIs loadings for felodipine (Figure 2). This was also observed with dog P. For almost all the simulations, TAUR was the component forming free monomers.

![Figure 2: Simulation box representing dog G with all three API loaded. The first line is felodipine (red), the middle line carvedilol(yellow) and the last line probucol(blue). For each API, from left to right, 1,10,20,50, and 100 molecules loading, respectively.](image)

**Radial distribution function (RDF)**

The results from RDF analysis showed how drugs and micelle components are distributed with respect to the micelle’s center of mass. As RDF calculates the density around molecules’ center of mass, and given that for the dog V, as mentioned before, only small aggregates were formed within the simulation time, we would not get a meaningful density distribution.

The positions of DIF components around the micelle observed the same tendency for both dogs, however, there seems to be a non-significant but noticeable difference between phospholipids (DIPC) and free fatty acids (FOLE) between the two dogs regardless of the API (Figure 3). Also, the presence of APIs widened the contact between the two components for the dog P only.
Figure 3: RDF for the dogs. The upper row represents the dog G and the lower represents the dog P. For each row, from left to right, without API, felodipine (black), carvedilol (yellow) and probucol (blue) 1 molecule loaded.

An increase in the number of API molecules widened the contact between the two components (DIPC and FOLE). Additionally, an increase in number of molecules increased the spreading from the center to the surface as molecules number become large to be accommodated by DIF components in the system (Figure A7). While an increase in carvedilol and felodipine did not show variation among dogs, it was for probucol. The center of micelle contained mainly probucol for dog G, whereas for dog P, DIPC and FOLE were also in the center alongside probucol for dog P until 100 molecules (Figure A8), (high loading snapshots not included).

The simulations showed that felodipine mostly and carvedilol slightly, tend to be placed at peripheral of micelle compared to probucol which lays mainly inside the micelle. These results, as shown in Figure 4, suggest the three APIs differed in the same dog. The study found that probucol was more available in the middle of the micelle (avoiding contact with water) than carvedilol and felodipine. Regardless the APIs loaded, an increase in number led to spreading of the colloidal components to go outside of the micelle core (occupancy of the outer layers) as expected and was similar for both dogs. Furthermore, with carvedilol and felodipine, it can be seen FOLE and DIPC being in the middle of the micelle along the drugs.
Figure 4: All the three APIs at different loading (10,20,50) in dog G. The upper line represents felodipine (FEL), the middle line carvedilol (CARV) and probucol (PRB) for the lower line.

The RDF comparing the density distribution of each component around the API demonstrated that all the components have affinity toward and interact better with phospholipids and free fatty acids than bile salts (Figure A9). Interestingly, carvedilol showed less interaction with fatty acids, which was unexpected. Overall, probucol interacts closely with all DIF components than other drugs. As expected, probucol interact more with itself and felodipine has the lesser affinity to itself. Moreover, no significant variability was observed between the dogs at same API.

Radius of Gyration
Overall, the shape of the micelles is spherical. The average sizes of gyrate around x, y and z axes showed close values between x,y,z. The radii of colloids raised from 1.15 nm for lower API loading to 1.87 nm for higher API loading on average. Reported values are mean of means ± standard deviation. P values < 0.0001 for ANOVA and Brown-Forsythe test (Figure A10, A11).

Prediction of solubility computationally.
The free energy of the system representing each dog was calculated. A lower energy indicating lower capacity of the system to solubilize the drug (Figure 5).
**Experimental section**

**Drug solubility in DIF samples**

Carvedilol and felodipine solubility in the three DIF samples are shown in the Figure 5. The lowest solubility for carvedilol was observed for the dog G at 0.682mg/ml while the highest was from dog V at 1.02mg/ml. The solubility for felodipine was 0.25 mg/ml, 0.28 mg/ml, and 0.594 mg/ml for dog G, V, P, respectively. Retention time for carvedilol was found to be 1.5 -2 min. There was not inter-day variation in carvedilol retention time, as it was very stable during each batch we used. Retention time for felodipine was 4 min.

![Figure 5: The left graph represents numerically calculated free energy and the right graph represents experimentally observed solubility.](image)

The samples’ pH was measured before and after 24hrs incubation and summarized in Table A7.

**Phospholipid quantification**

Quantified for phospholipids in DIF sample were performed. Phospholipids’ calibration was done and resulted in a nonlinear curve. Consequently, with curve, we could not predict accurately the composition; we can speculate our no conclusive results as shown in the Figure A12.

**Discussion**

The MD simulations performed in this work using CG martini force field in system boxes representing individual dogs has moderate computational cost, enabling fair understanding how DIF components behave and solubilize different poorly soluble drugs.

The micelle formation is the core for solubilization (Vinarov et al., 2018) and the data analysis focused on molecules within or in the vicinity of micelle. As it was expected, the results from the dog V differed from the other two dogs. This discrepancy could be attributed to the critical micelle concentration (CMC), the lowest concentration a micelle can form. The CMC has been estimated to be between 1.2 mM and 2.1mM (Tamesue and Juniper, 1967; Simonović and Momirović, 1997; Wüstner, Herrmann and Müller, 2000; Dong et al., 2013; Ravichandran and Gopinath, 2014; Verde and Frenkel, 2016). According to our data (0.62 mM for BS and 0.23 mM for FFA), we can infer that at the very low concentration in bile salts, phospholipids and FFA, there would
be no micelle formation necessary to solubilize the drug. Consequently, the dog V was removed to study interindividual variability in solubilizing the three drugs.

The shape and structure of micelles did not differ significantly among the dogs G and P. The observed size and shape of micelles were expected because the system contained a small number of molecules. The simulation snapshots as well as radius of gyration function determined that the micelles appeared to be spherical which is in line with the study that found BS micelle tend to form spherical if small micelle and prolate for larger micelle (Verde and Frenkel, 2010). The simulation also observed that taurocholate is the least aggregating among the bile salts, in agreement to what Verde et al. (Verde and Frenkel, 2010) observed, where they found taurocholate having a low aggregation number compared to taurodeoxycholate. To our understanding, in 128 simulations, almost all free monomers observed after equilibrium reached were taurocholate. The less availability of free monomers in probucol and carvedilol maybe explained by their hydrophobicity, which we can hypothesize to the increase in aggregation.

Radial distribution functions were used to analyze the positions of each DIF components, and the drugs positions with respect to the micelles’ centers of mass. There is a clear indication that phosphatidylcholine (DIPC) and free fatty acid (FOLE) are located mainly in the middle of the micelle, while taurodeoxycholate (TAUD) and taurocholate (TAUR) are in the outer layer of the micelle. As mentioned in previous studies, bile salts do not only solubilize drugs (Malik, 2016), but also they increase slightly the solubility of relatively nonpolar molecules (such as cholesterol or fatty acids)(Carey and Small, 1972), this can explain why the density distribution around the micelle and around the drugs observed DIPC and FOLE inside while BS were found mostly at the outer layer of the micelles as well BS having less affinity with drugs than the other components.

The finding that probucol avoid contact with water more than carvedilol and felodipine was expected because probucol is more hydrophobic than the other two drugs. An increase in number of molecules loaded expected to show more molecules moving outside the micelle. All probucol molecules tend to occupy the central part of the micelle, which can either prevent re-crystallization or initiate it by accumulating drug molecules. Also, transport from the center of the micelle to the cell might be less likely, except for the case of the entire micelle fusion (Kabedev et al., 2021). For carvedilol and felodipine, colloidal structures seem to be a more efficient drug delivery systems, as the molecules are still mostly placed at the interface and slightly separated by the components of colloids. Affinity of the drugs to themselves are then in competition with affinity to the intestinal fluid components, which is defined by the ratio of bile salts, phospholipids, and free fatty acids.
Regardless the drug and with both dogs G and P, 50 API molecules seemed to be the point where a DIF micelle components no longer capable to accommodate more drug molecules. That can be further translated to moles and used for prediction of drugs’ solubilization.

The values of free energy changes were in agreement with experimental data, meaning that solubility can be qualitatively predicted in computer simulations for small colloids. In this regard, as predicted computationally, carvedilol was more soluble than felodipine experimentally. Carvedilol experimental solubility values were in agreement of what (Alskär et al., 2019) reported. The difference in solubility between the three dogs was not as good reflected in simulations, but it might be because of the rather statistical factor - we only studied very small micelles. In reality, the colloids have many various sizes bigger than those we simulated; thus, our computational study would benefit from simulations of bigger colloids to address interindividual variability. Given the results of such calculations are in a qualitative agreement with experimentally observed solubilities, the computational model can be considered validated to some extent.

Based on that we propose that varying concentration of the DIF components, and as a result varying composition of the colloids, might trigger the solubilization to different extends for the three drugs.

The micelle formation is done periodically and systematically by removal and addition of monomers in the system before equilibrium reached and the fusion of monomers to aggregate is dependent on the concentration (Verde and Frenkel, 2016). The increase in concentration also involves fusion of small micelle to larger ones. However, this cannot be mimicked (for now) in vivo because BS are constantly released with higher amount at prandial state.

**Conclusion**

This project was undertaken to predict and evaluate the solubility of three drugs in DIF computationally and experimentally. The results from our computational model qualitatively predicted the solubility of two drugs that was confirmed experimentally. In addition, the model managed to demonstrate how different DIF components behave vis a vis of the three drugs, which to some extend can clarify experimental observations documented in literature. It was shown also that there is a slight difference among studied three dogs and how DIF components proportion may affect the solubilization of a particular drug. However, the model was not accurate enough to completely explain how solubility of the same drug varied between the dogs. A further improvement of the model might be beneficial for the study on interindividual variability effects.
References


**Appendix**

Figure A1: Model drugs and corresponding CG topologies.

Figure A2: RDF calculations (A and B are particles) (source: Gromacs documentation)  
(Radial distribution functions — GROMACS 2021.2 documentation, no date)

Figure A3: Felodipine Calibration Curve
Figure A4: Carvedilol Calibration Curve

Figure A5: Simulated DIF for dog V loaded with felodipine (red). From left to right (1, 10, 20, 50, 100 molecules respectively)

Figure A6: Simulation box representing dogs with felodipine (red) (10 and 100 molecules respectively). The upper row represents the dog G and lower row represent dog P.
Figure A7: Increase in API loading presented by 20 and 100 felodipine molecules. The upper panels represent dog G and the lower represent dog P.

Figure A8: Probucol compared to G and P dogs at 10 and 20 molecules loading. The first line represents G and the second represents P.
Figure A9: Dog G interactions of the APIs with all the components. The first line is 10 and the second line is 20 molecules. Felodipine (black) carvedilol (yellow), probucol (blue).

Figure A10: Gyrate mean values representing 0, 10, 20 and 100 probucol loading for the dogs (P values < 0.0001 for ANOVA and Brown-Forsythe test).
Figure A1: Gyration of dogs. The upper line represents the dog G and the lower dog P. Felodipine (red) and probucol (grey). The value is mean of means in each triplicate. With P value < 0.0001 (ANOVA and Brown-Forsythe test).

Figure A12: Quantified phospholipid in DIF on the left and PL calibration curve on the right.
**Table A1: Intermediate dog-QC samples for carvedilol**

<table>
<thead>
<tr>
<th></th>
<th>QC0 (µL)</th>
<th>DIF Blank (µL)</th>
<th>ACN (µL)</th>
<th>Conc (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC1</td>
<td>5</td>
<td>45</td>
<td>450</td>
<td>5.05</td>
</tr>
<tr>
<td>QC2</td>
<td>10</td>
<td>40</td>
<td>450</td>
<td>20.2</td>
</tr>
<tr>
<td>QC3</td>
<td>15</td>
<td>35</td>
<td>450</td>
<td>60.6</td>
</tr>
<tr>
<td>Blank</td>
<td>0</td>
<td>50</td>
<td>450</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table A2: Final dog-QCs and blank samples for carvedilol**

<table>
<thead>
<tr>
<th></th>
<th>Supernatant (µL)</th>
<th>Mobile Phase (µL)</th>
<th>Conc (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC1</td>
<td>100</td>
<td>900</td>
<td>5.05</td>
</tr>
<tr>
<td>QC2</td>
<td>200</td>
<td>800</td>
<td>20.2</td>
</tr>
<tr>
<td>QC3</td>
<td>400</td>
<td>600</td>
<td>60.6</td>
</tr>
<tr>
<td>Blank</td>
<td>100</td>
<td>900</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table A3: Intermediate dog-QCs for felodipine**

<table>
<thead>
<tr>
<th></th>
<th>QC0 (µL)</th>
<th>DIF Blank (µL)</th>
<th>cold ACN (µL)</th>
<th>Conc (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC1</td>
<td>6</td>
<td>44</td>
<td>450</td>
<td>62</td>
</tr>
<tr>
<td>QC2</td>
<td>10</td>
<td>40</td>
<td>450</td>
<td>103.33</td>
</tr>
<tr>
<td>QC3</td>
<td>20</td>
<td>30</td>
<td>450</td>
<td>206.668</td>
</tr>
</tbody>
</table>

**Table A4: Intermediate Recovery (RCs) for felodipine**

<table>
<thead>
<tr>
<th></th>
<th>DIF Blank (µL)</th>
<th>Cold ACN (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC1</td>
<td>50</td>
<td>450</td>
</tr>
<tr>
<td>RC2</td>
<td>50</td>
<td>450</td>
</tr>
<tr>
<td>RC3</td>
<td>50</td>
<td>450</td>
</tr>
</tbody>
</table>
**Table A5: Final dog-QCs samples for felodipine**

<table>
<thead>
<tr>
<th>QC</th>
<th>Supernatant QC (µL)</th>
<th>Mobile Phase (µL)</th>
<th>Conc (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC1</td>
<td>100</td>
<td>900</td>
<td>6.2</td>
</tr>
<tr>
<td>QC2</td>
<td>200</td>
<td>800</td>
<td>20.66</td>
</tr>
<tr>
<td>QC3</td>
<td>400</td>
<td>600</td>
<td>82.66</td>
</tr>
</tbody>
</table>

**Table A6: Final RC samples for felodipine**

<table>
<thead>
<tr>
<th>QC 10-fold</th>
<th>RCs Supernatant</th>
<th>MP (µL)</th>
<th>Conc (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC1</td>
<td>12</td>
<td>100</td>
<td>888</td>
</tr>
<tr>
<td>RC2</td>
<td>40</td>
<td>200</td>
<td>760</td>
</tr>
<tr>
<td>RC3</td>
<td>160</td>
<td>400</td>
<td>440</td>
</tr>
</tbody>
</table>

**Table A7: Table showing the pH measured from the Sample before and after 24 hours incubation.**

<table>
<thead>
<tr>
<th>Dogs</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>6.4</td>
<td>6.9</td>
</tr>
<tr>
<td>P</td>
<td>6.8</td>
<td>7.1</td>
</tr>
<tr>
<td>V</td>
<td>7.6</td>
<td>7.0</td>
</tr>
</tbody>
</table>