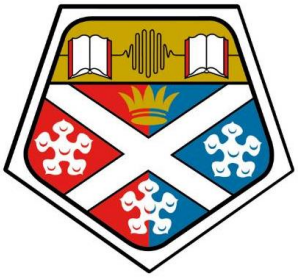

University of Strathclyde Glasgow

Novel *in vitro* Method to Study the Structured Solubility of Bioequivalent Fasted Intestinal Media with Other Biopharmaceutical Applications

By

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Declaration of Authenticity and Author's Rights

Signed: *Qamar Abuhassan*

Date: 19 – 1 - 2024

Acknowledgment

By the name of Allah

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List of Abbreviations

2D	Two-Dimensional
3D	Three-Dimensional
A	Surface Area
a	Acid
ACAT	Advanced Compartmental Transit Absorption
ACN	Acetonitrile
ADME	Absorption, Distribution, Metabolism and Elimination
An	Absorption Number
AP	Absorption Potential
API	Active Pharmaceutical Ingredients
AUC	Area Under the Curve
b	Base
B:P	Blood to Plasma Ratio
BCS	Biopharmaceutics Classification System
BS	Bile Salt
CAT	Compartmental Absorption Transit
CL	Clearance
Cmax	Maximum Concentration
CMC	Critical Micelle Concentration
Css	Supersaturation Concentration
D	Diffusion Coefficient
Do	Dose Number
DCS	Developability Classification System
DMSO	Dimethyl Sulfoxide
Dn	Dissolution Number
DoE	Design of Experiment
DS	Degree of Supersaturation
F	Bioavailability
F_{non}	Fraction Non-Ionised
FA	Fatty Acid

Fa%	Fraction Absorbed
Fa9SIF	Nine Fasted Simulated Intestinal Fluid
FaHIF	Fasted Human Intestinal Fluid
FaSSIF	Fasted Simulated Intestinal Fluid
FaSSIF-v1	Fasted Simulated Intestinal Fluid-version 1
FeHIF	Fed Human Intestinal Fluid
FeSSIF	Fed Simulated Intestinal Fluid
FPE	First Pass Effect
Fub	Unbound Fraction
GC	Glycocholate
GI	Gastrointestinal
GIT	Gastrointestinal Tract
GMO	Glyceryl Mono-Oleate
HCl	Hydrochloric Acid
HIF	Human Intestinal Fluid
HPLC	High Pressure Liquid Chromatography
IVIV	In Vitro In Vivo
IVIVC	In Vitro In Vivo Correlation
K	Potassium
$K_{o/w}$	n-Octanol Water Partition Coefficient
Ka	Absorption Rate Constant
KCl	Potassium Chloride
KH_2PO_4	Potassium Dihydrogen Monophosphate
KOH	Potassium Hydroxide
MAD	Maximum Absorbable Dose
MEC	Molar Extinction Coefficient
MeOH	Methanol
MG	Monoglyceride
MMC	Migrating Motor Complex
MP	Mobile Phase
Mp	Permeability Dependent Multiplier
n	Neutral

NA	Not Available
Na	Sodium
NaCl	Sodium Chloride
$NaH_2PO_4.H_2O$	Sodium Phosphate Monobasic Monohydrate
NaOH	Sodium Hydroxide
ns	Non-Significant
NSF	Non-Significant Factor
NT	Not Tested
ODS	Octadecylsilane Coated
OrBiTo	Oral Biopharmaceutical Tools
P	Permeability
P_{eff}	Effective Permeability
PBPK	Physiology Based Pharmacokinetic
PC	Phosphatidylcholine
PC S	Phosphatidylcholine from Soybean
PK	Pharmacokinetic
PL	Phospholipids
PO	Per Oral
pred/obs	Predicted/Observed Ratio
R	Intestinal Radius
r_p	Particle Radius
S	Physiological Solubility
S_0	Intrinsic Solubility
SIF	Simulated Intestinal Fluid
SIWV	Small Intestinal Water Volume
SLAD	Solubility Limited Absorbable Dose
SP	Stationary Phase
SS	Supersaturation
S_{si}	Small Intestine Solubility
TAC	Total Amphiphilic Concentration
TC	Taurocholate
THF	Tetrahydrofuran

Tmax	Maximum Time
T_{si}	Small Intestinal Transit Time
UV	Ultraviolet
V	Intestinal Fluid Volume
V_L	Lumenal Content Volume
V_0	Volume of Water Taken with the Dose
Vd	Volume of Distribution
X_0	Dose Administered
ρ	Drug Density

Abstract

Drug solubility is a key parameter controlling oral absorption, but intestinal solubility is difficult to assess *in vitro*. Human intestinal fluid (HIF) aspirates can be applied but they are variable, difficult to obtain and expensive. Simulated intestinal fluids (SIF) are a useful surrogate but multiple recipes are available, and the optimum is unknown. This situation creates difficulties during drug discovery and development research. A recent study characterized fasted HIF (FaHIF) aspirates using a multidimensional approach and determined 9 fasted simulated intestinal fluid (Fa9SIF) media recipes that represented over ninety percent of HIF compositional variability. These Fa9SIF recipes have been applied to determine the equilibrium solubility of twenty-one drugs. The solubility measurements enclose literature solubility values in both FaHIF and SIF, and are statistically equivalent to the previous design of experiment (DoE) studies. However, they have a narrower solubility range, suggesting an improved equivalence to *in vivo* solubility. This also highlights that intestinal solubility is a range and not a single value. The Fa9SIF media was examined and provided in the majority of cases a structured solubility behavior, that is consistent with physicochemical properties and previous solubility studies. The study also indicates that the use of two appropriate bioequivalent fasted intestinal media from the nine will identify *in vitro* the maximum and minimum fasted solubility boundaries for drugs and due to the media derivation, this is probably applicable *in vivo*. Statistical comparisons were further carried out, of the Fa9SIF media system against FaHIF, and do not detect a difference, and individual drug analysis produces an 85% correlation. An innovative *in vitro* vs FaHIF correlation window was determined, which enclosed 94% of solubility values from an additional data set, further validating equivalence. The Fa9SIF media system represents a new methodology for *in vitro in vivo* solubility correlation, this radically transforms predictive ability which will benefit drug discovery, development and formulation studies. The equilibrium solubility of the twenty-one drugs were also used for further biopharmaceutical applications including the developability classification system, supersaturation techniques, and physiology based pharmacokinetic (PBPK) modelling.

Chapter 1: General Introduction

1.1. Human Body

Human body consists of many organs and tissues, which are classified into systems depending upon their importance and function for example: respiratory, digestive, cardiovascular, urinary, nervous, musculoskeletal, and blood vascular systems (Paul, 2019). In this thesis, the digestive system which is also named as the gastrointestinal tract (GIT) is examined from a pharmaceutical point of view since it has the role of processing and absorbing the drugs administered by the oral route.

1.1.1. Gastrointestinal Tract Divisions

The digestive system consists of many parts, starting from the mouth, to oesophagus, stomach, small/large intestine and ending with rectum and anus (Paul, 2019), as seen in Figure 1.1. The GIT has a tubular like shape, each part has its own function (Paul, 2019):

1- The mouth:

Is where the food enters is physically processed by the tongue, teeth, and salivary glands, which are responsible for tasting, grinding and digesting food respectively. Saliva secreted by the salivary glands contains ptyalin, a digestive enzyme, which is responsible for carbohydrate processing.

2- The oesophagus:

The second part after the mouth is a connection canal to deliver food to the stomach by propulsion movement.

3- The stomach:

Is a reservoir where the chemical conversion of food from solid to semi-solid state occurs. The parietal cells lining the stomach are responsible for the secretions of gastric acid into the stomach lumen, also it secretes intrinsic factor which is responsible for B12 absorption in the ileum (Hsu et al., 2022). The chief cells, found in the stomach, are responsible for the secretion of pepsinogen, which is converted to its active form (pepsin) by the gastric acid secreted from the parietal cells. Pepsin is responsible for protein digestion into smaller particles (polypeptides) (Hsu et al., 2022).

4- The intestine:

a. Small intestine: consists of three parts: the duodenum, jejunum, and ileum. The first function is the completion of the physical and chemical digestion of the food particles, aided by the pancreas and gallbladder secretions. Pancreas secrets important enzymes such as amylase, trypsin with chymotrypsin, and lipase, which are responsible for the digestion of carbohydrates, proteins, and fat respectively. The fed state induces the gallbladder to secret bile into the duodenum, via the bile duct, which in turn is responsible for the conversion of fat into fat droplets. Both gallbladder and pancreas are connected and situated above the small intestine. The second main function of the small intestine is for absorption of the digested food and drugs, due to the large surface area available by the microvilli and villi, which line the small intestine walls.

b. Large intestine: consists of seven parts: the cecum (connects the small intestine to the colon), the colon (ascending, transverse, descending, and sigmoid), rectum (stores faeces before being moved to the anus), and anal canal (the last part of the GIT, where the faeces are expelled out of the body). Large intestine is responsible for the water absorption from faecal matter, and the movement of the faeces into the rectum through the peristalsis movement, which occurs throughout the intestine.

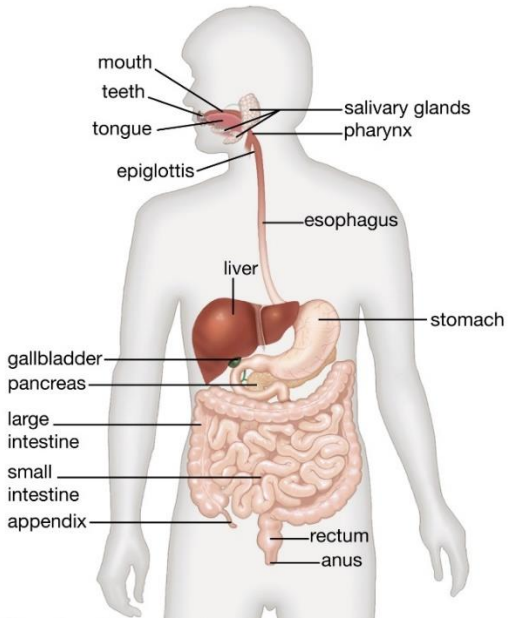


Figure 1.1: The human digestive system (Encyclopaedia Britannica).

1.1.2. Gastrointestinal Tract pH

The pH of the stomach in the fasted state is highly acidic, ranging from 1 - 2, and 3 - 7 in the fed state (Mudie et al., 2010), but the duodenum has a pH of 6.4, which increases up to 7.5 at the last portion of the small intestine; the ileum (Evans et al., 1988). While the pH of large intestine has a mean of pH 7 (Evans et al., 1988). This will be discussed later in more details.

1.2. Drug Delivery Systems

Drug delivery systems have many routes of administration. The most common and desirable route is per oral (PO), as it is considered to be non-invasive, cheap, and easy to administer (Talegaonkar and Bhattacharyya, 2019). This is reflected by the high usage of tablets and capsules, more than seventy percent, compared to other pharmaceutical dosage forms (Khadra et al., 2015). Since solid drugs are not absorbed from the gastrointestinal tract, the processes of drug disintegration and dissolution are a critical stage during oral absorption (Khadra et al., 2015).

This creates a huge interest for formulation scientists to study the factors affecting the absorption of oral dosage forms, along with the vast challenges related to the drug's bioavailability in the human body (Talegaonkar and Bhattacharyya, 2019). The bioavailability (F) is defined as the rate and extent of the drug and its derivatives reaching the systemic circulation (Block, 1992). The greatest limitations to oral bioavailability are drug permeability through gastrointestinal (GI) membranes, and its dissolution and solubility in the GI media (Talegaonkar and Bhattacharyya, 2019). Oral bioavailability is actively studied because of the increased discovery of poorly water-soluble drugs, which accounts for the low and variable concentration profiles reaching the systemic circulation corresponding to poor oral bioavailability, and different therapeutic responses (Sabnis, 1999). Also, this consumes time and money from drug developers, as well as, it exposes the patient to higher side effects due to higher doses taken (Savjani et al., 2012).

1.3. Drug Solubility

1.3.1. Definitions

Solubility, in general, is the solutes' maximum ability to dissolve in a solvent, in order to make a fully saturated solution (Savjani et al., 2012). From a biopharmaceutical point of view, the aqueous solubility is an essential physicochemical feature for any drug molecule (Bou-Chacra et al., 2017), and it can be defined from different aspects. The intrinsic solubility is the solubility of the non-ionized form of a molecule, present in its free acid or free base (Stuart and Box, 2005). Whereas, the equilibrium (saturated) solubility is the measurement of the maximum reached capability of a molecule using fixed conditions (temperature, media components and concentrations, time of agitation, and others), until it reaches a stable saturation point over a period of time (Lachman et al., 1986, Myrdal and Yalkowsky, 2002). On the other hand, the supersaturated solubility is exceeding the equilibrium concentration limit by changing one or more conditions, which will cause the solution to oversaturate and present in a metastable state (this will be discussed further below) (Myrdal and Yalkowsky, 2002).

1.3.2. Methods to Enhance Drug Solubility

Different methods can be applied to enhance the drugs solubility (Savjani et al., 2012):

a. Physically: by changing the organisation of molecules' interactions, as in crystallization state.
b. Chemically: by pH change, salt formation, and usage of buffers.
c. Miscellaneously: adding solubilizers or surfactants.

The utilisation of surfactants is one of the basic techniques applied to increase drug solubility, as well, it's commonly used in this research. Surfactants are molecules which have both hydrophilic and hydrophobic parts (amphiphiles) (Savjani et al., 2012). When a surfactant concentration exceeds a specific point, which is around 0.05 - 0.1 % of the solvent volume, the formation of a micelle will take place, this point is called the critical micelle concentration (CMC) (Savjani et al., 2012). Most of the intestinal components such as phospholipids (PL), fatty acids (FA), bile salts (BS), and cholesterol are good examples of surfactants, and it will start to self-assemble above the CMC point (Ghezzi et al., 2021). The hydrophilic part of the micelle will be exposed to the aqueous medium, while the drug will be entrapped in the hydrophobic core, limiting its exposure to the aqueous medium, thus increasing the drug solubility

(Ghezzi et al., 2021), see Figure 1.2. The stability state of the micelles above its CMC point is either thermodynamically unstable, where there is a continuous movement between the micelles formation and the aqueous system it sets within, or kinetically stable (Ghezzi et al., 2021).

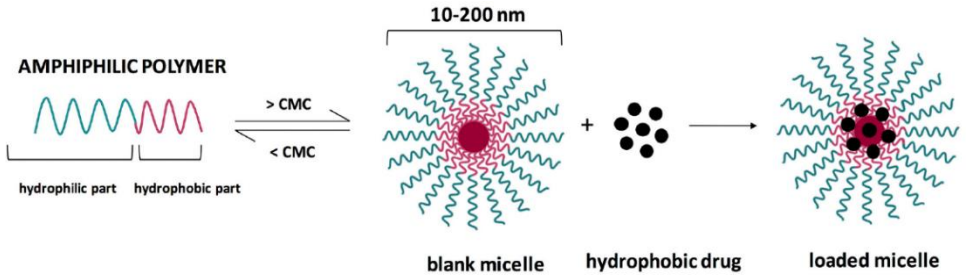


Figure 1.2: Micelles formation by the amphiphiles assembly and entrapping of low aqueous solubility drugs, after reaching the critical micelle concentration (CMC) (Ghezzi et al., 2021).

The supersaturation (SS) technique is one of the methods to overcome the low solubility of oral drugs by increasing the concentration of the free drug (Brouwers et al., 2009) at the absorption site (Palmelund et al., 2016). This approach depends on exceeding the thermodynamic equilibrium solubility of a compound, which will meta-stabilize for a period of time, before shifting to a more thermodynamically stable state and precipitation takes place (Palmelund et al., 2016). SS techniques were studied by two main methods, either by pH shift, or more often, by the solvent shift methods (Palmelund et al., 2016). The solvent shift method is preferred especially if limited quantities of a compound are available and its performed by dissolving the tested compound in a highly soluble organic solvent like the dimethyl sulfoxide (DMSO) (Palmelund et al., 2016). A small volume of this prepared organic solution is added into the desired aqueous medium, where SS is intended to be induced (Palmelund et al., 2016). The ratio between the SS concentration and the thermodynamic equilibrium concentration is called the degree of supersaturation (DS), and by this the SS can be defined (Palmelund et al., 2016). Each compound has its own inherent capability to supersaturate, so the DS can vary significantly (Palmelund et al., 2016). Some compounds with a high DS, will stay supersaturated for a long time before

precipitating, while others precipitate immediately once reaching the SS point (Palmelund et al., 2016).

The limitation to use the solvent shift method was the different DS presented by each drug (Palmelund et al., 2016). Palmelund et al. used a standard method to test the effect of each concentration addition on the supersaturation stability, this was done by measuring the highest concentration achieved before immediate precipitation ($Css_{100\%}$), with further three measurements of 87.5%, 75%, and 50% of the $Css_{100\%}$ concentration (Palmelund et al., 2016). Whereas the time needed to induce precipitation, was the point where a 2.5% decrease of the $Css_{100\%}$ occurs (Palmelund et al., 2016). The solvent shift method was carried out by many researchers with the same method mentioned above, as in one study, the SS was determined by setting the DS to 20, where one DS unit was equivalent to the equilibrium solubility of the compound in the same tested media (Bevernage et al., 2010). Where in another study, the DS was determined by adding multiple small quantities of the pre-dissolved DMSO and the tested compound into a defined volume of fasted simulated intestinal fluid (FaSSIF) media, until reaching the $Css_{100\%}$ point (Plum et al., 2020a). The $Css_{100\%}$ point was determined by monitoring the ultraviolet (UV) signal of the concentrations added, using a microDiss® profiler device (Plum et al., 2020a). Any baseline shift of the UV signal or a deviation from linearity was indicating a precipitation action (Plum et al., 2020a). The precipitation rate was calculated based on the average concentrations between the supersaturated plateau point, and the concentration of the plateau formed after precipitation (Plum et al., 2020a). Other devices were also used to measure the drugs SS state, such as the InForm®, which used the same techniques used by the microDiss® profiler device.

1.4. Factors Influencing Bioavailability

Four main factors will be discussed here, which play a major role in affecting oral drug bioavailability: physiological, physicochemical, pharmacokinetic, and the formulation factors.

1.4.1. Physiological Factors

1.4.1.1. Gastrointestinal pH

The different pH values of the GIT segments can affect drug ionization and thus solubilization within the tract (Vinarov et al., 2021, Sabnis, 1999). Poorly soluble weak acidic drugs, have low dissolution rate in acidic stomach, but a higher dissolution capability in intestine due to the intestine's high pH (Dressman et al., 2007). Whereas, poorly soluble weak bases are mainly dissolved in the stomach (Dressman et al., 2007). After dissolution, the dissolved drug permeates through the GIT membranes, this can be accomplished by the non-ionized form of the drug (Williams et al., 2013, Sabnis, 1999). The last step is the drug movement to the systemic circulation to have the desired therapeutic effect, see Figure 1.3.

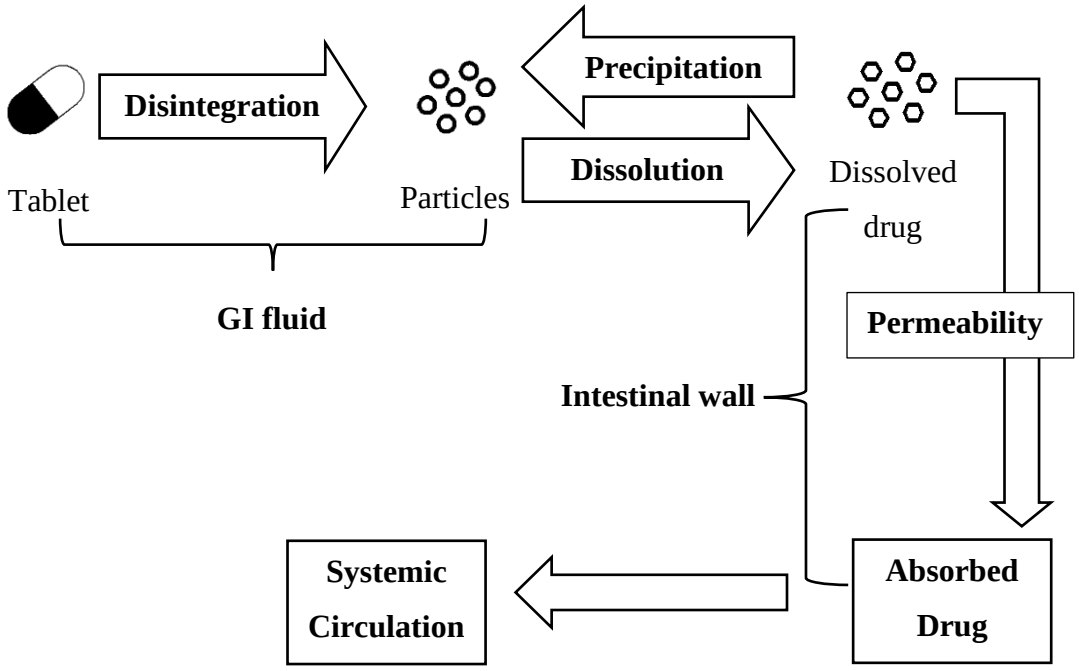


Figure 1.3: The oral drugs absorption process (Sugano, 2009).

More advanced techniques are used to monitor the GI pH continuously, with high precision, and in less than seconds, by using *in vivo* pH recording capsules (Korostynska et al., 2007). This technique, of monitoring pH, is important to study the metabolism of tissues, keep a track of the peripheral blood flow in diabetic patients, and other benefits (Korostynska et al., 2007). Different methods and materials are used for this purpose, and are chosen depending on their sensitivity, resolution at various pH ranges, lifetime, and robustness (Korostynska et al., 2007). One of the examples to

monitor pH change is the fiber optic pH sensors, which depend on the absorption or the florescence of a chromophore (Wolfbeis, 2006). This method is considered to be safe for usage, as it involves no electrical current to process, as many other methods, yet it needs analysis equipment which forms bulky sensors hard to be inserted in human body (Korostynska et al., 2007).

1.4.1.2. Gastric Emptying

The drug absorption mainly occurs in the small intestine, so any delay in emptying the drug from the stomach to the small intestine will affect the absorption capability (Sabnis, 1999, Vinarov et al., 2021). Gastric emptying can be controlled by many factors:

a. Food absence or existence

In a fasted stomach, small intestine and colon will all go through repetitious contractions called inter-digestive migrating motor complex (MMC) processes (Minami and Mccallum, 1984, Takahashi, 2012), lasting for around 130 min (Deloose et al., 2012). MMC has a main role in propelling the stomach content, and protecting the intestinal lumen from any growth of bacteria (Ma and Lee, 2020). It consists of three motility phases, first is a resting phase with no contractions, whereas during phase two, irregular contractions start to take place (Vantrappen et al., 1977). Lastly, phase three, lasts for 10 - 20 minutes, and it's called 'the housekeeper wave', which transfers all the gastric ingredients to the pylorus opening (the distal end of the stomach which opens to the small intestine) (Gleysteen et al., 1985, Sabnis, 1999). As a result, the administered dose reaches the intestine depending on the housekeeper wave phase (Gleysteen et al., 1985).

On the other hand, the inter-digestive MMC is interrupted by food, and its controlled by different mechanical, hormonal, and neuronal processes which help with gastric emptying during food existence (Kelly, 1974, Sarna, 1985). Each process is controlled by a different method, the mechanical mechanism is mediated by receptors which surround stomach, duodenum, and jejunum, such as acidic, osmotic, mechanical, and L-tryptophan receptors (Stephens et al., 1976). Whereas the hormonal mechanism is controlled by cholecystokinin (Jin et al., 1994), gastrin (Lenz, 1988), secretin (Debas et al., 1977, Lafontaine et al., 1983), and motilin (Debas et al., 1977). Lastly, the neural

mechanism is regulated by the inhibitory vagal system (Gleysteen et al., 1985, Lenz, 1989).

b. The dosage form state

The size of the drug particles can vary from very fine (1 – 5 mm in diameter) which are emptied with fluids, to larger particles which are emptied via the incorporation with the housekeeper wave, discussed above (Vinarov et al., 2021, Sabnis, 1999). Gastric emptying is also changed with the various types of drug formulation, and the elimination half-lives for oral dosage forms range between 19 - 87 minutes (Kaniwa et al., 1988). For example, nitrofurantoin drug when taken as a solid dosage form have a longer gastric emptying half-life compared to when taken as a solution (Niazi et al., 1983).

1.4.1.3. Intestinal Transit

The small intestine forms eighty percent of the whole length of intestine, its connected via the ileocecal valve down to the cecum, which is linked to the colon (Ma and Lee, 2020, Sabnis, 1999). After the gastric emptying takes place, the drug is moved, by a consistent time, to the small intestine, where the absorption process takes place (Ma and Lee, 2020, Sabnis, 1999), see Figure 1.4. On the contrary of the gastric emptying characteristics, the intestinal transit doesn't depend on the state of the material moved, nor the existence of food (Davis et al., 1986). So, the bioavailability of fast-solubilized drugs can only be affected by the gastric emptying process, whereas the intestinal transit time affects the absorption rate of drugs which need a carrier transporter to be systematically absorbed (Leesman et al., 1988).

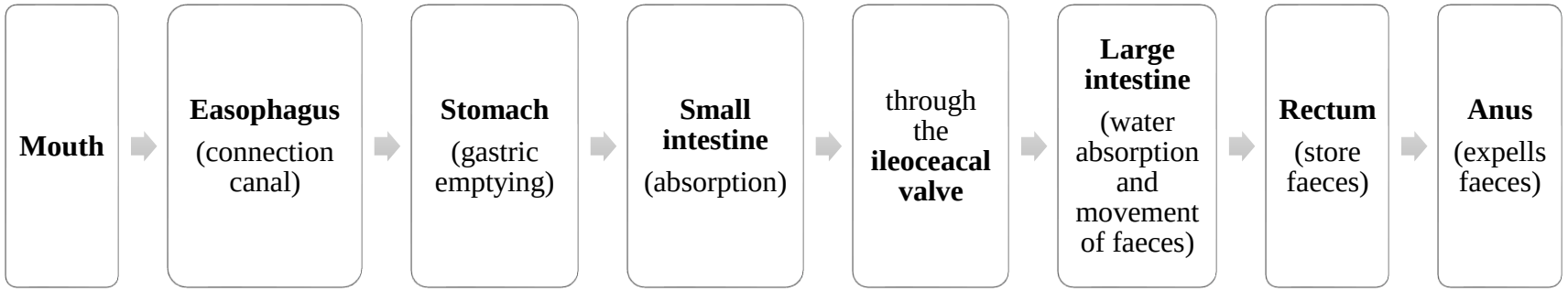


Figure 1.4: The gastrointestinal tract main parts.

1.4.1.4. Diet

The effect of diet can be an enhancer, delayer, or reducer to the drugs dissolution process (Koziolek et al., 2016, Sabnis, 1999). The absorption of drugs was studied by many scientists, and most of them found that with feeding status, more drug is absorbed (Koziolek et al., 2016, Sabnis, 1999). In addition, the metabolism process of drugs was studied with and without food, and was found that a meal saturated with proteins and low carbohydrates level can increase the metabolism state of drugs, whereas a low protein with high carbohydrate meal can do the opposite (McKellar et al., 1993, Williams et al., 1993). Fatty meals act variably, and is hugely affected by the drugs properties and formulation (Sabnis, 1999). Water soluble drugs have lower absorption with the consumption of a saturated fatty meal. On the other hand, fatty meals can increase the absorption of poorly soluble drugs, such as griseofulvin, by partitioning into the oily phase preventing the drug exposure to stomach acids (Khalafalla et al., 1981). Whereas, highly rich fatty meals could reduce the drug bioavailability, by delaying stomach emptying process, or acting as an absorption barrier due to the highly partitioning process of the drug into the oily phase (Pao et al., 1998).

1.4.2. Physiochemical Factors

1.4.2.1. Chirality of the Drug

In 1848, Louis Pasteur was the first scientist considering the stereochemistry importance, based on an assumption that the highest activity of an organic molecules is because of its asymmetrical shape which forms a non-superimposable mirror image (Mayerson, 1995). Stereoisomers are molecules with the same physicochemical features, which is hard to be differentiated in the symmetrical (achiral) environments, but easier in the asymmetrical environments (Landoni and Soraci, 2001, Sabnis, 1999). Stereoisomers can result in different drug bioavailability values, which is refereed to various reasons, first, the different pharmacokinetic rate constants of each isomer (Duddu et al., 1993). Second, the interaction of the drug's isomer with other chiral centres of the media excipients. Third, the alteration of an isomer to other form (Duddu et al., 1993). Lastly, the stereoselective metabolism which is developed during the drug absorption phase (Duddu et al., 1993).

1.4.2.2. Partition Coefficient and pKa

Each drug has its own chemical properties, the majority of drugs are either weak acids or weak bases, which will get ionized depending on their pKa and the media pH it reacts with (Mayerson, 1995), which can be calculated using the Henderson Haselbach equation (Bhagavan, 2002):

pH = pKa + Log [Conjugate base] ÷ [Acid]

The drug partition coefficient between n-octanol and water ($K_{o/w}$) is a major contributor to the drug diffusion through body membranes, which affects the drug ionization degree, and thus the absorption process (Navia and Chaturvedi, 1996). $K_{o/w}$ is also called Log P (Cumming and Rücker, 2017). The ionized drugs will have less $K_{o/w}$ value compared to the non-ionized form (Miller et al., 1985, Sabnis, 1999).

1.4.2.3. Complex Formation

There are different types of chemical interactions (complexing), one of the main types is the inclusion interaction (Bai et al., 2009, Sabnis, 1999). The inclusion complex is formed by the molecule's arrangement style rather than its chemical bonding (Bai et al., 2009, Sabnis, 1999). One component of the complex structure will be merged in the molecular structure of the other components, resulting in a more stable architecture (Bai et al., 2009, Sabnis, 1999). The most popular example for inclusion complex is cyclodextrins, which merges the hydrophobic drugs into it, to make a higher overall hydrophilic properties (Albers and Muller, 1995), and therefore increase the drug solubility.

1.4.3. Pharmacokinetic Factors

1.4.3.1. First Pass Effect (Pre-Systemic Elimination)

It's a process caused by the high metabolic degradation of the drug in the liver before its movement to the systemic circulation (Herman and Santos, 2019, Sabnis, 1999). Pre-systemic elimination lowers the amount of drug reaching the circulation, and as a result it affects the drug bioavailability (Herman and Santos, 2019, Sabnis, 1999).

1.4.3.2. Drug Interaction

There are 2-ways of interactions, the direct and indirect modes (Sabnis, 1999). The direct interactions occur when two drugs interacts physically or chemically and change their GI absorption behaviour (Sabnis, 1999). For example, the administration of 4-flouroquinilones antibiotics (such as ciprofloxacin) with an aluminium hydroxide-based antacids, which will cause the quinolone to chelate with the metallic cations, and thus significantly lower their bioavailability.

Whereas the indirect interaction occurs when one drug had an effect on the GI affecting another drug absorption. For example, metoclopramide, diamorphine, and diphenhydramine were seen to affect the GI motility, resulting in changed absorption behaviour of other drugs in the GIT (Welling, 1988). Also, macrolides (such as clarithromycin) interaction in the GIT increases the contractile activity of GIT affecting the bioavailability of other co-administered drugs (Nakayoshi et al., 1992).

1.4.4. Formulation Factors

1.4.4.1. Types of Dosage Forms

The bioavailability of solutions, emulsions and suspensions, need no disintegration process to be absorbed, thus gives higher bioavailability (Pentikäinen, 1986). On the other hand, solid dosage forms like tablets and capsules need disintegration (Pentikäinen, 1986, Sabnis, 1999).

1.4.4.2. Excipients

As some solid dosage forms don’t give the maximum bioavailability, and as its more convenient to be taken by patients, so other excipients are being added to enhance the absorption action (Jackson et al., 2000, Sabnis, 1999). One of the most important excipient are the absorption enhancers, which enters and interacts with the GIT's lipid bi-layer and the protein-filled regions, thus increasing drug absorption (Muranishi, 1990, Sabnis, 1999). Examples of such enhancers are surfactants (Florence, 1981), BS (Fagundes-Neto et al., 1981), mixed micelles (Muranishi, 1985), and FA (Tokumura et al., 1987).

1.5. Oral Drug Absorption Models and Classifications

Solubility as a factor in oral absorption permitted, by using a range of assumptions, the development of drug absorption models that could be applied to calculate or estimate the absorption of drugs. One of the first values proposed was the Absorption Potential (AP, Eq. (1)) (Dressman et al., 1985):

$$AP = \log (P \times F_{non} \times S_0 \times V_L \div X_o) \quad \text{(Equation 1)}$$

Where P is the drug permeability to the drug, F_{non} is the non-ionised fraction at pH 6.5, S_0 is the intrinsic solubility at 37°C, V_L is luminal content volume, and X_o is the dose administered.

A further variation is the Maximum Absorbable Dose (MAD), which could be calculated using equations 2 (Curatolo, 1998) and 3 (Sun et al., 2004):

$$MAD = S \times K_a \times SIWV \times T_{si} \quad \text{(Equation 2)}$$

$$MAD = P_{eff,\,human} \times S \times A \times T_{si} \quad \text{(Equation 3)}$$

Where, S is the physiological solubility (at pH 6.5), K_a is the transintestinal absorption rate constant (min^{-1}), SIWV is the small intestinal water volume (mL), T_{si} is the small intestinal transit time (min) (3.32 hr), P_{eff} is the human effective drug permeability ($cm.s^{-1}$), and A is the surface area of small intestinal absorption (7.54×10^4 cm^2).

These equations utilise aqueous solubility at pH 6.5, however, it was recognised that aqueous solubility is not identical to intestinal solubility due to the presence of solubilising agents such as BS and PL (Dressman et al., 2007). Also, the permeability and the solubility act in compensation, as the drugs with high permeability could offset their low solubility value, which will mislead determining the highest dose, where above the absorption is limited (Butler and Dressman, 2010).

The MAD approach was further modified with three dimensionless parameters: dose number (Do), dissolution number (Dn), and absorption number (An). This model considered a drug to be solubility limited, if not all the drug dose was dissolved in the GI fluid volume of 250 mL and the physiological pH range. In addition, a drug is dissolution rate limited, if not all the drug particles dissolved within the small intestine transit time (3.32 hr). Lastly, the drug will be permeability limited if there was a low rate of moving from the gut lumen into gut wall.

The three dimensionless parameters were incorporated in the Biopharmaceutic Classification System (BCS), which considered different key elements for drug absorption such as the drug's effective intestinal permeability, and the volume required

to dissolve the highest dose of a drug, see Figure 1.5 (Amidon et al., 1995). This enabled the usage of *in vitro* data instead of *in vivo* data to classify oral drugs and to study their *in vivo* performance (Amidon et al., 1995). The BCS has four categories: Class I drugs have high solubility between pH (1 - 8), and high permeability properties, whereas class II drugs can cross all the gut membranes easily, but has limited solubility properties in aqueous media (Amidon et al., 1995). On the other hand, class III drugs have poor permeability, but high solubility properties, and lastly, class IV drugs is the worst category which is for drugs having both poor solubility and permeability (Amidon et al., 1995).

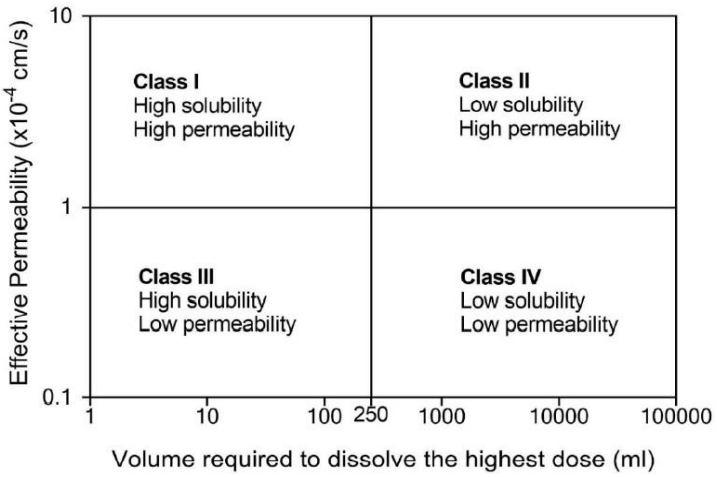


Figure 1.5: The Biopharmaceutics Classification System (Amidon et al., 1995).

The BCS system was classified upon the lowest aqueous solubility of the drug in 250 mL at the physiological pH range, but this was not an ideal classification, as some drugs are pH-dependent, affected by the GIT's solubilizers, and/or influenced by the presence of food, which would underestimate the drug solubility (Butler and Dressman, 2010). This issue was resolved by the Developability Classification System (DCS) by measuring the drugs solubility in a biorelevant dissolution media which contained intestinal solubilizers such as bile acids (Dressman et al., 1998, Galia et al., 1998). Also, the GI fluid volumes were adjusted to more representative volumes (500 mL), as well as class II was divided into two categories; IIa dissolution rate limited, and IIb solubility limited, focusing on the extent of the drug's absorption, see Figure 1.6.

The Dose Number (Do, equation 4 (Lawrence et al., 1996b), introduced the dose/solubility ratio concept, which was further expanded in the DCS (Butler and Dressman, 2010), and also required the use of a "physiological" solubility value rather than a simple aqueous value, which led to the solubility limited absorbable dose (SLAD) (equation 5) concept.

$$Do = X_o / V_0 \times S \quad \text{(Equation 4)}$$

Where V_0 is the volume of water taken with the dose.

$$SLAD = S_{si} \times V \times Mp \quad \text{(Equation 5)}$$

The solubility value required to calculate SLAD was the intestinal equilibrium solubility (S_{si}) (Butler and Dressman, 2010), which can be measured in intestinal fluid or simulated intestinal fluids. V is the intestinal fluid volume (500 mL), and Mp is the permeability dependent multiplier.

The SLAD is represented by the boundaries found between class IIa and class IIb (see Figure 1.6) for drugs with high permeability, and between class II and IV for the drugs with low permeability (Butler and Dressman, 2010).

For a high permeability drug, Mp is equal to the absorption number (An, equation 6); for low permeability drugs is set equal to 1. R is the intestinal radius (1.25 cm (Lawrence et al., 1996b)).

$$An = P_{eff} \times T_{si} / R \quad \text{(Equation 6)}$$

Incorporating the dissolution rate instead of the dose/solubility ratio, has introduced the usage of the dissolution number (Dn, equation 7) which could be rearranged to calculate the drug particle radius (r_p, equation 8) below which the absorption of drugs is not limited (Lawrence et al., 1996b). This will be useful for drugs of class I, IIa, and III, as other classes (IIb and IV) are solubility limited, and both are more controlled by the dose/solubility ratio (Butler and Dressman, 2010).

$$Dn = (3D / r_p^2) \times (S_{si} / \rho) \times T_{si} \quad \text{(Equation 7)}$$

$$r_p^{\,2} = (3D / Dn) \times (S_{si} / \rho) \times T_{si} \quad \text{(Equation 8)}$$

Where D is the diffusion coefficient ($5*10^{-6}$ cm^2/s), r_p is the particle radius (μm), and ρ is the drug density (1.2 g/cm^3), the diffusion coefficient and density are representative values.

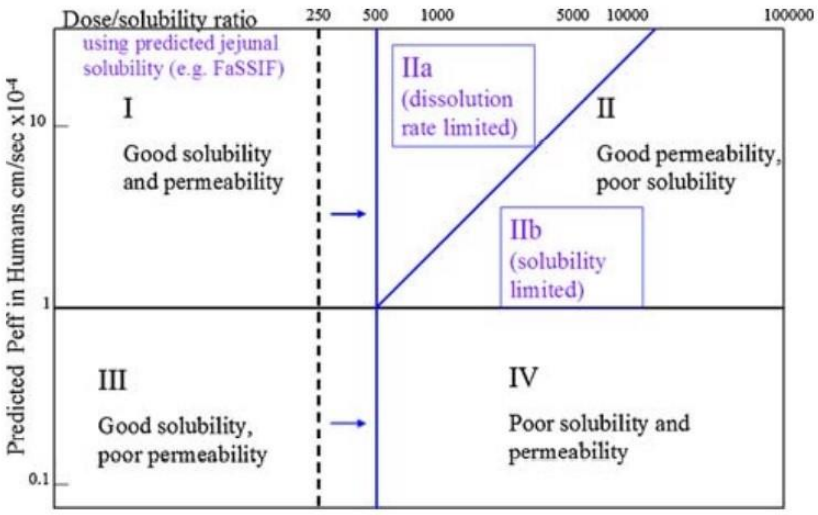


Figure 1.6: The Developability Classification System (Butler and Dressman, 2010).

The last and the most complex oral absorption model to be discussed here is GIT compartmental models. It was first introduced by the compartmental absorption transit (CAT) model (Lawrence et al., 1996b), which was used to mathematically predict the fraction of a drug absorbed, and evaluate the plasma concentration profiles (Lawrence et al., 1996b). The transit flow of the CAT model in the small intestine was described by seven compartments, namely duodenum, upper and lower jejunum, and four compartments for ileum (Lawrence et al., 1996a). The CAT model led to the advanced compartmental transit absorption (ACAT) model, see Figure 1.7, which included the same seven compartments with the addition of the stomach and colon, to enable gastric emptying and colonic absorption processes (Agoram et al., 2001).

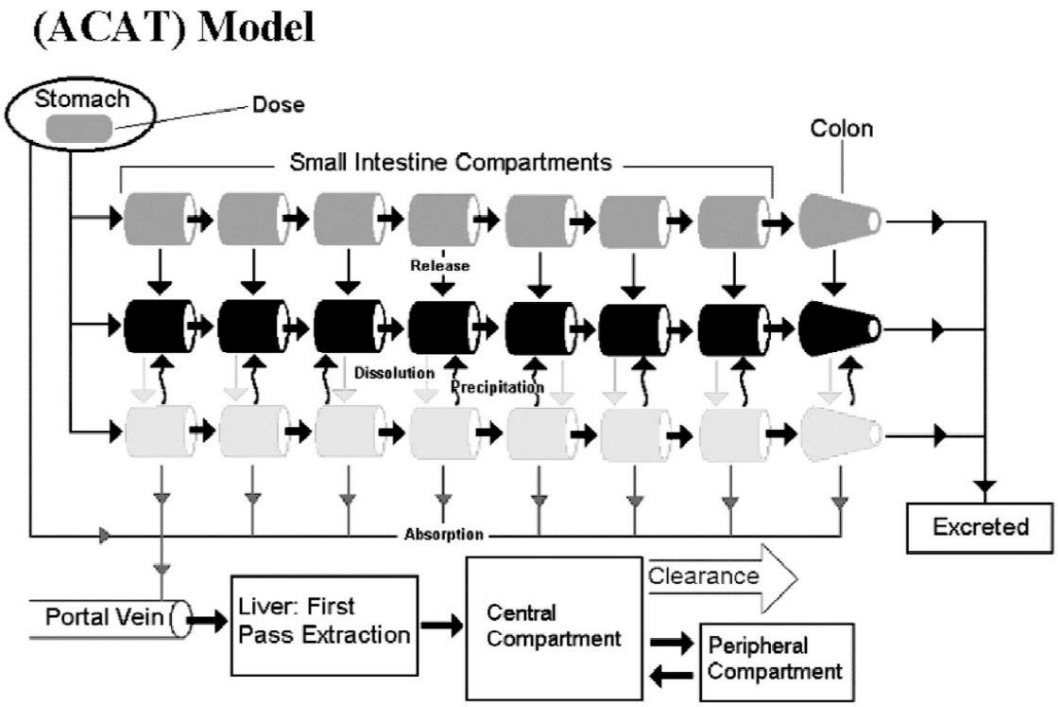


Figure 1.7: The advanced compartmental transit absorption (ACAT) model, with 9 compartments (Agoram et al., 2001).

This modelling approach is applied by a computational technology during developing and discovering compounds (Agoram et al., 2001), which requires a good knowledge in both mathematical and biopharmaceutical modelling (Butler and Dressman, 2010). However, this complexity reduces the time needed to submit a new drug application, and reduces the number of experiments needed to develop and select compounds (Agoram et al., 2001). This included the collection of literature *in vivo*, and *in vitro* data and applying statistical measurements to estimate some biopharmaceutical properties, by the usage of two-dimensional (2D) and three-dimensional (3D) molecular structures. Then, incorporating different *in silico* mathematical equations for the ACAT model to predict the rate, extent, and GI absorption by a physiology based pharmacokinetic (PBPK) approach (Agoram et al., 2001).

Human PBPK is a description of the absorption, distribution, metabolism and elimination (ADME) processes in the human body (Gerlowski and Jain, 1983). This approach involves dividing the body organs into different compartments, which are connected by the body fluids (circulatory system) (Gerlowski and Jain, 1983). Three main factors are considered to affect the compartmental oral drugs absorption: the

physiological factors such as the blood flow rate, and tissue volumes, physicochemical factors such as ionization, lipophilicity, particle size and solubility, and pharmacological factors such as the site of action, mechanism of transport, and the dosage form (Gerlowski and Jain, 1983, Lawrence et al., 1996b).

GastroPlus® software, which will be used in Chapter 6, was the first software programmed to comprehensively describe the PBPK of the GIT, and it was modelled based on the ACAT model with 9 compartments, each has its own transit time and volume (Agoram et al., 2001).

1.6. Media used to Determine Drug Equilibrium Solubility

Drug discovery and development can result in variable physicochemical properties, and behaviour in human body, as a result of this, different tests were needed to detect each drug's physicochemical activity, and to discover the properties which give an acceptable bioavailability after the administration of oral drugs (Galia et al., 1998). Oral drug solubility was tested in different buffers and media, which will be discussed in details.

1.6.1. Physiologically Adapted Media and Buffers

The first method used to determine the equilibrium solubility was the shake-flask method, where the drug is added into a specific medium, at a fixed temperature and agitation type, until equilibrium is achieved (Higuchi, 1965). The saturated solution is separated from the undissolved drug via centrifugation or filtration, before the equilibrium solubility is determined (Higuchi, 1965). The mostly used buffer media for the shake-flask method are phosphate buffer pH (5.8 – 8), and acetate buffer (Bou-Chacra et al., 2017). Both buffers were considered to have comparable ionic strength and osmolality properties as the *in vivo* physiological fluids (Bou-Chacra et al., 2017). Bicarbonate-based buffer (Krebs buffer) (Fadda et al., 2009), was used to study the intestinal absorption, but needed sensitive storage conditions (Bou-Chacra et al., 2017). Other buffers, less commonly used, are for example, maleate (Ottaviani et al., 2010), and glycine buffers (Charkoftaki et al., 2010).

Different types of buffers was used, as a representative of the physiological fluids, to measure the drug solubility, but even the most commonly used buffer (phosphate) failed to predict the *in vivo* solubility of drugs, because of its poor capacity of solubilization (Klein, 2010, Li and He, 2015).

1.6.2. Human Intestinal Fluid (HIF)

This method included testing the new drug entities in a real HIF medium, by withdrawing the HIF samples either before or after administering the drug. This technique required huge quantities of HIF media, which is hard to be collected from healthy volunteers (Finholt and Solvang, 1968). In addition, HIF sampling faced different obstacles related to the high costs, and the questioned ethical experiments tested on human beings with no assured treatment (Dressman et al., 2007).

HIF sampling was carried out in fasted and fed states:

a. Fasted state (FaHIF)

HIF sampling was collected from healthy fasted individuals from the first start point of the jejunum by different researchers (Brouwers et al., 2005, Brouwers et al., 2006, Brouwers et al., 2007a, Brouwers et al., 2007b, Kalantzi et al., 2006, Lindahl et al., 1997, Pedersen et al., 2000b, Persson et al., 2005, Moreno et al., 2006). This location was the most convenient place for the aspiration tubes to be installed, in addition, more volumes could be taken from this near spot, rather than the distal parts (Dressman et al., 2007). When FaHIF samples were collected they were either frozen at -70° C without the addition of any substance (Kalantzi et al., 2006, Lindahl et al., 1997, Pedersen et al., 2000b, Persson et al., 2005, Moreno et al., 2006), or some substances were added for specific purposes. For example, phenylmethylsulfonylfluoride was added to the aspirated sample, to eliminate trypsin activity which would break down the FaHIF sample's proteins (Kalantzi et al., 2006). Whereas, to stop the activity of lipase enzymes, which break down the lipid content of the FaHIF sample, one of the following techniques was carried out. First, by adding 5% v/v of 100 mM di-isopropyl fluorophosphate with 50 mM acetophenone, and 250 mM phenylboronic acid (Armand et al., 1996). Second, by adding tetra-hydrolistatin (orlistat) (Carrière et al., 2001, Persson et al., 2005), and third, by using p-bromo phenylboronic acid (Fine et al., 1990). In addition, to prevent any microbial growth, the addition of 6 mM NaN_3 and

0.01 mM chloramphenicol were used (Pedersen et al., 2000b). The FaHIF aspirates were then centrifuged for 10 - 20 minutes at 5000-10,000 rpm (Kalantzi et al., 2006, Pedersen et al., 2000b, Persson et al., 2005), and transferred to High Pressure Liquid Chromatography (HPLC) vials (Dressman et al., 2007) to analyse the solubility of each aspirate (Ran and Yalkowsky, 2001).

b. Fed state (FeHIF)

The aspirates were withdrawn from healthy volunteers who were previously given a liquid food, and then added to a previously prepared vial containing lipase and trypsin inhibitors, as well as, a microbial growth inhibitor (Dressman et al., 2007). However, those inhibitors were assumed to affect the solubility profile of the administered drugs (Dressman et al., 2007), so only Orlistat was added (Persson et al., 2005).

1.6.3. Animal Intestinal Fluid

Another way to detect the dissolution and solubility properties of oral dosage forms was by taking the intestinal fluids from animals as a replacement for HIF fed media (Dressman et al., 2007). The relation between dogs and human aspirates, were previously studied, and found a good acceptance between both species by the small meal intake (lower than 200 mL) (Persson et al., 2005), whereas an overestimation in dogs was noticed with high volume meals, and that was clearly caused by the high bile salts available in canine's small intestine after eating (Dressman et al., 2007).

1.6.4. Simulated Intestinal Fluid (SIF)

Drug formulators studied the *in vivo* performance and solubility of drugs by preparing simulated gastric and intestinal media, in both fasted and fed states (Dressman et al., 1998, Galia et al., 1998), with the use of the BCS guidance (Amidon et al., 1995). This was accomplished by different protocols, substances, and concentrations, starting by adding GIT solubilizers such as BS and PL to the SIF media (Galia et al., 1998). However, the drug solubility depended on other variables, such as pH, buffer capacity, ionic strength, the volume available for dissolution (Dressman et al., 1998), and also the lipid degradation products (Jantratid et al., 2008). As well, in the fasted state, the concentrations of BS and PL in human intestinal fluids are normally low (Clarysse et

al., 2009), but in the fed state, the concentrations are high, which increases drug solubility (Clarysse et al., 2011).

For the validation of the SIF media chosen, a simple and stable media was needed (Clarysse et al., 2011), and the studied SIF media was stable for one day only (Söderlind et al., 2010). From this, another surfactant was introduced to the media, which depended on the surface-active compound; D-α-tocopheryl polyethylene glycol 1000 succinate, which was easily used compared to the previous surfactants (Söderlind et al., 2010). Also, it overcame the physical instability problem found in FaSSIF media, where the lipids aggregates started to build up after one day of the prepared media (Söderlind et al., 2010). Lastly, the solubility results found by using the SIF media, were validated and statistically compared with the solubility results found in the HIF aspirates (Clarysse et al., 2011).

1.6.5. Design of Experiments of Simulated Intestinal Fluid

There have been multiple efforts to prepare the best SIF media recipe, covering all the variables tested through the years (Bou-Chacra et al., 2017, Clarysse et al., 2011, Dressman et al., 2007). This includes understanding the consequences of multiple SIF variables on drug dissolution properties, mixing them together, and discovering the conditions which give the best results (Khadra et al., 2015). Khadra et al., was the first to study such effects together by applying a statistical design of experiment (DoE) technique in the fasted state (Khadra et al., 2015). The fasted SIF media was tested first, because of its simplicity compared to the fed state (Khadra et al., 2015). This was further followed by multiple DoE investigations of the fed (Zhou et al., 2017b), full range (Perrier et al., 2018), dual range (Ainousah et al., 2017), and a small-scale (McPherson et al., 2020).

1.6.5.1. Fasted SIF DoE

This statistical DoE was tested by using two extreme levels in the fasted state, high and low, as seen in Table 1.1, testing seven components of the intestinal fluid, including BS (sodium taurocholate), PL (lecithin), buffer (sodium phosphate), salt (sodium chloride- NaCl), pH, enzyme (pancreatin), and digestion product (sodium oleate), all concentrations were based on literature studies as seen in Table 1.2.

Table 1.1: Values of fasted DoE for seven intestinal components (Khadra et al., 2015).

Component	**Substance**	**Lower value (mM)**	**Centre point (mM)**	**Upper value (mM)**
Bile salt	Sodium TC	1.5	3.7	5.9
Phospholipid	Egg phosphatidylcholine	0.2	0.6	1
Buffer	NaH_2PO_4	15	30	45
Salt	NaCl	68	87	106
pH	NaOH/HCl	5	6	7
Enzyme (U/mL)	Pancreatin	270	465	660
Fatty acid	Sodium oleate	0.5	5.25	10

TC: taurocholate, enzymes concentrations were based on (Armand et al., 1996).

Table 1.2: Representation of the values tested in previous literature studies with different gastrointestinal components in fasted simulated intestinal fluid.

Component (mM)	**(Dressman et al., 1998)**	**(Galia et al., 1998)**	**(Pedersen et al., 2000a, Pedersen et al., 2000b)**	**(Vertzoni et al., 2004)**	**(Sunesen et al., 2005)**		**(Jantratid et al., 2008)**	**(Brinkmann-Trettenes and Bauer-Brandl, 2014)**
					low	**high**		
Sodium TC	5	3	0	3	2.5	6.3	3	3
Lecithin	1.5	0.75	0.9	0.75	0.5	1.25	0.2	1.5
Sodium GC	-	-	3.7	-	-	-	-	-
NaH_2PO_4	(K) 29	(K) 28.6	50	28.66	(K) 29	(K) 29		32.9
NaCl	(K) 220	(K) 103.3	150 (Total Na)	106	No salt (KCl)	No salt (KCl)	68.62	105
NaOH				~13.8			34.8	98
Osmolarity (mOsmole)	280-310	270±10	-	270±10	-		180	-
Pancreatin	-	-	-	-	-	-	100 (U/mL)	32 (mg/mL)
Tris/maleic acid	-	-	-	-	-	-	19.12	
pH	6.8	6.5	6.5	6.5	6.8	6.8	6.5	6.5

GC: Glycocholate, TC: Taurocholate, K: potassium, Na: sodium.

In this fasted DoE, twelve drugs; acidic (indomethacin, naproxen, phenytoin, and piroxicam), basic (aprepitant, carvedilol, tadalafil, zafirlukast), and neutral (felodipine, fenofibrate, griseofulvin, and probucol) were analysed (Khadra et al., 2015). The equilibrium solubility of all the drugs were measured using a quarter fraction factorial design requiring 32 media compositions to be measured in duplicate, along with a centre point also measured in duplicate (Khadra et al., 2015). The statistical analysis was conducted by Minitab® 16.0 program for main significant effects, along with the significant effects of the 2-way interactions between the components (Khadra et al., 2015).

All the fasted DoE solubility results were superimposed or in the same range of the previous literature either from FaHIF or FaSSIF systems (Augustijns et al., 2014, Khadra et al., 2015). Some drugs exhibited high solubility variability, such as zafirlukast and fenofibrate, which indicates that they were affected by different components (Khadra et al., 2015). However, griseofulvin and tadalafil showed a lower variability range (Khadra et al., 2015). The standard deviation of literature FaHIF solubility result of fenofibrate was 132% (Clarysse et al., 2011), while griseofulvin was 29% (Annaert et al., 2010), which is comparable with the fasted DoE ranges (Khadra et al., 2015). The components effects were also investigated by grouping the drugs into acidic, basic, and neutral (Khadra et al., 2015). Five out of seven components significantly affected the acidic drugs solubility, exception was for pancreatin and salt components (Khadra et al., 2015). Whereas, six out of seven components significantly affected the basic and neutral drugs solubility, exception was for pancreatin only. The solubility of acidic drugs was affected by pH by a strength of ten times more than the other significant effects (Khadra et al., 2015), which is the same result reported previously (Clarysse et al., 2009). The effect of pH was followed by FA, BS, and buffer components, and this was also the same literature result found for indomethacin (Clarysse et al., 2009). PL was the lowest significant effect, but in previous studies it was studied in combination with bile salt (Clarysse et al., 2009). The basic and neutral drugs solubility effects were comparable, with FA to be the highest effect, followed by pH but to a lower magnitude compared to acidic drugs (Khadra et al., 2015). The next two effects, with relatively the same level of influence, were BS and PL, followed by, but with much lower impacts, buffer and salt (Khadra

et al., 2015). As an overall, the solubility of basic and neutral drugs were affected by a combination of the solubilising factors (FA, BS, and PL) along with pH, which is a comparable result found for the basic drugs in a previous multiple linear regression analysis (Clarysse et al., 2009). However, the neutral drugs solubility was previously studied at a fixed pH value, since the focus was only on the solubilising capacity factors (Kleberg et al., 2010, Pedersen et al., 2000b, Söderlind et al., 2010). Yet, one study reported the pH effect on hydrocortisone (neutral drug) solubility in HIF samples (Pedersen et al., 2000a), which was attributed to a change to the media components ionization behaviour (Khadra et al., 2015).

The 2-way interaction effects were 54 interactions, only one third of the interactions were statistically significant (Khadra et al., 2015). Acidic drugs displayed three significant interactions, first two were between pH with both FA and BS, which is caused by ionization (pKa of acids around 5 (Holm et al., 2013), DoE pH 5-7), and thirdly between pH and buffer interaction, which is due to the salt effect (Khadra et al., 2015). Basic drugs had twice the number of the significant interactions compared to acidic drugs (Khadra et al., 2015). pH with FA is the highest effect in all interactions, which is referred to the ionization effect discussed above, also an interaction between pH with both salt and PL, BS with both FA and buffer, and lastly with a lower effect between PL and salt (Khadra et al., 2015). The effect of PL with salt is due to the effect of salt on changing the CMC limit, thus affecting solubilisation of the basic drugs (Khadra et al., 2015). Neutral drugs had a higher significant interactions, eight, which corresponded to a complex control on solubility (Khadra et al., 2015). The interactions were between pH with three factors (FA, BS, and salt), BS with three factors (FA, PL, and buffer), PL with salt, and FA with salt (Khadra et al., 2015). As in acidic and basic drugs, the pH and FA was the highest significant interaction, in addition to the significant effect of pH with both salt and BS, which reveals the importance of testing pH in neutral drugs, as discussed above (Khadra et al., 2015). The interaction effect of BS with PL resembles the previously reported result (Söderlind et al., 2010), but with a lower interaction value, and that is probably due to the high effects made by pH, which swamped any other interaction effects in this fasted DoE (Khadra et al., 2015).

1.6.5.2. Fed SIF DoE

A continuation work from the fasted DoE (Khadra et al., 2015) was carried out to see the applicability of the DoE protocol in the fed state, and if any differences were present (Zhou et al., 2017b). The factors tested were the same fasted DoE seven components: pH, BS, PL, FA, buffer, salt, and pancreatin, but using higher concentrations, and adding monoglyceride (MG). The fed DoE was tested on thirteen poorly soluble drugs acidic (ibuprofen, indomethacin, phenytoin, valsartan, and zafirlukast), basic (aprepitant, bromocriptine, carvedilol, and tadalafil), and neutral (felodipine, fenofibrate, itraconazole, and probucol) (Zhou et al., 2017b). To study the fed DoE, the concentrations used (Table 1.3) were based on literature concentrations ((Bergström et al., 2014) Figures 1,6,9,10) and Table 1.4 (Zhou et al., 2017b).

Table 1.3: Representation of the values used in fed DoE (Zhou et al., 2017b).

Component	**Substance**	**Lower value (mM)**	**Upper value (mM)**
Bile salt	Sodium TC	3.6	24
Lecithin	Egg PL	0.5	4.8
Buffer	Maleic acid	28.6	58.09
Salt	NaCl	125	203
pH	NaOH/HCl	5	7
Enzyme (U/mL)	Pancreatin	100	150
Fatty acid	Sodium oleate	0.8	52
Monoglyceride	GMO	1	6.5

TC: taurocholate, PL: phospholipid, GMO: glyceryl mono-oleate.

Table 1.4: Summery of previous literature values of fed SIF components (FeSSIF).

Component	**(Dressman et al., 1998)**	**(Galia et al., 1998)**	**(Vertzoni et al., 2004)**	**(Jantratid et al., 2008)**	**(Kleberg et al., 2010)**
pH	5	5	5	5.8	6.5
Buffer (mM)	Acetate	Acetate	Citrate	Maleate	Maleate
Sodium TC (mM)	15	15	15	10	5 - 20
Lecithin (mM)	4	3.75	3.75	2	1.25 - 5
BS/PL	3.75	4	4	5	4
Salt	0.19 M (KCl)	0.20 M (KCl)			
Sodium oleate (mM)	-	-	-	0.8	0 - 45
Mono oleate (mM)	-	-	-	5	0 - 10

TC: taurocholate, BS: bile salt, PL: phospholipid.

In order to have the same statistical power of the studied fasted DoE (Khadra et al., 2015) with the addition of the MG component, without doubling the number of samples, the DoE was converted into a D-optimal design, which resulted in a higher resolution for the main effects, and a lower resolution for the 2-way interactions (Zhou et al., 2017b). The design required 92 samples (44 conditions, all in duplicate, and 4 repeating centre points) (Zhou et al., 2017b). Where available, the results were compared with literature solubility values of FeHIF and fed SIF (Augustijns et al., 2014, Clarysse et al., 2011), and found all the results to be in the same range of the fed DoE solubility results (Zhou et al., 2017b). The fed DoE solubility ranges were found to be highly variable, and in some cases it was a three log range (Zhou et al., 2017b), which is greater than the results found in the fasted DoE (Khadra et al., 2015).

Each drug acted differently towards the eight SIF components tested, the salt was the lowest significant effect to one drug only, followed by buffer and MG to affect two drugs, pancreatin to effect three drugs (Zhou et al., 2017b). This was comparable with the fasted DoE which found the pancreatin and salt to be the least significant factors (Khadra et al., 2015). In contrast, the higher effects resulted from BS (twelve drugs), and pH, FA, and PL (ten drugs) (Zhou et al., 2017b). For acidic drugs, pH was the main factor influencing solubility (Zhou et al., 2017b), which is the same as the fasted DoE results for acidic drugs (Khadra et al., 2015), but with a decrease of the maximum significant effect value from ninety to fifteen, and that could be because of the higher surfactants concentrations present in this fed DoE, and thus the higher solubilising capacity of the surfactants (Zhou et al., 2017b). All the acidic drugs were positively affected by the pH component, which is comparable to the positive effect found of the HIF pH on the acidic drugs solubility (Clarysse et al., 2009). The pH effect was followed by FA, BS, and PL but no significant result was seen from buffer, as it was significant in the fasted DoE (Khadra et al., 2015, Zhou et al., 2017b). The solubility of the basic drugs had the BS to be the most significant effect, followed by the FA, pH, and PL which was just significant (Zhou et al., 2017b). Finally, the neutral drugs have a mix of significant effects for all the factors except the salt effect, with a maximum effect from the FA followed by the BS (Zhou et al., 2017b). On the third place was the pH effect and PL to be relatively similar to each other (Zhou et al., 2017b).

1.6.5.3. Full Range DoE

Further experiments were carried out by combining the fasted and fed DoE, but with removal of the non-significant components such as pancreatin, to lower the experimental media number, and to examine the drug's GI solubility in a single DoE (Perrier et al., 2018). This was achieved by covering the whole range between the lowest limit of the fasted DoE (Khadra et al., 2015) to the highest limit of fed DoE component concentrations (Zhou et al., 2017b). This full range DoE tested seven SIF components: FA, BS, pH, PL, buffer (using phosphate buffer instead of maleic acid), salt and MG, via a fractional DoE including 32 measurements made in duplicate, giving 64 values for the statistical analysis (Perrier et al., 2018). The values are presented in Table 1.5.

Table 1.5: Representation of the values used in the full range; fed and fasted DoE (Perrier et al., 2018).

Component	**Substance**	**Lower value (mM)**	**Upper value (mM)**
Bile salt	Sodium TC	1.5	24
Lecithin	Phosphatidylcholine	0.2	4.8
Buffer	Monophosphate buffer (KH_2PO_4)	15	45
Salt	NaCl	68	203
pH	NaOH/HCl	5	7
Fatty acid	Sodium oleate	0.5	52
Monoglyceride	GMO	0.5	6.5

TC: taurocholate, GMO: glyceryl mono-oleate.

The experiment was performed on nine BCS class II drugs: two acidic (indomethacin, and phenytoin), four basic (aprepitant, carvedilol, tadalafil, and zafirlukast), and three neutral drugs (felodipine, fenofibrate, and probucol). The full range DoE solubility results were within the fasted and fed DoE results (Khadra et al., 2015, Zhou et al.,

2017b), and literature FaHIF, and FeHIF solubility ranges (Augustijns et al., 2014). Each drug presented a different variability range, ranging from one to three orders of magnitude, as for the acidic drugs the solubility of indomethacin was highly variable, but not the phenytoin's solubility (Perrier et al., 2018). The reason behind this difference is due to the ionisation behaviour of each drug in the DoE, as the pKa of indomethacin and phenytoin is 4.6 and 8.3 respectively, and the DoE pH range was between 5 - 7, therefore, phenytoin was predominantly unionized, whereas indomethacin was predominantly ionized (Perrier et al., 2018). Another reason is the higher lipophilicity of indomethacin (log P 4.27) compared to phenytoin (log P 2.47), resulting in a higher interaction with the micellar components (Perrier et al., 2018). For the basic drugs, the solubility of carvedilol and tadalafil was comparable with fasted (Khadra et al., 2015), and fed (Zhou et al., 2017b) DoE solubility ranges, but not aprepitant and zafirlukast (Perrier et al., 2018). Zafirlukast had the highest solubility range (four magnitude orders), and the most homogenous distribution points (Perrier et al., 2018). This compound has the highest Log P value of 5.56, and a pKa of 4.3, which corresponds to the unionized form predominance over the DoE pH range (5 – 7) (Perrier et al., 2018). Carvedilol (pKa 7.8), and tadalafil (pKa 10) were both ionized, while aprepitant (pKa 2.8 (b), 9.7 (a)) acted as a neutral compound in the DoE pH range (5 – 7) (Perrier et al., 2018). The lipophilicity difference between aprepitant (Log P 4.2), and carvedilol (Log P 4.5), corresponds to the higher carvedilol solubility range (Perrier et al., 2018). Since the neutral drugs are not ionizable, lipophilicity was the major contributor to the drugs solubility (Perrier et al., 2018). Felodipine (log P 3.8) and fenofibrate (log P 5.2), both behaved similarly, whereas probucol (log P 10.9) showed low solubility results, which could be a result of the very high lipophilicity value which limited its solubility (Perrier et al., 2018). The full range DoE covered majority of the studied drugs' equilibrium solubility ranges, which indicated that the reduced experimental size DoE was enough to discover the solubility variability in SIF media (Perrier et al., 2018).

A statistical comparison was carried out to test the normality of the data distribution, and it showed a non-normal distribution which could be a result of the DoE structure, or that the drug solubility is actually not normally distributed (Perrier et al., 2018). This latter reason agrees with the FaHIF characterization study of BS and PL, which

found skewed concentration distribution results (Riethorst et al., 2016), also previously measured mean and media solubility differences in HIF studies, showed a non-normal distribution (Psachoulias et al., 2011). A Mann-Whitney test was therefore performed to evaluate the differences between the fasted and fed solubility distributions, which displayed a higher statistically significant solubility results in fed compared to the fasted DoE (Perrier et al., 2018). The same result was also found in previous studies (Augustijns et al., 2014, Bevernage et al., 2010, Clarysse et al., 2011). Six out of nine drugs of the full DoE were statistically equivalent compared to the combined fasted and fed DoE solubility results (Khadra et al., 2015, Zhou et al., 2017b), which both should represent the full solubility range if added together, but three drugs (aprepitant, tadalafil, felodipine) were significantly different (Perrier et al., 2018). This difference, in one third of the studied drugs, could be explained by the DoE approach, which samples a structured solubility space, rather than a random method, so the validity of the statistical comparison may fail (Perrier et al., 2018).

As an overall, all the groups of drugs in this full DoE resulted in the same highest significant factors (Perrier et al., 2018), along with the 2-way interactions found in fasted and fed DoE studies (Khadra et al., 2015, Zhou et al., 2017b). For the acidic drugs, the highest three significant factors were pH, FA, and BS (Perrier et al., 2018), which is the same result found in fasted and fed DoE (Khadra et al., 2015, Zhou et al., 2017b). For basic drugs, also three significant factors were observed: BS, pH, and FA, but not PL (Perrier et al., 2018), which was significant in fasted DoE (Khadra et al., 2015). For neutral drugs, only the solubility of felodipine and fenofibrate was influenced by FA and BS as the most affecting significant factors, while pH and PL had an effect on felodipine only (Perrier et al., 2018), which is the same result found in previous fasted and fed DoE (Khadra et al., 2015, Zhou et al., 2017b).

The 2-way interactions for the acidic drugs were between FA with pH (Perrier et al., 2018), as also seen in fasted and fed DoE (Khadra et al., 2015, Zhou et al., 2017b). Whereas, other significant interactions were seen in full DoE only, salt with MG, and only for indomethacin BS with three factors (FA, pH, and PL) (Perrier et al., 2018). The basic drugs (except zafirlukast) had two significant 2-way interactions, FA with pH found in aprepitant and carvedilol, which is the same result found in the fasted and fed DoE (Khadra et al., 2015, Zhou et al., 2017b). Whereas, BS with pH was a

significant effect in tadalafil for full DoE only (Perrier et al., 2018). Finally, the neutral drugs had two significant 2-way interactions mainly between FA with both pH and BS (Perrier et al., 2018).

1.6.5.4. Dual Range DoE

The dual range DoE included two studies for fasted and fed states, by testing seven factors affecting solubility: BS, PL, FA, MG, cholesterol, pH, and the ratio between BS and phospholipid (BS/PL). This was carried out by removing the salt and buffer components, which both showed no significant effects in previous DoE studies (Khadra et al., 2015, Perrier et al., 2018, Zhou et al., 2017b). As shown in Table 1.6, the lower and upper levels were constructed for the seven factors each for the fasted and fed states (Ainousah et al., 2017).

Table 1.6: Representation of the upper and lower values used in the dual range fed and fasted DoE (Ainousah et al., 2017).

Component	**Substance**	**Fasted**		**Fed**	
		Lower value (mM)	**Upper value (mM)**	**Lower value (mM)**	**Upper value (mM)**
Bile salt	Sodium TC	1.5	5.9	3.6	15
Lecithin	Phosphatidylcholine	0.2	0.75	0.5	3.75
Fatty acid	Sodium oleate	0.5	15	0.8	25
Monoglyceride	GMO	0.1	2.8	1	9
Cholesterol	Cholesterol	0.1	0.26	0.13	1
pH	NaOH/HCl	5	7	5	7
BS:PL ratio		7.5	7.9	7.2	4

TC: taurocholate, BS: bile salt, PL: phospholipid, GMO: glyceryl mono-oleate.

Two designs (8 samples each) were tested with lower and upper limits, and two centre points for each design, then both fasted and fed data were combined in a one DoE (Ainousah et al., 2017). In this study, the solubility measurements were carried out on nine drugs, two acidic (indomethacin and phenytoin), four basic (aprepitant,

carvedilol, tadalafil, and zafirlukast), and three neutral drugs (felodipine, fenofibrate, and probucol) (Ainousah et al., 2017).

The data of the dual level DoE were analysed for normality and found 9 data sets, out of 18 possibilities, to be normally distributed (Ainousah et al., 2017). This was not consistent with the previous DoE studies, which had a non-normal distribution of all the solubility data (Khadra et al., 2015, Perrier et al., 2018, Zhou et al., 2017b). The 9 normally distributed data were found in the fasted design for aprepitant, carvedilol, and tadalafil, and in the fed design for carvedilol, felodipine, phenytoin, probucol, tadalafil, and zafirlukast.

The results showed variability in solubility of each drug either in the fasted or fed designs, for example; the fasted design showed a lower solubility variability of tadalafil compared to fenofibrate (Ainousah et al., 2017). The effects of each individual component on the dual DoE (fasted and fed) were analysed, and 29 out of 126 effects determined to be significantly different (P value < 0.05), but interestingly, each drug was acting different from other drugs, some were highly affected by the factors like felodipine, while others were not affected at all, such as carvedilol and tadalafil (Ainousah et al., 2017). For the acidic drugs, the solubility of indomethacin was mostly affected by pH in fasted and fed dual designs (the same result found previously (Khadra et al., 2015, Perrier et al., 2018, Zhou et al., 2017b)), which is related to its pKa 4.6 (ionized in the DoE pH range 5 - 7) (Ainousah et al., 2017). For phenytoin (pKa 8.3 - unionized in the DoE pH range), its solubility was significantly affected in the fasted dual design only, first by pH, but in a negative direction, which could be correlated to the changes in the composition of the media (the cholesterol addition) (Ainousah et al., 2017). Second, also in a negative direction by cholesterol, which is a result not found before (Khadra et al., 2015, Perrier et al., 2018, Zhou et al., 2017b), third by FA and finally by BS/PL ratio (Ainousah et al., 2017). For the basic drugs, the fed state had no statistically significant factors affecting solubility, but in the fasted state, the solubility of zafirlukast had significant effects from pH, cholesterol, and MG, and the solubility of aprepitant was affected by FA, PL, and MG (Ainousah et al., 2017). The solubility of neither carvedilol or tadalafil was significantly affected by the media components, which was affected in previous DoE studies (Khadra et al., 2015, Perrier et al., 2018, Zhou et al., 2017b). This discrepancy

reflects a drug dependent behaviour (Ainousah et al., 2017). The positive effect of cholesterol on the solubility of zafirlukast, along with the negative MG effect, were not found in literature (Ainousah et al., 2017), but both components were not included in the fasted DoE protocols (Khadra et al., 2015, Perrier et al., 2018). Finally for the neutral drugs, more complex effects were observed, and the solubility of each drug was affected by multiple media components (Ainousah et al., 2017), which is in agreement with previous fasted and fed DoE studies results (Khadra et al., 2015, Zhou et al., 2017b). FA significantly affected the solubility of all 3 drugs (felodipine, fenofibrate, and probucol) in the fasted and fed dual states (Ainousah et al., 2017), which is the same result found in (Khadra et al., 2015) and (Zhou et al., 2017b). PL impacted felodipine and fenofibrate significantly in both fasted and fed states, whereas pH affected the fasted and fed states for fenofibrate, and only the fasted state of felodipine, and this solubility effect of pH was due to the indirect ionization effect to the media factors (Ainousah et al., 2017). The solubility of felodipine was also affected by MG in both fasted and fed dual DoE, which doesn't correlate to the results found in previous DoE studies, and by BS in fed state only, which was also affecting both felodipine and fenofibrate in previous fasted DoE (Khadra et al., 2015, Perrier et al., 2018, Zhou et al., 2017b). Probucol was affected by BS/PL ratio significantly, but without any effect from PL or pH which were significant in previous DoE studies (Ainousah et al., 2017).

For the components 2-way interactions, 6 out of 9 of the drugs showed no significant effect, and the rest of the three drugs, phenytoin (acidic), zafirlukast (basic), and probucol (neutral) were affected by all of the eight studied component interactions (accounting for around 32% of the effects possibilities) (Ainousah et al., 2017). This was found between BS with either pH, FA, PL, MG, cholesterol, or BS/PL, and between PL with either FA or MG (but the effect between PL with MG was not significant in probucol). This 32% significancy was relatively close to the previous percentage 33% (Khadra et al., 2015) and 28% (Zhou et al., 2017b), but not with the same components (Ainousah et al., 2017).

As an average of all the drug's effects (regardless of the direction), the acidic drugs in the dual DoE were affected by pH (Ainousah et al., 2017), which is the same result found in previous designs, but were previously also affected by FA, PL, and BS

components (Khadra et al., 2015, Perrier et al., 2018, Zhou et al., 2017b). For the basic drugs, FA was affecting both the fasted and fed dual DoE (Ainousah et al., 2017), the same result found in previous designs which were also affected by pH, PL, and BS (Khadra et al., 2015, Perrier et al., 2018, Zhou et al., 2017b). For the neutral drugs, fasted and fed designs were affected significantly by pH, FA, and PL, along with MG in both fasted and fed states, and only fed by FA, and PL (Ainousah et al., 2017). This was comparable with previous designs (Khadra et al., 2015, Perrier et al., 2018, Zhou et al., 2017b).

1.6.5.5. Small Scale DoE of Fasted and Fed

This DoE was carried out by minimising the number of experiments to be ten for fasted and nine for fed state, and reducing the studied components to four for the fasted media (BS, PL, FA, and pH), and five for the fed media (adding MG) (McPherson et al., 2020). The components which had no significant effects in the previous DoE such as salt and buffer were added in constant proportions (McPherson et al., 2020). Each component was studied in 3 levels: low, mid, and high depending on the previous DoE concentrations, see Table 1.7 (McPherson et al., 2020). The experiment was carried out on twelve poorly water soluble drugs four acidic (ibuprofen, indomethacin, valsartan, and zafirlukast), five basic (aprepitant, bromocriptine, carvedilol, and tadalafil), and three neutral drugs (felodipine, fenofibrate, and probucol) (McPherson et al., 2020).

Table 1.7: Upper and lower values used in the small range fasted and fed DoE (McPherson et al., 2020).

Component	**Fasted (mM)**						**Fed (mM)**				
	All low	**All high**	**Mid 1**	**Mid 2**	**FaSSIF-v1 with oleate**	**FaSSIF-v1**	**All low**	**All high**	**Mid 1**	**Mid 2**	**FeSSIF-v2**
Bile salt	1.5	5.9	1.5	5.9	3	3	3.6	24	3.6	15	10
Lecithin	0.2	1	0.75	0.75	0.75	0.75	0.5	4.8	2	2	2
Fatty acid	0.41	3.2	1.64	1.64	1.64	-	6.6	32.8	19.7	19.7	0.8
Monoglyceride	-	-	-	-	-	-	1	6.5	5	5	5
Buffer	Phosphate 28.4						Phosphate 45				Maleic acid (19)
Salt	NaCl 105.9						NaCl 125.5				
pH	5 and 7				6.5	6.5	5 and 7				5.8

The results were compared with previous fasted (Khadra et al., 2015), fed (Zhou et al., 2017b) DoE studies, and HIF results (Augustijns et al., 2014), and detected only a significant difference in 3 comparisons out of 20 (in fed state ibuprofen and probucol, and in fasted state tadalafil), resulting in a match of 85% of the results to have no significant difference (McPherson et al., 2020).

Upon statistical analysis, only few components revealed a significant effect on solubility, for acidic drugs: indomethacin was affected by pH in both fasted and fed states, while valsartan and zafirlukast were affected only in fasted state by pH. In addition, zafirlukast was also affected by FA, BS, and PL (McPherson et al., 2020). This major pH effect was also seen in previous fasted and fed DoE studies (Khadra et al., 2015, Zhou et al., 2017b), and as this was found in 3 out of 4 acidic drugs in fasted state, it was due to the ionisation impact of pH on solubility, along with the drug's pKa and the DoE pH range (McPherson et al., 2020). In the fed state, the higher amphiphile concentration overwhelmed the pH induced ionization (McPherson et al., 2020). For basic drugs, in the fasted state, tadalafil was only affected by pH, whereas carvedilol was affected by BS then pH, and this result matches the previous full fasted DoE (Khadra et al., 2015). In the fed state, no significant effects were found, and this may be due to the reduced number of experiments resulting in a lower statistical power (McPherson et al., 2020). For the neutral drugs, fenofibrate (fasted and fed) and felodipine (fasted) were affected by FA, also felodipine was affected by BS (fed), and probucol (fasted) by pH (McPherson et al., 2020), which were considered primary or secondary effects compared to the previous full fasted and fed DoE studies (Khadra et al., 2015, Zhou et al., 2017b).

Where available, a comparison between the reduced DoE (McPherson et al., 2020) and the full DoE (Khadra et al., 2015, Zhou et al., 2017b) found the following: in fasted state, a total of 11 significant effects was found in the reduced DoE compared to 25 significant effects in full DoE (McPherson et al., 2020). While in fed state, only 3 significant effects were found in the reduced DoE compared to 12 effects in the full DoE (McPherson et al., 2020). 9 out of 11 of the solubility effects in the reduced DoE resulted in a single factor effect with no 2-way interactions, while 18 out of 19 of the full DoE drugs effects resulted in multiple factor effects, with 2-way interactions (McPherson et al., 2020).

As an overall, this reduced DoE was helpful in defining the drug's solubility ranges, by a minimal matrix of solubility determinations (McPherson et al., 2020). Also, it helped with finding the most significant factors affecting the oral drugs solubility, but this had a statistical limitation due to the small samples number, and possibly due to the inherent drug physicochemical properties and behaviour (McPherson et al., 2020).

1.6.6. Multidimensional Analysis of HIF Composition

The DoE studies were very useful and highlighted a range of conclusions, for example the solubility should be studied as a range not a single point as in previous literature, also the key media components driving solubility were identified. Generally, a consistent drug solubility envelope was found, because of the similar concentrations of amphiphile components and pH used. Furthermore, solubility distributions in the DoE systems were not normal, indicating the complex solubility behaviours, along with the individual solubility behaviours, displayed by the drugs, which appear to be related to the drug's physicochemical properties and molecular structure. A reduced DoE was applied which confounded some 2-way interactions and could not detect higher interactions. Also, the reduction in the media number in the DoE, has reduced the ability to statistically determine significant factors, however it was easier to handle experimentally. The DoE, as a statistical construct, probably produces media system that are not biologically sensible, therefore, a small-scale bioequivalent system was required. This was presented by a recent publication which examined HIF composition using a multidimensional mathematical analysis that treated the fluid as a 5-dimensional system (Pyper et al., 2020).

Table 1.8: The original values of the 8 points + a centre point, which were applied in this fasted SIF media (Pyper et al., 2020)[1].

Media number	Bile Salt (mM)	Phospholipid (mM)	Free fatty acid (mM)	Cholesterol (mM)	pH
1	1.06	0.16	1.04	0.01	6.64
2	11.45	2.48	2.88	0.38	7.12
3	3.4	0.33	2.88	0.09	8.04
4	3.56	1.18	1.04	0.06	5.72
5	3.62	1.25	3.43	0.03	7.14
6	3.35	0.31	0.87	0.17	6.62
7	5.33	0.4	2.96	0.07	6.42
8	2.27	0.96	1.01	0.08	7.34
9	3.46	0.52	1.64	0.032	6.54

The matched data sets (fasted 152, and fed 172) of the 5 variables were visually plotted in 2D figures, where BS was chosen as a constant x-axis with the other 4 components on the y-axis (Pyper et al., 2020). The data showed an ellipsoid distribution, with a generally positive slope (Pyper et al., 2020). The mean of the ellipsoid shape was close to the higher concentration points, whereas the median and the Euclidean centre points where close to the centre of the distribution (Pyper et al., 2020). The literature fasted and fed solubility results (HIF, SIF, and DoE) were also plotted on the four 2D figures, to observe their distribution and relevance to the solubility results (Pyper et al., 2020). Despite the variable aspiration techniques of literature HIF samples (due to the pooled sampling procedures, and varying protocols, such as the variable food type taken before sampling, and the pre-water administration), the HIF data fitted in the distribution cloud (Pyper et al., 2020). For literature SIF recipes, as an overall, there was a good agreement between the central distribution values with the fasted data set, and all the recipes were inside the solubility cloud, but the central distribution values of the fed state SIF indicated the possibility to refine the SIF recipes to match the multidimensional concentrations (Pyper et al., 2020). The DoE protocols were in part

[1] Values presented are copied directly from original literature.

affected by the limitations of the HIF pooled samples, as some data points were within the distribution space, such as in PL versus BS in the fasted state, but other comparisons especially in the fed state were totally outside the cloud distribution, which correspond to the urge to refine the previous DoE protocols concentrations for a better coverage (Pyper et al., 2020).

This multidimensional analysis of HIF identified 8 bioequivalent media compositions that statistically characterised the variation within the sample set in the fasted (96%) and fed (98%) states. In addition, a centre point was identified in each state using a Euclidean approach in 5-dimensional space, rather than the mean (or similar) value for each component since the component distributions were not normal. The 8 points (with a centre point) will be used and applied in the following chapters to find a better linkage between the *in vitro* measurements and the *in vivo* data (Pyper et al., 2020).

1.7. Aims and Objectives of this Research

The aim of this project is to determine the equilibrium solubility of twenty-one drugs using the multidimensional analysis of fasted HIF compositions (Pyper et al., 2020). The drugs are acidic (furosemide, ibuprofen, indomethacin, mefenamic acid, naproxen, phenytoin, piroxicam, and zafirlukast), basic (aprepitant, atazanavir, carvedilol, dipyridamole, posaconazole, and tadalafil), and neutral (acyclovir, carbamazepine felodipine, fenofibrate, griseofulvin, paracetamol, and probucol), where twelve of them were investigated in the original fasted DoE study (Khadra et al., 2015). The equilibrium solubility data and the drug's significant effects will be compared, to the original fasted DoE (Khadra et al., 2015) and where appropriate, to the reduced experiment fasted DoE distributions (Ainousah et al., 2017, McPherson et al., 2020), and literature fasted HIF and SIF values. This comparison allows to investigate the solubility of drugs in different fasted simulated intestinal fluid systems, as presented in Chapter 2.

In addition, to apply the fasted equilibrium solubility of the twenty-one drugs determined in the nine media recipes, along with a value in simulated fasted simulated intestinal fluid version 1 (FaSSIF-v1), to the original DCS grid and its associated calculations (Butler and Dressman, 2010). This will predict the 21 drugs' absorption

potential and provide the limits for the likely *in vivo* solubility behaviour. Also, to determine a solubility frequency distribution within those limits, to assess solubility behaviour across the population range, based on the twenty volunteers sampled in the original study (Riethorst et al., 2016), as presented in Chapter 3.

This project also aims to investigate the solubility behaviour of the nine fasted media recipes, to determine if this is consistent between the drugs and drug categories (Chapter 3) and examine possible correlations between the *in vitro* measurements and the *in vivo* data, by finding the solubility boundaries, as presented in Chapter 4.

An automated system will be introduced to measure the supersaturation (SS) concentration, time needed to induce SS, and where possible, the precipitation rate, of ten selected drugs. This will be carried out using different fasted SIF media recipes and different path lengths, using the inForm® instrument. Moreover, to compare the resulting SS measurements with available literature findings, as presented in Chapter 5.

The last part of this project is to predict the pharmacokinetic (PK) parameters and the fraction absorbed, of selected six poorly soluble drugs, using *in silico* modeling approach (GastroPlus® software) with the equilibrium solubility data. Furthermore, to evaluate the predictions of the simulated models with literature *in vivo* observed data, and other *in silico* simulated models, as presented in Chapter 6.

To be noted that four research papers were published by the results found in this research work (Abuhassan et al., 2021, Abuhassan et al., 2022a, Abuhassan et al., 2022b, Abuhassan et al., 2024), see appendix for details.

Chapter 2: Small Scale *in vitro* Method to Determine a Bioequivalent Equilibrium Solubility Range for Fasted Human Intestinal Fluid

2.1. Introduction

Oral biopharmaceutical studies are critical for pharmaceutical companies during drug development (Moscicki and Tandon, 2017). Drug solubility is one of the most important biopharmaceutical parameters which is influenced by the gastrointestinal tract (GIT) environment in addition to the drug's chemical properties (Khadra et al., 2015). The GIT, where solid oral drugs dissolve, is a heterogeneous environment with variable components, such as enzymes, electrolytes, bile salt (BS), proteins, and many others (Washington et al., 2000). Through the years, multiple research groups have studied the effect of such intestinal components on the oral drugs solubility, by either aspirating human intestinal fluid (HIF) samples, or preparing *in vitro* simulated intestinal fluid (SIF) media, depending on the HIF data (Bergström et al., 2014, Clarysse et al., 2011, Lindahl et al., 1997). The HIF data was important as a 'gold' standard, for checking the SIF solubility results (Dahlgren et al., 2021). The issues found with HIF solubility results are that HIF protocols used different sampling techniques, and others used variable volunteer ages and numbers, along with the high variability and difficulty of HIF aspiration, which requires correct intubating and locating of the catheter's position, and then collecting the HIF samples (Bergström et al., 2014, de la Cruz-Moreno et al., 2017). This led to the development of multiple SIF media to study the drug solubility, but most were based on different readings of the HIF measurements, and the usage of variable GIT component concentrations (Bou-Chacra et al., 2017). This provides variability in the solubility value determined by the different SIF media, and presents an additional question of which media recipe is more appropriate (Fuchs et al., 2015).

As part of the EU IMI Oral Biopharmaceutical Tools (OrBiTo) research program (Lennernäs et al., 2014) this group conducted a design of experiment (DoE) study into equilibrium solubility in simulated fasted media (Khadra et al., 2015), which aims to statistically determine solubility variation, using conditions that are hypothesized to reflect the component variation within the experimental system or simulated fluid.

Statistically, this links a high concentration value of one component with a low concentration value of another, a combination in the SIF system (for example BS with phospholipid (PL)) that may not arise in *in vivo* HIF and therefore be bioequivalent. DoE approaches therefore do not have a direct relationship to HIF.

To address the issues found with SIF and DoE approaches, a recent publication has examined HIF composition using a multidimensional mathematical analysis, that treated the fluid as a 5-dimensional system, consisting of the following components, pH, BS, PL, fatty acid (FA), and cholesterol (Pyper et al., 2020). This statistical study relied on a previous study which measured the HIF component concentrations in twenty healthy volunteers, 10 males and 10 females, under consistent conditions (Riethorst et al., 2016). The age of the individuals was between 18 - 31 years, BMI between 19 - 25 Kg/m^2, and all were overnight fasted for more than 12 hours prior to sampling (Riethorst et al., 2016). All the individuals were given 250 mL of water before the samples were aspirated every 10 minutes, for 90 minutes (Riethorst et al., 2016). This multidimensional analysis utilized a data set of fasted HIF (FaHIF) samples and identified eight Fa9SIF points, which statistically covered more than 96% of the HIF composition variability, plus a centre point, which could enable results to better correlate *in vitro* data to the *in vivo* environment (Pyper et al., 2020). To achieve the multidimensional analysis, the measured concentrations of components were summed and treated as a single variable, for example, six BS species were analysed but only a single concentration was calculated (Table 2.2). This simplification applies to BS, PL, and FA where in HIF multiple species will be present. This is a situation also applicable to SIF, and for BS it is known that the concentration has a greater influence on solubilization than species (Zughaid et al., 2012). However, it does represent an ever-present challenge between simulation by simplification and the native fluid. The chemical structures of the components used in this research are presented in Figure 2.1.

In this protocol, the BS used as a representative of the bile salts analysed (Pyper et al., 2020) is sodium taurocholate, with a molecular weight of 537.7 g/mole, consisting of sodium, taurine, and cholic acid. Bile acids are synthesized by the liver from cholesterol, and it's responsible for the digestion process in the small intestine, especially the solubilization of dietary lipids, and the digestion of solubilized fat

nutrients (Lennarz and Lane, 2013). Second, is cholesterol which is a product of animal metabolic processes, it's found in the plasma lipid bilayer membrane, and responsible for different structural activities, mainly the plasma membrane's physical integrity (Paukner et al., 2022). It is synthesized in the liver, and eliminated from the body mostly by its conversion to bile (Engelking, 2010). Third is PL, which is a lipid mixture, one of its types is phosphatidylcholine (PC) (lecithin) (Caballero et al., 2003), which is used in this fasted protocol. Lecithin's role is mainly used for the metabolism and protection of cells from pathogens or chemicals (Caballero et al., 2003), and it has amphipathic properties, with a hydrophilic head, and a hydrophobic tail (Narváez-Rivas and León-Camacho, 2016). Lastly, FA is represented by sodium oleate, which is a monounsaturated free fatty acid with a long chain of 18 carbons, and by the interaction with Na, its molecular formula and molecular weight are, $C_{18}H_{33}NaO_2$ and 304.4 g/mole (PubChem). Na oleate is the salt form of oleic acid, which can be ionized, and the Na is linked at the position of the deprotonated oxygen, as shown in Figure 2.2 below, which gives an alkaline pH in aqueous media (Windholz et al., 1976).

This research tested the eight points, plus a centre point, using previous laboratory techniques used with the DoE studies (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020), to study the effects of the five intestinal components on the solubility of twenty-one Biopharmaceutics Classification System (BCS) class II and IV drugs mainly. Acidic drugs (furosemide, ibuprofen, indomethacin, mefenamic acid, naproxen, phenytoin, piroxicam, and zafirlukast). Basic drugs (aprepitant, atazanavir, carvedilol, dipyridamole, posaconazole, and tadalafil). Neutral drugs (acyclovir, carbamazepine felodipine, fenofibrate, griseofulvin, paracetamol, and probucol). See Table 2.1 for the details of each drug's physicochemical properties. Salt and buffer were also added, but in constant amounts due to its minor statistical impact on solubility found in the DoE studies (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020, Zhou et al., 2017b).

In this research, wherever the SIF abbreviation is used, it will be representative of the literature simulated intestinal fluid, Fa9SIF is the fasted media used in this research study, and HIF is the human intestinal fluid. Whereas the FaSSIF-v1 represents the purchased material from Biorelevant.com, which is the Fasted Simulated Small Intestinal Fluid version 1, also this could be abbreviated as FaSSIF, which will

represent the two tested samples of FaSSIF-v1, and FaSSIF-v1 with Na oleate. The solubility term used in the context is referred to the equilibrium solubility.

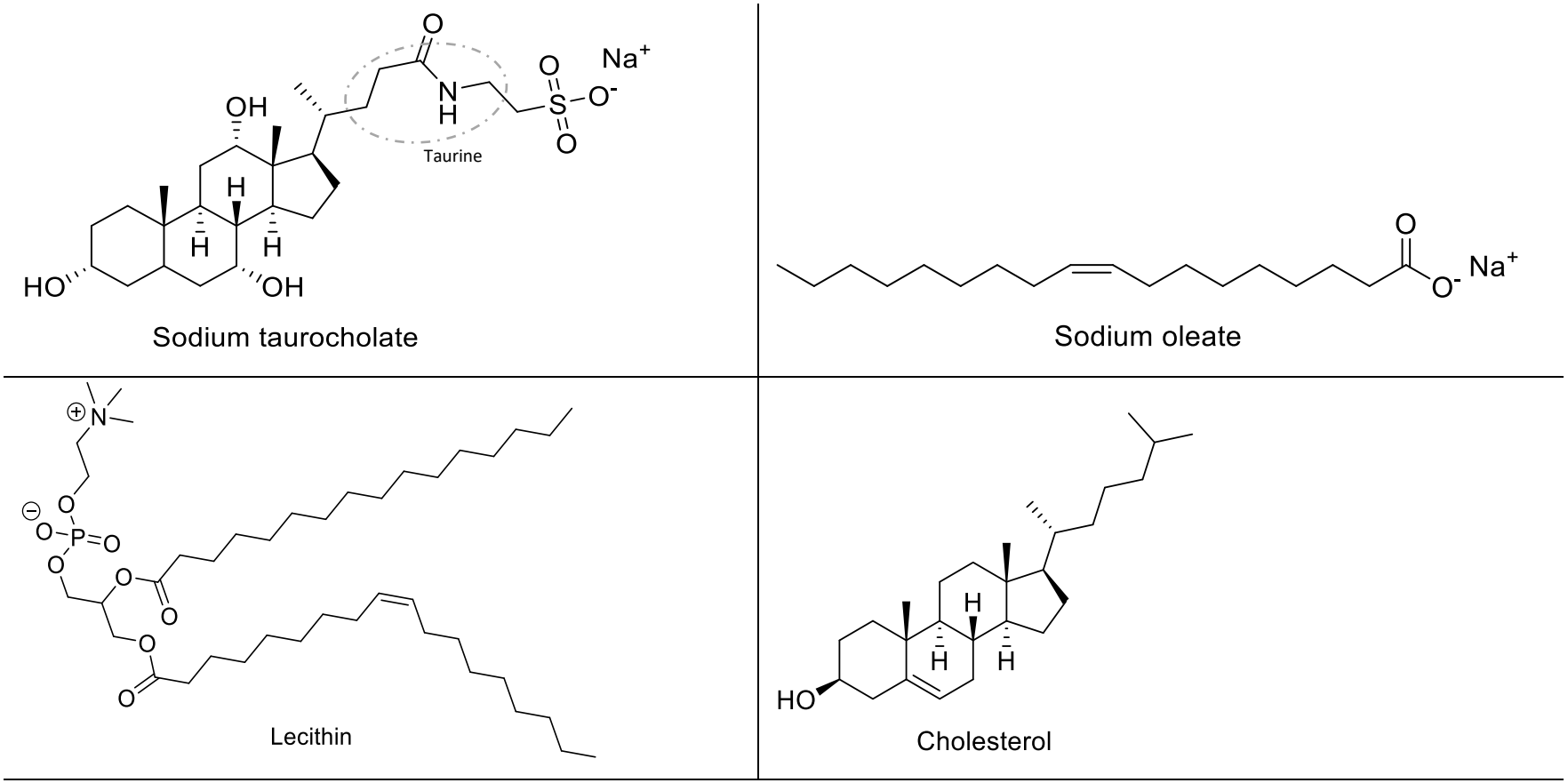
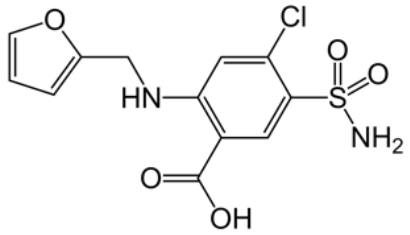
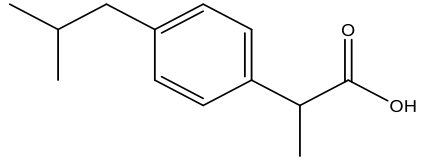
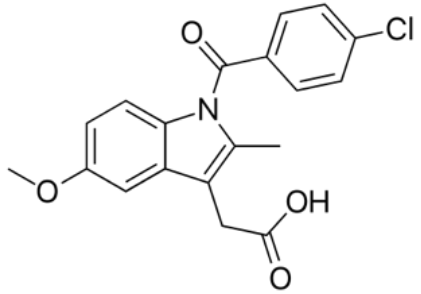
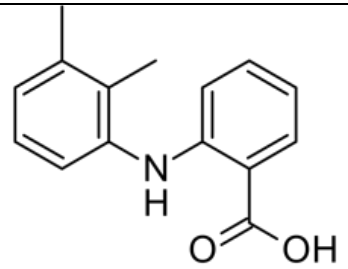
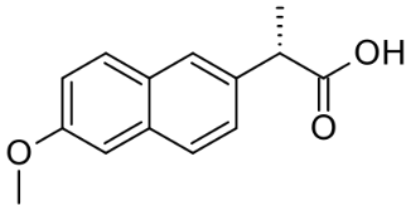
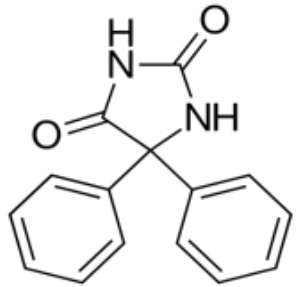
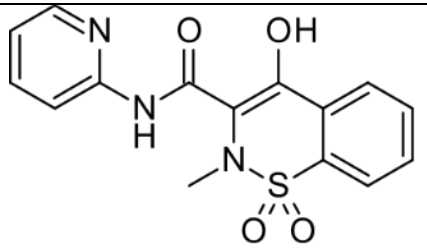
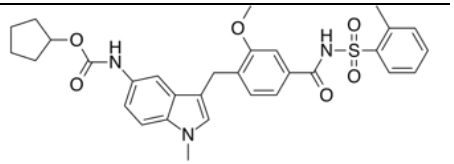
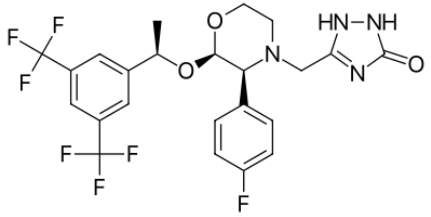


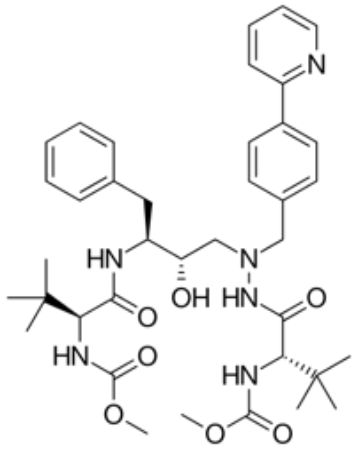
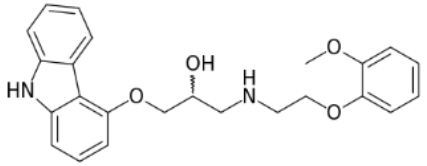
Figure 2.1: The chemical structures of the four components used in this Fa9SIF study.

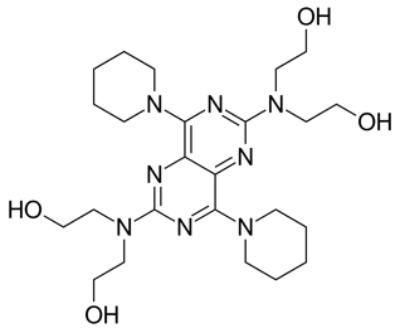
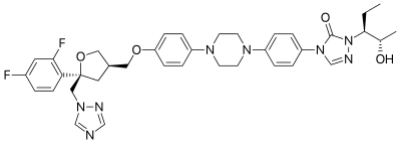
Table 2.1: Summary of the basic physicochemical properties of the acidic, basic, and neutral drugs used in this Fa9SIF study.

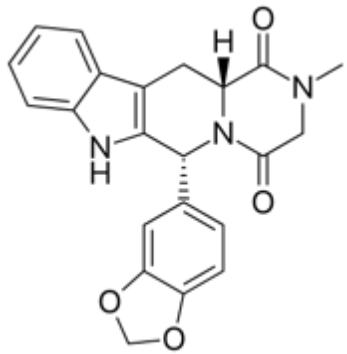
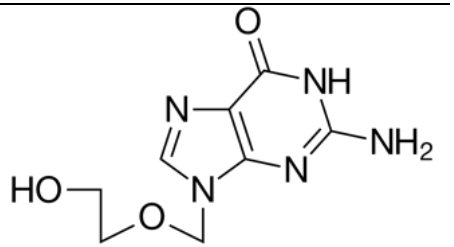
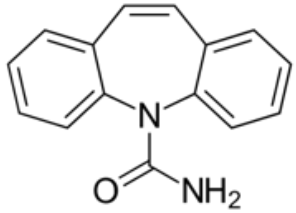
Drug	**a/b/n**	**pKa**	**Log P**	**Molecular weight (g/mole)**	**Molecular formula**	**Medical usage**	**Structure**
Furosemide	a[4]	3.9[4]	2.03[18]	331	$C_{12}H_{11}ClN_2O_5S$	Diuretic for congestive heart failure	
Ibuprofen	a[1]	5.3[2]	3.97[18]	206	$C_{13}H_{18}O_2$	Anti-inflammatory, analgesic	
Indomethacin	a[3]	4.6[3]	4.27[18]	358	$C_{19}H_{16}ClNO_4$	Anti-inflammatory	

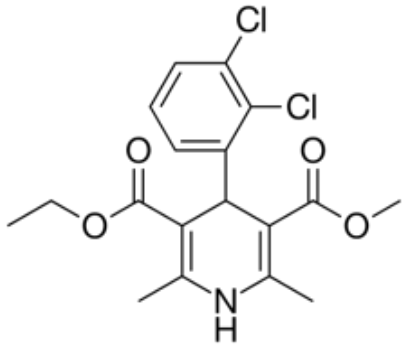
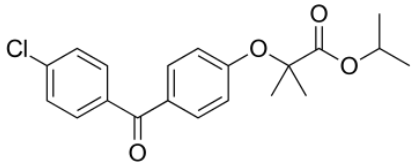
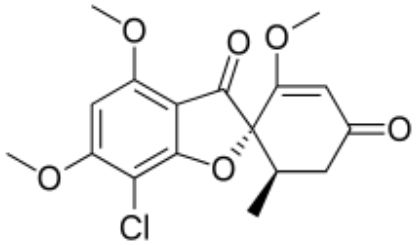
Drug	a/b/n	pKa	Log P	Molecular weight (g/mole)	Molecular formula	Medical usage	Structure
Mefenamic acid	a[5]	4.2[5]	5.12[18]	241	$C_{15}H_{15}NO_2$	Anti-inflammatory, antipyretic and analgesic	
Naproxen	a[6]	4.2[6]	3.18[18]	230	$C_{14}H_{14}O_3$	Anti-inflammatory, analgesic	
Phenytoin	a[7]	8.3[7]	2.47[18]	252	$C_{15}H_{12}N_2O_2$	Antiepileptic, anticonvulsant	

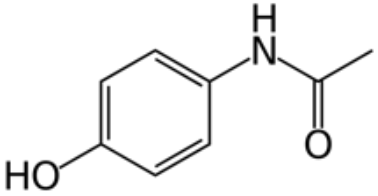
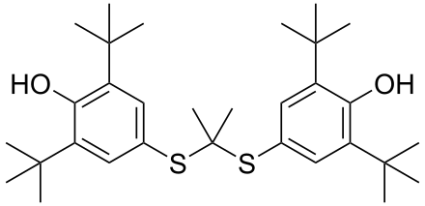
Drug	a/b/n	pKa	Log P	Molecular weight (g/mole)	Molecular formula	Medical usage	Structure
Piroxicam	a[1]	6.4[8]	3.06[18]	331	$C_{15}H_{13}N_3O_4S$	Anti-inflammatory, antipyretic and analgesic	
Zafirlukast	a[9]	4.1[9]	5.56[24]	576	$C_{31}H_{33}N_3O_6S$	Anti-asthmatic	
Aprepitant	b[10]	9.7[10]	4.5[19]	534	$C_{23}H_{21}F_7N_4O_3$	Antiemetic	

Drug	a/b/n	pKa	Log P	Molecular weight (g/mole)	Molecular formula	Medical usage	Structure
Atazanavir	b[11]	4.5[11]	5.9[22]	705	$C_{38}H_{52}N_6O_7$	Treatment and prevention of HIV-1 infection and AIDS	
Carvedilol	b[1]	8.0[12]	4.19[18]	406	$C_{24}H_{26}N_2O_4$	Antihypertensive	

Drug	a/b/n	pKa	Log P	Molecular weight (g/mole)	Molecular formula	Medical usage	Structure
Dipyridamole	b[1]	6.4[12]	3.95[21]	505	$C_{24}H_{40}N_8O_4$	Antiplatelet	
Posaconazole	b[13]	3.6, 4.6[13]	4.6[20]	701	$C_{37}H_{42}F_2N_8O_4$	Antifungal	

Drug	a/b/n	pKa	Log P	Molecular weight (g/mole)	Molecular formula	Medical usage	Structure
Tadalafil	b[14]	3.5[15]	1.70[19]	389	$C_{22}H_{19}N_3O_4$	Vasodilatory activity	
Acyclovir	n[16]	-	-1.56[18]	225	$C_8H_{11}N_5O_3$	Antiviral agent	
Carbamazepine	n[4]	-	2.45[18]	236	$C_{15}H_{12}N_2O$	Anticonvulsant and analgesic	

Drug	a/b/n	pKa	Log P	Molecular weight (g/mole)	Molecular formula	Medical usage	Structure
Felodipine	n^{1}	-	3.86[18]	384	$C_{18}H_{19}Cl_2NO_4$	Antihypertensive agent	
Fenofibrate	n^{4}	-	5.3[23]	361	$C_{20}H_{21}ClO_4$	Antihyperlipidemic	
Griseofulvin	n^{1}	-	2.18[18]	353	$C_{17}H_{17}ClO_6$	Fungistatic agent	

Drug	a/b/n	pKa	Log P	Molecular weight (g/mole)	Molecular formula	Medical usage	Structure
Paracetamol	n[17]	-	0.46[23]	151	$C_8H_9NO_2$	Analgesic and antipyretic	
Probucol	n[1]	-	11.3[19]	517	$C_{31}H_{48}O_2S_2$	Antilipidemic activity	

RT; room temperature. a/b/n: acid/base/neutral. Storage depends on each drug company's storage conditions.

References: 1- (Söderlind et al., 2010) 2- (Oh et al., 2016) 3- (Annaert et al., 2010) 4- (Clarysse et al., 2011) 5- (Prasad et al., 2022) 6- (Fillet et al., 1998) 7- (Hassel, 1981) 8- (Khadra et al., 2015) 9- (McPherson et al., 2020) 10- (Liu et al., 2015) 11- (Indulkar et al., 2015) 12- (Bergström et al., 2004) 13- (de Alencar Danda et al., 2019) 14- (Mohamad et al., 2022) 15- (Polat et al., 2019) 16- (Wang et al., 2015) 17- (Mechnou et al., 2022) 18- (Benet et al., 2011) 19- (Perrier, 2019) 20- (Hens and Bolger, 2019) 21- (Girdhar et al., 2018) 22- Chemaxon 23- PubChem 24- (Zhou et al., 2017a).

2.2. Aims and Objectives

- Measure the fasted intestinal equilibrium solubility using more relevant simulated fasted intestinal media recipes derived from a multidimensional analysis of FaHIF.
- Check statistical differences between current solubility results and literature solubility results, along with the distribution ranges, to link with *in vivo* data and have more reliable correlations.
- Study the media components' significant effect on drug solubility, to enable any SIF media composition refinement.
- Study the importance and effect of changing the SIF components composition.

2.3. Materials and Methods

2.3.1. Materials

Sodium taurocholate, cholesterol, sodium chloride (NaCl), sodium oleate, ammonium formate, potassium hydroxide (KOH), hydrochloric acid (HCl), acyclovir, carbamazepine, carvedilol, dipyridamole, fenofibrate, furosemide, griseofulvin, indomethacin, mefenamic acid, naproxen, phenytoin, piroxicam, probucol, and tadalafil were purchased from Merck Chemicals Ltd, Germany. Aprepitant and felodipine were previously provided through the OrBiTo by Dr. Holm, Head of Pre-formulation, Lundbeck, Denmark. Zafirlukast was purchased from Stratech Scientific Limited, UK. Ibuprofen from BASF chemical company. Paracetamol was provided by Mallinckrodt Pharmaceuticals, Ireland. Atazanavir and posaconazole from ChemShuttle, USA. Phosphatidylcholine from soybean (PC S) was purchased from Lipoid, Germany. Chloroform and formic acid from Rathburn chemical company, UK. FaSSIF-v1 media was purchased from Biorelevant.com, UK. Sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$) was purchased from Fisher Scientific, Germany. All acetonitrile (ACN) and methanol (MeOH) solvents were high-pressure liquid chromatography (HPLC) gradient. All water is ultrapure Milli-Q water.

2.3.2. Methods

2.3.2.1. Samples Preparation

As most of the component's concentrations are below the limits of measuring using an analytical balance, BS, PL, and FA original concentrations were multiplied by 15. While cholesterol concentration was multiplied by 1500 times to provide a stock solution. All the concentrations used were identical to the FaHIF statistical analysis concentrations (Pyper et al., 2020), as in Table 2.2.

Table 2.2: Fasted concentration values of the 8 points + a centre point (Pyper et al., 2020), which were applied in this Fa9SIF study.[2]

Media number	Bile Salt (mM)	Phospholipid (mM)	Fatty acid (mM)	Cholesterol (mM)	pH	pH*TAC (mM)
1	1.06	0.16	1.04	0.01	6.64	15.07
2	11.45	2.48	2.88	0.38	7.12	31.11
3	3.4	0.33	2.88	0.09	8.04	31.71
4	3.56	1.18	1.04	0.06	5.72	33.40
5	3.62	1.25	3.43	0.03	7.14	36.96
6	3.35	0.31	0.87	0.17	6.62	53.87
7	5.33	0.4	2.96	0.07	6.42	56.24
8	2.27	0.96	1.01	0.08	7.34	59.48
9	3.46	0.52	1.64	0.032	6.54	122.4

TAC: total amphiphilic concentration.

The desired quantities of sodium taurocholate, lecithin PC S, and sodium oleate were weighed into 9 beakers, labelled as stock A (1 - 9), and dissolved in 3 mL chloroform. Stock B was prepared by adding the cholesterol to 10 mL of chloroform, in another 9 beakers labelled as stock B (1 - 9). 100 µL of each of stock B (1 - 9) were transferred into each of stock A (1 - 9), followed by evaporating the chloroform, using a nitrogen gas source. The dried lipids were re-suspended with water and made up to 5 mL in volumetric flasks. Two pre-stocks of phosphate buffer ($NaH_2PO_4.H_2O$) of a concentration of 28.4 mM, and salt (NaCl) of concentration 105.9 mM were prepared

[2] Values presented are copied directly from original literature.

in two 5 mL volumetric flasks, the concentrations were taken from (McPherson et al., 2020). The final media samples were prepared in nine centrifuge tubes (final media volume 4 mL), to reach the desired concentrations mentioned in Table 2.2. Each tube contained five substances: an addition of 267 µL of each stock solution (containing the four components), two additions of 267 µL salt and phosphate buffer stock solutions, an excess of a solid drug, and made to 4 mL with water (3199 µL). Finally, the fifth component was measured by adjusting the pH of each tube, as shown in Table 2.2, using 1 M KOH and 1 M HCl. The pH was adjusted with a ±0.05, and the KOH/HCl volume not exceeding 1% of the total sample volume. The 9 tubes were placed on the shaker for 1 hour at room temperature. A further pH adjustment was conducted after one hour to make sure pH isn't changed, then incubated in the 37 °C room for 24 hours on the orbital shaker. After incubation, the tubes were checked for the presence of a solid drug, a 1 mL sample was extracted into a 1.5 mL Eppendorf tube and then centrifuged for 15 minutes, at 10000 rpm. 0.5 mL of the supernatant was transferred to an HPLC vial, for concentration determination using the Shimadzu Prominence LC-2030C HPLC.

2.3.2.2. Fasted Simulated Small Intestine Fluid (FaSSIF) Media

The FaSSIF-v1 media from Biorelevant.com contains BS and lecithin with a small proportion of salt and buffer. Also, another sample was prepared, by adding Na oleate to the FaSSIF-v1 prepared sample, the desired concentrations needed for this Fa9SIF study are shown in Table 2.3 (McPherson et al., 2020).

Table 2.3: Fasted media compositions and concentrations (McPherson et al., 2020).

Media number	Composition	Bile salt (mM)	Phospholipid (mM)	Fatty acid (mM)	pH
10	FaSSIF-v1	3	0.75	-	6.5
11	FaSSIF-v1 + Na oleate	3	0.75	1.64	6.5

Preparation was according to a published literature method (McPherson et al., 2020). Buffer ($NaH_2PO_4.H_2O$) and salt (NaCl) stocks were prepared, by dissolving 40 and 60

mg of each respectively with water, in a 10 mL volumetric flask, and adjusting to pH 6.5. 20mg of FaSSIF-v1 media powder was added and mixed until completely dissolved then adjusted to the final volume with water. 4 mL of this stock (FaSSIF-v1) was added to a centrifuge tube containing an excess of a solid drug, pH was adjusted to 6.5, and labelled as sample 10. For the preparation of sample 11, 3863 µL of FaSSIF-v1 stock was added to a centrifuge tube containing an excess of a solid drug, with 137 µL of sodium oleate stock solution, and a final step was pH adjustment to 6.5. Note: Na oleate was prepared by dissolving 73 mg of Na oleate with water, into a 5 mL volumetric flask, heat was required to aid solubilization. Both media 10 and 11 were handled the same as the previous samples from 1 – 9, by being shaken for 1 hour, pH adjusted, then both incubated in the 37 °C room for 24 hours on an orbital shaker, centrifuged, and analysed by the Shimadzu HPLC instrument.

2.3.2.3. HPLC Conditions

The analysis of the 11 samples was performed by applying a gradient elution for all the drugs (except probucol), following literature protocols (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020) starting with the two-mobile phases' (MP) preparation:

a- MP A, is the aqueous MP which consisted of 100% water, with 10 mM ammonium formate, and adjusted to pH 3 using formic acid.

(Note: formic acid was added in less than 0.002% of the mobile phase volume)

b- MP B, is the organic MP which consisted of acetonitrile (ACN) and water as 9:1 proportion (900 mL of ACN and 100 mL water), with 10 mM ammonium formate concentration.

The liquid chromatography elution gradient is in Table 2.4.

Table 2.4: The liquid chromatography timetable run.

Time (minutes)	Percentage of MP B (%)
0	30
3	100
4	100
4.5	30

The HPLC method of probucol was utilized by a previous isocratic method, of 45:45:10 ACN, MeOH, and water (Khadra et al., 2015). The column used for probucol, acyclovir, furosemide, and dipyridamole was Speck and Burke, ODS-H optimal 5 µm (30 x 150 mm), and for paracetamol was Kromasil 60-5-SIL (3mm, 15cm). For the rest of the drugs, Xbridge® C18 5 µm, dimensions 2.1 x 50 mm was used. The run time for each sample was 8 minutes, the column temperature used was 30 °C, the maximum pressure was 4000 psi, and the flow rate for all the drugs was 1 mL/min, except for carvedilol 0.7 mL/min, and acyclovir and carbamazepine 0.5 min/mL. See Table 2.5 below for more details on the HPLC conditions used.

Table 2.5: The HPLC conditions used in this Fa9SIF media.

	Drug	Injection volume (µL)	Retention time (minute)	Wavelength (nm)	Calibration curve range (mg/mL)	R^2
1	Furosemide	10	2.5	291	0.07-2	0.99
2	Ibuprofen	100	2	254	1-7	0.99
3	Indomethacin	10	2.1	254	0.01-0.3	0.99
4	Mefenamic acid	10	2.3	291	0.008-0.6	0.99
5	Naproxen	10	1.6	254	0.03-1	1
6	Phenytoin	20	1.1	254	0.01-0.09	0.99
7	Piroxicam	10	1.1	254	0.04-0.8	0.99
8	Zafirlukast	25	2.6	254	0.001-0.025	0.99
9	Aprepitant	50	2.3	254	0.004-0.09	0.99
10	Atazanavir	10	1.7	254	0.0008-0.008	0.99
11	Carvedilol	10	1.6	254	0.04-0.4	0.99
12	Dipyridamole	10	2.5	291	0.007-0.07	0.99
13	Posaconazole	10	1.9	254	0.0008-0.008	0.99
14	Tadalafil	50	1.4	291	0.001-0.02	0.99
15	Acyclovir	10	1.5	254	0.24-0.3	0.99
16	Carbamazepine	10	1.9	291	0.09-0.25	0.99
17	Felodipine	10	2.4	254	0.004-0.3	0.99
18	Fenofibrate	10	3	291	0.002-0.06	0.99
19	Griseofulvin	10	1.5	291	0.008-0.04	0.99
20	Paracetamol	10	1.1	254	0.045-0.058	0.99
21	Probucol	100	4.9	220	0.0006-0.02	0.99

2.3.2.4. Calibration Curve

Five or six concentration standard points were prepared covering the samples AUC range, above, below, and in-between, to make a calibration curve ($R^2 > 0.99$), to allow calculation of the samples' concentrations, see Table 2.5.

2.3.2.5. Statistical Analysis

Each solubility experiment was performed as a triplicate, and the mean was calculated and processed using Microsoft Excel. For more detailed analysis, two programs were used, Minitab®18, and Graph Pad Prism® 5, using Windows 11. The Minitab software analysed a custom factorial design of experiment, for the 5 components concentrations, mentioned in Table 2.2. Whereas, Graph Pad Prism software, used non-parametric Kruskal-Wallis test with Dunn's multiple comparison correction, or Mann-Whitney test (for drugs with less than three comparison groups), where only results of P value < 0.05 were considered significant. For further details please refer to (Abuhassan et al., 2021).

2.4. Results and Discussion

2.4.1. Equilibrium Solubility Findings and Comparison

The equilibrium solubility ranges resulted from the eight points and a centre point were plotted and compared, where available, with previous fasted DoE studies: DoE 66 points (Khadra et al., 2015), DoE 10 points (Ainousah et al., 2017), and DoE 9 points (McPherson et al., 2020). Figures 2.2, 2.3, and 2.4 present the solubility ranges of acidic, basic, and neutral drugs respectively. Literature values of FaHIF and SIF solubility results (Augustijns et al., 2014), plus two FaSSIF values from this study, were also plotted for visual comparison but are not included in the statistical analysis (only three or more HIF or SIF values were statistically analysed, and all were not significantly different (results not presented)).

A statistical comparison of the Fa9SIF equilibrium solubility and the other DoE results carried out for 31 possible cases, resulted in a statistically equivalent relationship for twenty-six of the cases, 84%. This corresponds that the Fa9SIF study is measuring the same solubility ranges as previous DoE studies (Ainousah et al., 2017, Khadra et al.,

2015, McPherson et al., 2020). For 12 out of 13 of the studied drugs, the FaSSIF two values; sample 10 and 11 of this Fa9SIF study, were both in the same range as previous studies (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020), and found to be close to the centred solubility; sample 9.

Literature FaHIF solubility data were available for 10 drugs, and in 80% of the cases, the HIF solubility lay within the Fa9SIF envelope, the exceptions were carvedilol and probucol. Whereas 9 drugs had 12 literature fasted SIF data, only 1 SIF solubility point for griseofulvin wasn't in the range of the Fa9SIF study, which counts for over 90% of the data. This comparison shows some errors due to different protocols used in the previous FaHIF/SIF studies (Augustijns et al., 2014). As an overall, the previous HIF and SIF data were comparable and with a good agreement with this novel five-dimensional Fa9SIF study, but all imply that the variable nature of HIF composition needs a range of data points to test a drug's solubility, not a single point.

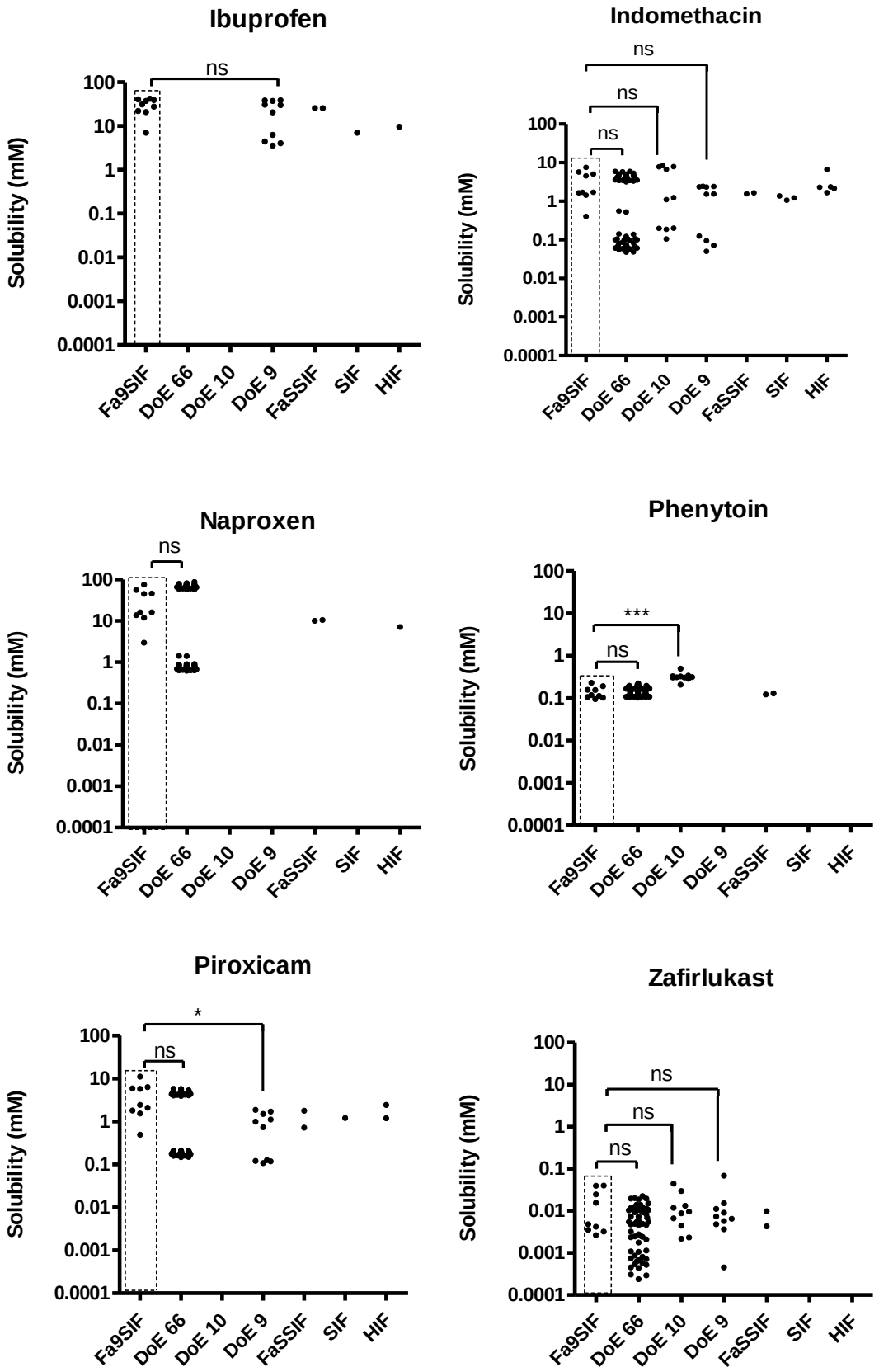

Ibuprofen
Indomethacin
Naproxen
Phenytoin
Piroxicam
Zafirlukast
Solubility (mM)
100
10
1
0.1
0.01
0.001
0.0001
ns
*
***
Fa9SIF
DoE 66
DoE 10
DoE 9
FaSSIF
SIF
HIF

Figure 2.2: Acidic drugs measured equilibrium solubility comparison of Fa9SIF– this study, DoE 66 (Khadra et al., 2015), DoE 10 (Ainousah et al., 2017), and DoE 9 (McPherson et al., 2020). The FaSSIF points are points 10 and 11 analysed in this Fa9SIF media. Literature fasted HIF (human intestinal fluid) and SIF (simulated intestinal fluid) (Augustijns et al., 2014). ns: non-significant. * $p = 0.0172$; *** $p = 0.0003$.

Four out of six acidic drugs of this Fa9SIF9 study were not significantly different compared to the literature fasted DoE results (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020). Exceptions are phenytoin (pKa 8.3) and piroxicam (pKa 6.4), which were respectively unionized and partially ionized compared to the other totally ionized acidic drugs studied in this pH range. pH was the main parameter controlling acidic drug solubility, as the pH values which were used previously was 5, 6, or 7 (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020), whereas in this Fa9SIF study, the pH was a range between 5.72 to 8.04. In previous DoE studies, the data points distribution was divided into two groups, upper and lower, depending on their pH values which were constructed by a DoE analysis, but this Fa9SIF study gave a better distribution and range of the solubility values, due to the multidimensional analysis pH range (Pyper et al., 2020).

Two out of three basic drugs from this Fa9SIF9 study were not significantly different compared to other DoE studies. The only exception was tadalafil, which had a significantly different solubility range compared to two DoE studies (Khadra et al., 2015, McPherson et al., 2020, Ainousah et al., 2017), but not DoE 10 (Ainousah et al., 2017). This result reflects a drug-dependent behaviour, as tadalafil is the only unionized drug in this pH range (5.72 – 8.04), with pKa of 3.5, and has the lowest Log P value (1.7).

Three out of four neutral drugs from this Fa9SIF study were not significantly different compared to other DoE studies (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020), exception is griseofulvin which was only tested in the first DoE (Khadra et al., 2015). This difference can also be correlated to the drug's lowest Log P value (2.18) compared to other neutral drugs, and to the media components variation.

In general, the distribution of the basic and neutral drugs solubility was smaller than previous DoE distribution results, and this is due to the different concentration used for PL, FA, and BS, which all contributed to micelle formation, the main solubility influencer, see Table 2.6.

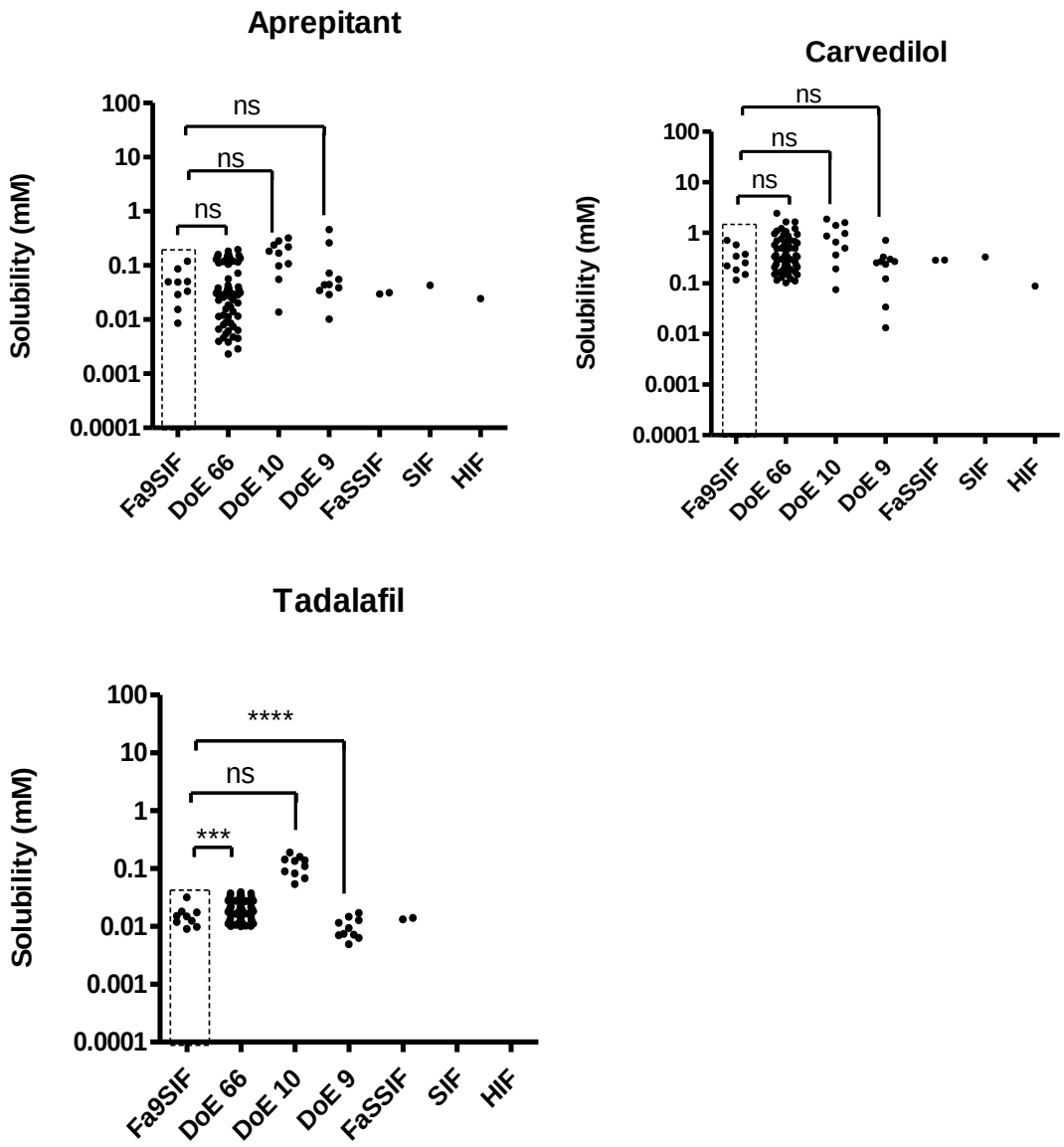


Figure 2.3: Basic drugs measured equilibrium solubility comparison of Fa9SIF–this study, DoE 66 (Khadra et al., 2015), DoE 10 (Ainousah et al., 2017), and DoE 9 (McPherson et al., 2020). The FaSSIF points are points 10 and 11 analysed in this Fa9SIF media. Literature fasted HIF (human intestinal fluid) and SIF (simulated intestinal fluid) (Augustijns et al., 2014). ns: non-significant. *** p = 0.0002; **** p = 0.0001.

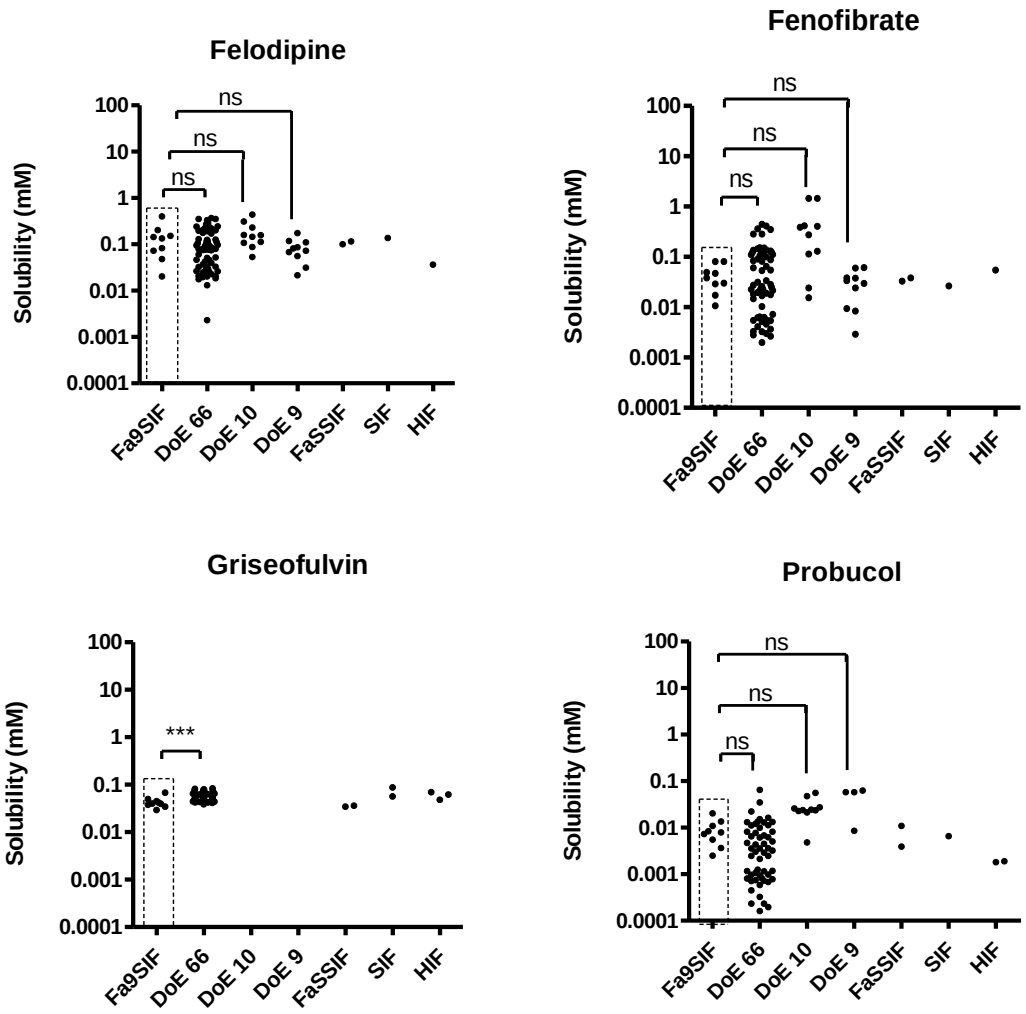


Figure 2.4: Neutral drugs measured equilibrium solubility comparison of Fa9SIF–this study, DoE 66 (Khadra et al., 2015), DoE 10 (Ainousah et al., 2017), and DoE 9 (McPherson et al., 2020). The FaSSIF points are points 10 and 11 analysed in this Fa9SIF media. Literature fasted HIF (human intestinal fluid) and SIF (simulated intestinal fluid) (Augustijns et al., 2014). ns: non-significant. *** p = 0.0006.

2.4.2. Significant Factors Effects and Comparison

The various acidic, basic, and neutral drugs were statistically analysed by Minitab software through a custom DoE, to determine the most statistically significant media components influencing solubility (only the 9 points were considered in this analysis). As seen by the results in Figure 2.5, the solubility of four out of eight of the acidic drugs: furosemide, indomethacin, naproxen, and piroxicam was significantly affected by media pH, but not any of the other media components. The significant effect resulted from the pH, was due to the ionization impact on those drugs. Whereas, phenytoin (pKa 8.3), was not ionized in the pH range of this study (pH 5.72 – 8.04), which accounted for a lower solubility effect from pH. However, the solubility of mefenamic acid (log P 5.12), and zafirlukast (log P 5.56), was not affected by the pH component as well, but this could be related to their high lipophilicity value (> 5) which masked the pH ionization effect. Lastly, ibuprofen showed no significant effect, which is comparable to a previous DoE study (McPherson et al., 2020).

Where available, this result was compared with the significant factors of the previous fasted DoE studied drugs (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020), as the pH component was also affecting the solubility of indomethacin, naproxen, and piroxicam. Whereas the solubility of phenytoin and zafirlukast were significantly affected by more than three intestinal components (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020), which is referred to the different concentrations and higher sample number used in the previous DoE approach, see Table 2.6. For example, higher FA concentration, in previous DoE studies, accounted for a higher overall solubility for the tested samples.

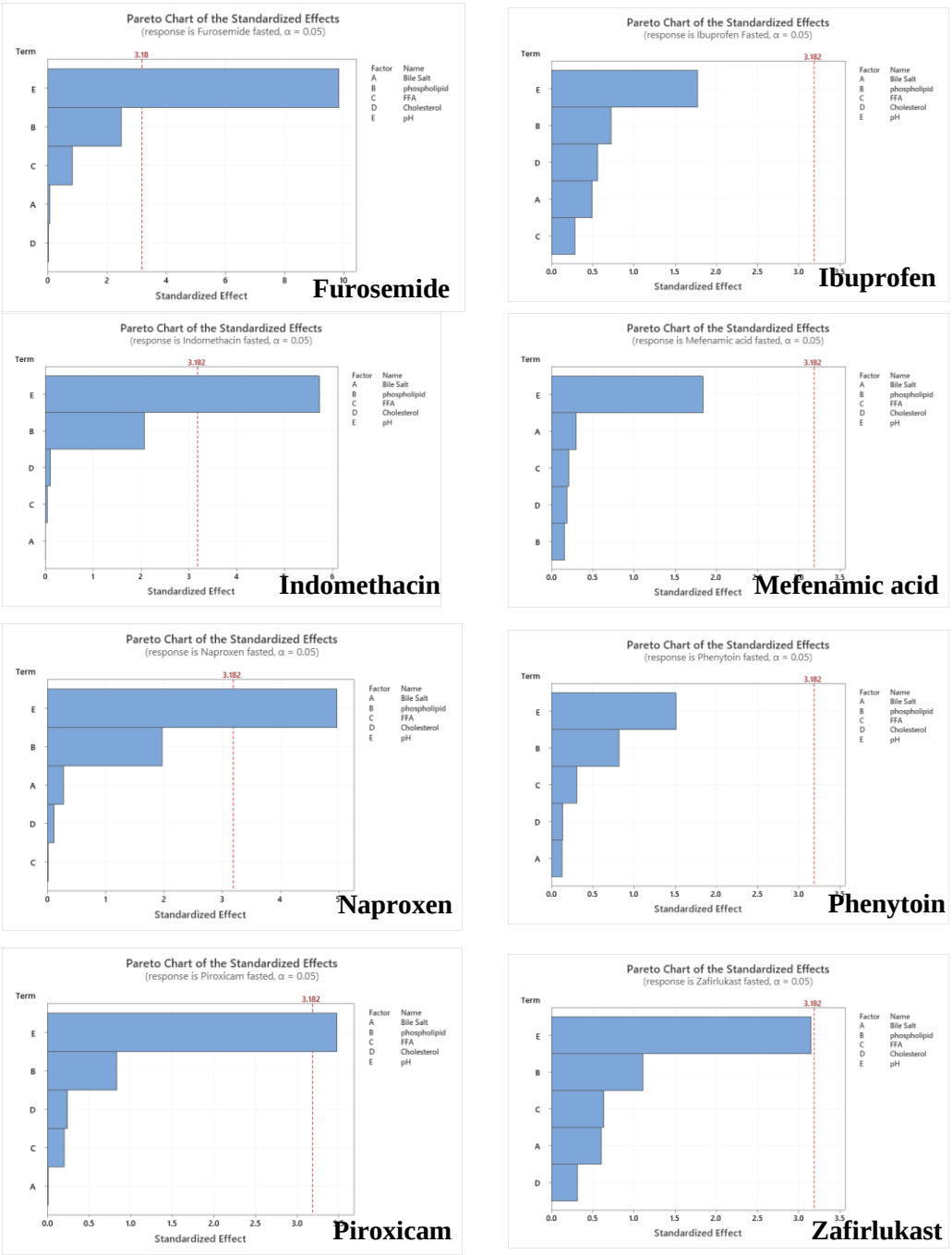


Figure 2.5: Acidic drugs – custom design of experiment analysis. Vertical red lines indicate statistical significance ($P < 0.05$).

The solubility of three basic drugs out of six (aprepitant, carvedilol, and tadalafil) was significantly affected by the PL component, which is due to the formation of micelles. Aprepitant solubility was also affected significantly by the FA component, see Figure 2.6. The solubility of three drugs (not studied in DoE studies) were not affected by any component, posaconazole (pKa 3.6 and 4.6), atazanavir (pKa 4.5), and dipyridamole (pKa 6.4), as the first two were not ionized in the prepared media, and the latter was partially ionized in the studied pH range (5.72 – 8.04), which accounted for a lower solubility.

Where available, this result was compared with the significant factors of the previous fasted DoE studied drugs, and was comparable with aprepitant results (Ainousah et al., 2017, Khadra et al., 2015), and with one literature study for tadalafil (Khadra et al., 2015), but not carvedilol (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020). Carvedilol solubility showed a different behaviour in each DoE study, see Table 2.7, which is related to the different components' concentrations used in each study.

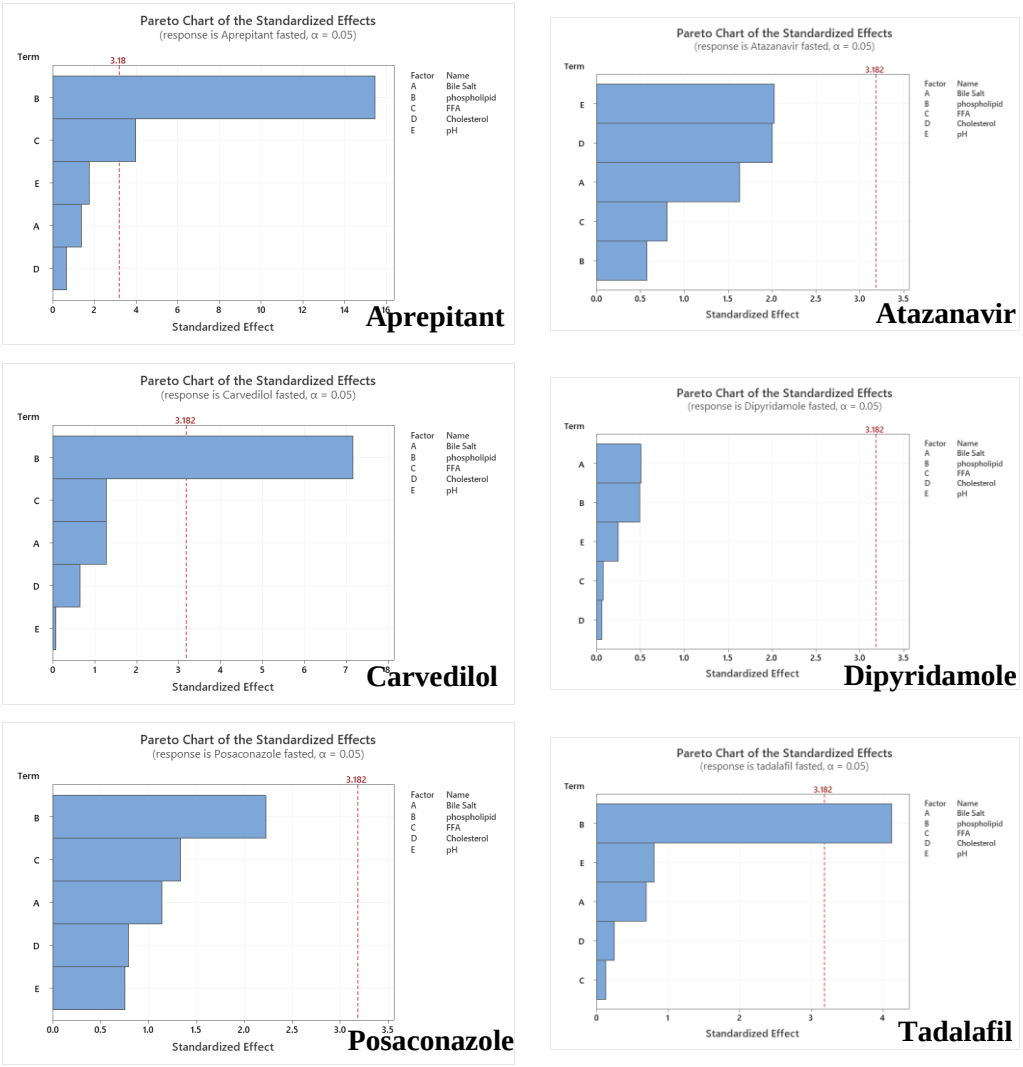
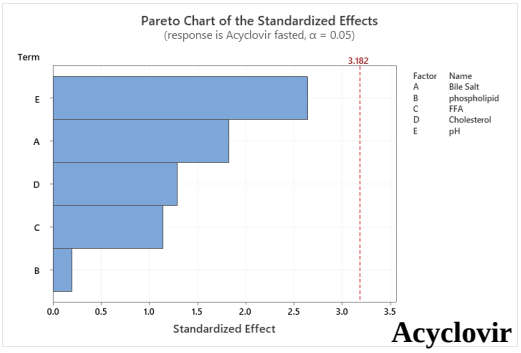
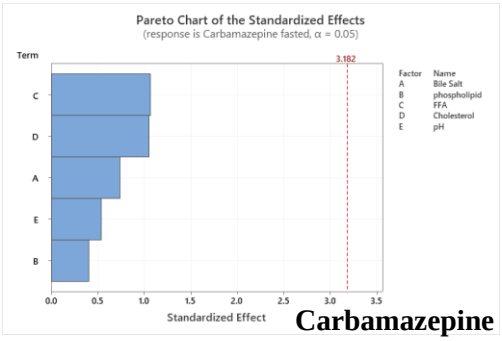


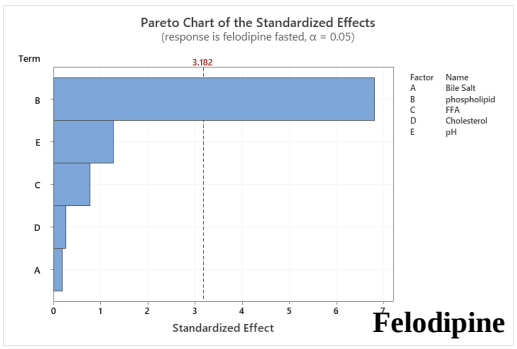
Figure 2.6: Basic drugs – custom design of experiment analysis. Vertical red lines indicate statistical significance ($P < 0.05$).

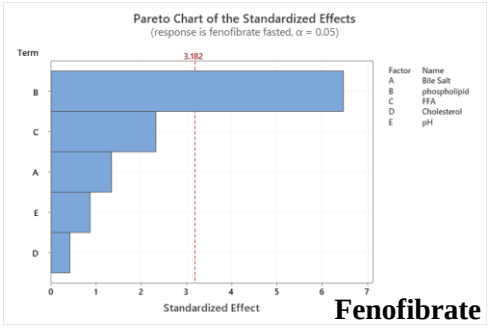
Lastly, for the solubility of the neutral drugs, only felodipine and fenofibrate were significantly affected by phospholipid, which is due to micelles formation, see Figure 2.7. Yet, the solubility of probucol was not affected by any intestinal component due to its high lipophilicity (log P 11.3).

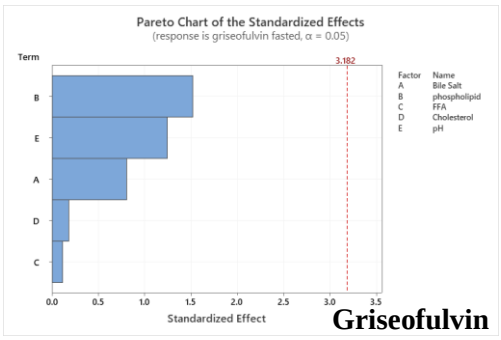
Where available, this result was compared with the significant effects of the previous fasted DoE studied drugs (Ainousah et al., 2017, Khadra et al., 2015), and was comparable with felodipine and fenofibrate. Whereas, the solubility of griseofulvin and probucol was significantly affected by other intestinal components (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020), but not in this study, which is referred to the different concentrations and sample numbers used in each study, see Table 2.6.

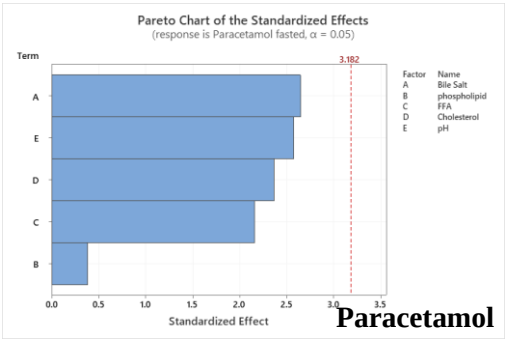












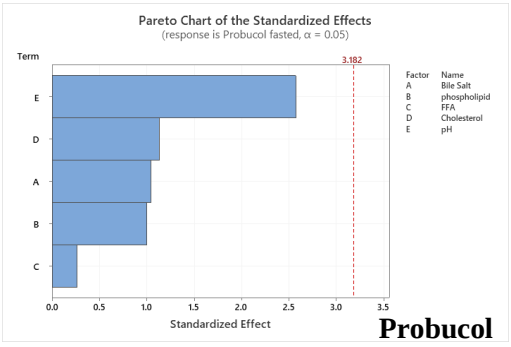


Figure 2.7: Neutral drugs – custom design of experiment analysis. Vertical red lines indicate statistical significance ($P < 0.05$).

As an overall, in the Fa9SIF study, significant factors were found in 8 out of 13 drugs, just over 60%. The 10 (Ainousah et al., 2017, McPherson et al., 2020) and 9 (McPherson et al., 2020, Ainousah et al., 2017) points DoE studies have detected the same number of drugs, 7 out of 9, 78%. Whereas, all the 12 drugs in the 66 points DoE (Khadra et al., 2015) were found to have significant effects. The lowered statistical resolution found by the custom DoE of this study, compared to the previous analysed DoE studies, is due to the lower number of sample points, the different components and concentrations used in each simulated media system, and because the Fa9SIF study was not statistically designed as a DoE. Therefore, due to those differences, small-scale studies using Fa9SIF media compositions are not useful for the identification of the media factors, or factor combinations, that significantly influence a drug's solubility, and to assess this property large-scale DoE studies are required.

Table 2.6: Media component concentrations in the Fa9SIF study and published fasted DoE studies (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020).

Component	**Substance**	**Fa9SIF (Pyper et al., 2020)**	**(Khadra et al., 2015)**	**(Ainousah et al., 2017)**	**(McPherson et al., 2020)**
		Low-High (mM)	Low-High (mM)	Low-High (mM)	Low-High (mM)
Bile Salt	Na taurocholate	1.06 - 1.45	1.5 - 5.9	1.5 - 5.9	1.5 - 5.9
Phospholipid	Soybean lecithin	0.16 - 2.48	0.2 - 1	0.2 - 0.75	0.2 - 1
Fatty acid	Na oleate	1.01 - 3.43	0.5 - 10	0.5 - 15	0.4 - 3.2
Cholesterol	Cholesterol	0.01 - 0.38	-	0.1 - 0.26	-
pH	HCl/KOH	5.72 - 8.04	5, 6, and 7	5, 6, and 7	5, 6, and 7

Note: Khadra et al., DoE used egg lecithin as a phospholipid.

Table 2.7: A comparison between the significant factors affecting the acidic, basic and neutral drugs in this Fa9SIF media versus the previous fasted DoE studies (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020).

Drug	**Fa9SIF (Pyper et al., 2020) 9 points**	**(Khadra et al., 2015) 66 points**	**(Ainousah et al., 2017) 10 points**	**(McPherson et al., 2020) 9 points**
Ibuprofen	NSF	NT	NT	NSF
Indomethacin	pH	pH, bile salt, buffer, fatty acid	pH	pH
Naproxen	pH	pH	NT	NT
Phenytoin	NSF	pH, bile salt, phospholipid, fatty acid, buffer, salt, pancreatin	pH, fatty acid, cholesterol, BS:PL ratio	NT
Piroxicam	pH	pH	NT	NT
Zafirlukast	NSF	pH, fatty acid, phospholipid, bile salt	pH, cholesterol, monoglyceride	pH, fatty acid, bile salt, phospholipid
Aprepitant	Phospholipid, fatty acid	Phospholipid, pH, fatty acid	Fatty acid, phospholipid, monoglyceride	NSF
Carvedilol	Phospholipid	Bile salt, fatty acid	NSF	Bile salt, pH

Drug	**Fa9SIF** **9 points**	**(Khadra et al., 2015)** **66 points**	**(Ainousah et al., 2017)** **10 points**	**(McPherson et al., 2020)** **9 points**
Tadalafil	Phospholipid	Bile salt, pH, buffer, phospholipid, fatty acid, salt	NSF	pH
Felodipine	Phospholipid	pH, fatty acid, phospholipid, bile salt	pH, fatty acid, phospholipid, monoglyceride	Fatty acid
Fenofibrate	Phospholipid	Fatty acid, bile salt, pH, phospholipid, buffer, salt	pH, fatty acid, phospholipid	Fatty acid
Griseofulvin	NSF	pH, bile salt, phospholipid, fatty acid, buffer, salt	NT	NT
Probucol	NSF	pH, fatty acid	Fatty acid, BS:PL ratio	pH

NT: not tested, NSF: non-significant factor, BS:PL is bile salt to phospholipid ratio.

2.4.3. Solubility Multiples

A striking feature of the original DoE 66 (Khadra et al., 2015) was equilibrium solubility variability, with some drugs exhibiting a greater than three log range between the lowest and highest values measured. In Figure 2.8, the calculated solubility multiple (highest solubility ÷ lowest solubility) is, where available, presented of each drug of the Fa9SIF study versus the 3 different fasted DoE studies (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020), to compare the distribution range of each. There is a major reduction in the solubility multiple in the Fa9SIF system compared to other DoE studies (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020). As, in the 66 points DoE (Khadra et al., 2015), over 80% of drugs had higher solubility multiple values compared to the Fa9SIF study. As well, in DoE 10 points (Ainousah et al., 2017), 67% of the drugs had a higher solubility multiple compared to the Fa9SIF study, and just over 20% were relevant (± 0.03). Finally, in the 9 points DoE study (McPherson et al., 2020), the solubility multiple of 60% of the studied drugs were higher than the Fa9SIF study, and 10% was equal (± 0.05).

Two reasons behind these different results, first is the variable media composition and concentrations, see Table 2.6, as the range of media factors and factor values assessed between the systems is not equivalent, and this will influence the solubility measurements. For example, the DoE 66 pH was 5, 6, or 7 (Khadra et al., 2015), whilst the Fa9SIF had a pH range between 5.72 to 8.04. In contrast, the FA range is lower in the Fa9SIF (0.9 – 3.4 mM) when compared to the DoE 66 (0.5 – 10 mM) (Khadra et al., 2015). In addition, cholesterol is present in the Fa9SIF system but not in the DoE 66 (Khadra et al., 2015). The combined solubility influence of these various differences is difficult to predict. Second, the Fa9SIF study didn't combine statistically driven measurement points, which linked a high value of one factor with a low value of another factor, as conducted in previous DoE studies (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020).

Some drugs in the three systems had a minimal difference in the solubility multiple measurements (< 2.5), such as griseofulvin and phenytoin (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020). These drugs, represent 2 drugs out of 4, and 2 out of 5 cases where there is a significantly statistical different result, between

the Fa9SIF data and the three fasted DoE studies (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020). Also, another three drugs showed the same narrow solubility behavior, acyclovir, carbamazepine, and paracetamol, but were not studied in previous DoE studies.

This multi-point assessment process reveals a behaviour that has not been previously reported in the literature, possibly since studies only examine a single point or SIF recipe but with multiple drugs (Fagerberg et al., 2015). The solubility distributions indicate that these drugs have a very low solubility variability within a simulated intestinal media system, and presumably therefore HIF, and the solubility window moves as the media factors and factor values are varied. The latter statement is self-evident, but the consistent low solubility range is not, and overall, this result is an example of a drug dependent solubility behaviour in these systems, which is present (Khadra et al., 2015, Zhou et al., 2017b), but is very difficult to visualize (Dunn et al., 2019, Zhou et al., 2017b). It is interesting that these three drugs have relatively a low molecular weight and log P values, and molecularly have similar compact structures with predominantly flat aromatic rings. This simple chemical property analysis could also be applied to other drugs, for example indomethacin (pKa 4.6), naproxen (pKa 4.2), and piroxicam (pKa 6.4), but the solubility multiple for these drugs is much larger (Figure 2.8). However, for the acidic drugs it is known that pH is the major solubility driver (Khadra et al., 2015) and these drugs have pKa values within the three fasted DoE studies (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020) or the Fa9SIF pH range. This is evident in the previous DoE studies (Figure 2.2) where points cluster in either high or low groups (pH values tested 5, 6 and 7), however the solubility multiple within a pH cluster is low. It is interesting that this low solubility multiple is present in both ionized and non-ionized states for indomethacin, naproxen and piroxicam. The limited solubility variability in the ionized state is understandable, since this represents aqueous solubility of the ionized molecule, but the tight solubility of the non-ionized which should partition into the amphiphilic micellar structure is comparable to the behaviour of phenytoin (pKa 8.33), and griseofulvin (neutral), with the two not ionized. This is most likely to be related to molecular structure and properties and indicates that molecular structure sits within the three categories in controlling solubility behaviour in fasted intestinal media systems. There are not

sufficient examples in this study to assess this effect, however this is an indication of a link between molecular structure or shape and solubility in the intestinal media systems over and above more general properties such as pKa and log P (Bergström and Larsson, 2018). Further focused studies will be required to fully elucidate this behavior.

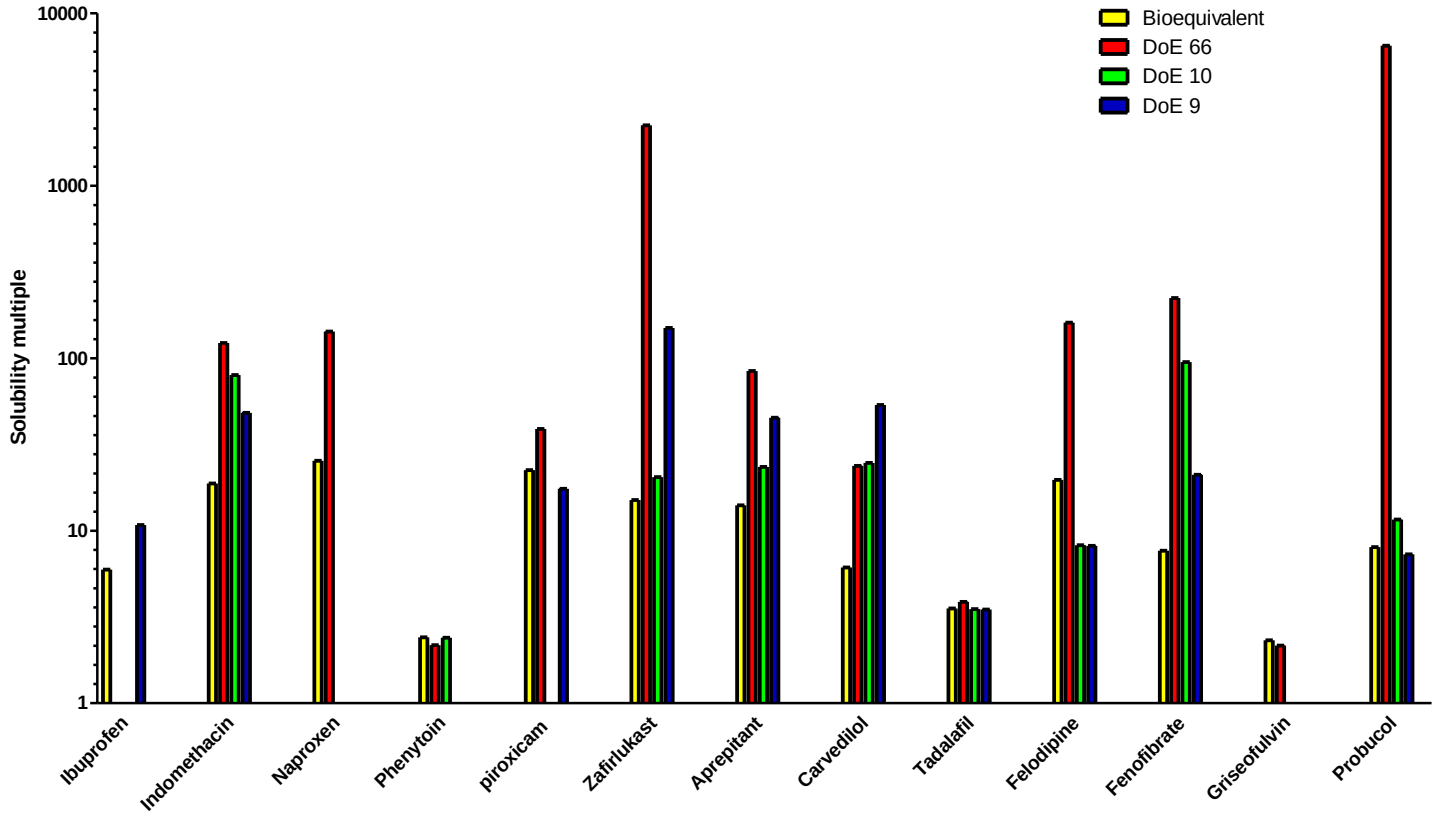


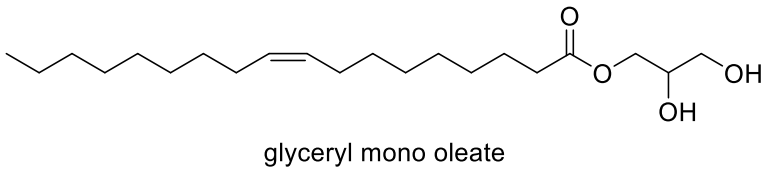
Figure 2.8: The solubility multiple findings of Fa9SIF vs DoE studies. Fa9SIF results with the other fasted DoE 66, 10, and 9 points solubility results (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020).

Solubility multiple = highest solubility ÷ lowest solubility.

2.4.4. The Solubility Effect Using a Fatty Acid Derivative

Six out of the twenty-one drugs, tested in section 2.1, were tested with glyceryl mono-oleate (GMO) as a FA derivative, instead of the free FA components (which was represented by Na oleate). The aim was to check if there would be any significant difference between the solubility results, using different components.

GMO was already used in previous fed DoE studies (Perrier et al., 2018, Zhou et al., 2017b), and was presented as a monoglyceride (MG) component to represent the fed state. GMO is a fatty acid derivative, which has different ionization patterns compared to Na oleate. As with pH change, GMO can't be ionized because it lacks a free exchangeable hydrogen. Its molecular formula and molecular weight are $C_{21}H_{40}O_4$, 356.5 g/mole. GMO is an interaction between glyceryl ester and oleate, where the glyceryl functional group links to the deprotonated oxygen, to form a structure which is insoluble in water with neutral properties, see Figure 2.9.



glyceryl mono oleate

Figure 2.9: The molecular structure of glyceryl monooleate (reference is PubChem).

2.4.4.1. Materials and Methods

Cithrol GMO was purchased from CRODA company and was used as the same concentrations used for Na oleate FA in the original study, see Table 2.2. The eight samples, a centre point (sample 9), and the two FaSSIV-v1 (sample 10), and FaSSIV-v1 with Na oleate (sample 11), were prepared and analysed with the same methods detailed in section 2.3.2. The significant effects were analysed using a custom DoE by Minitab software. Prism software version 5 was used to perform Wilcoxon matched pairs t-test (P value < 0.05) of the resulting solubilities, refer to section 2.3.2 for further details.

2.4.4.2. Results and Discussion

a. HPLC Results

The injection volumes and wavelengths used were the same as the original study with Na oleate, refer to Table 2.5. The only difference was in the retention time of each drug, as the usage of GMO with the other components of the Fa9SIF study: Na taurocholate, lecithin, cholesterol, and pH, had reduced the components polarized nature. This effect was seen by the small increase of the retention time, 0.1 - 0.3 minutes, in over 80% of the studied drugs, see Table 2.8. The MP used in this study was a combination of two MP in gradient elution, same as the one used before.

Table 2.8: The retention time resulted using the glyceryl mono oleate as a fatty acid derivative, instead of Na oleate.

	Drug	**Retention time (minute)**
1	Ibuprofen	2.3
2	Piroxicam	0.98
3	Tadalafil	1.5
4	Felodipine	2.67
5	Fenofibrate	3.31
6	Griseofulvin	1.67

b. Equilibrium Solubility Findings and Comparison

The solubility results, of six drugs, with either Na oleate or GMO are presented in Figure 2.10. Three out of six of the analysed drugs, were significantly different. This difference proves the importance of using biorelevant SIF components, and also reflects the complexity of the GIT behavior.

This comparison was not conducted in previous literature, so it’s difficult to study the results effects, also, a higher number of drugs is needed to increase the study accuracy and understand the solubility effects.

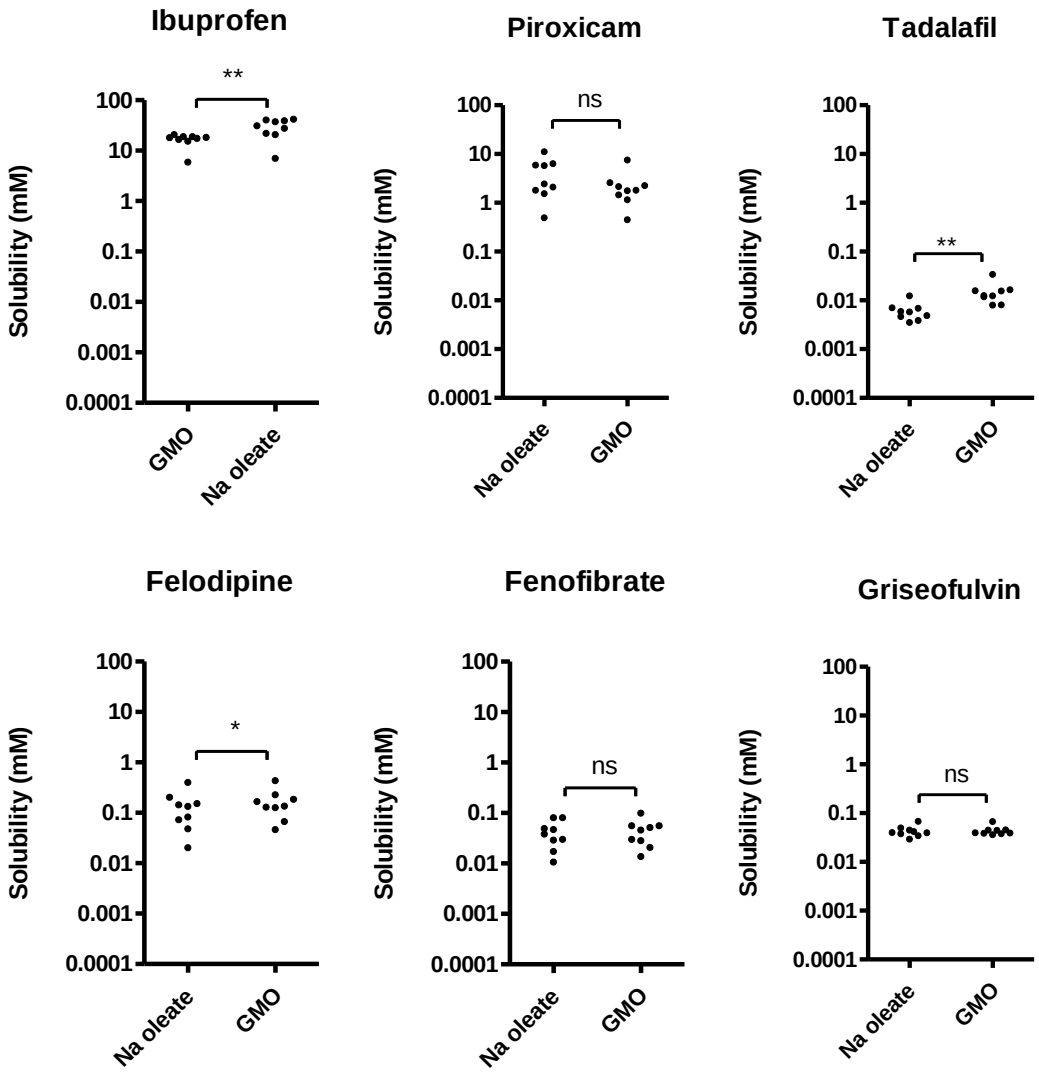


Figure 2.10: Statistical comparison for the solubility results between Na oleate fatty acid or GMO fatty acid derivative solubility results, for 6 drugs. * P= 0.0195, ** P= 0.0039 – 0.0078.

c. Significant Factors Effects and Comparison

This experiment studied 2 acidic, 1 basic, and 3 neutral drugs, ibuprofen, piroxicam, tadalafil, felodipine, fenofibrate, and griseofulvin respectively. The significant effects on solubility of the 5 media components are presented in Figure 2.11.

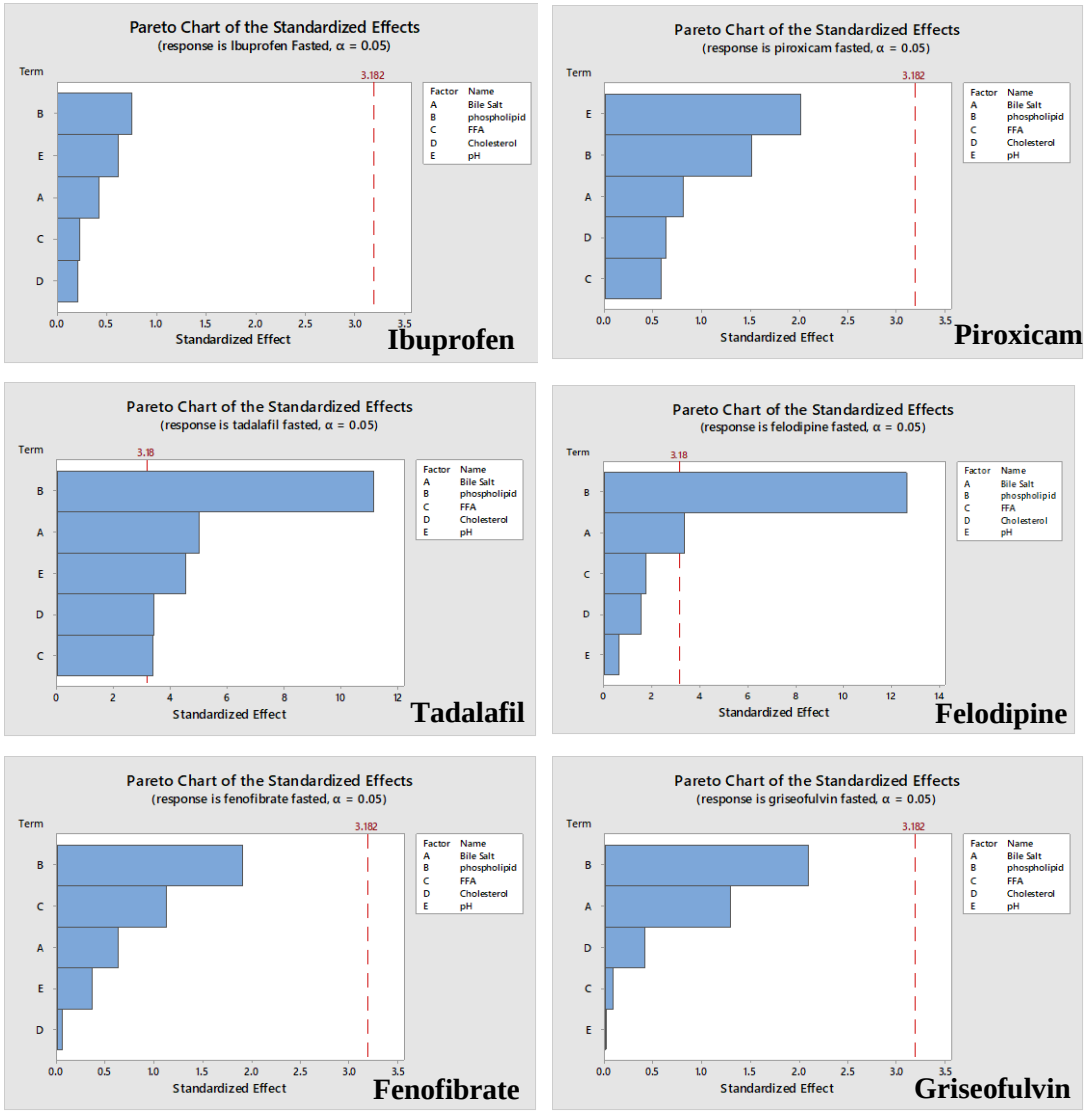


Figure 2.11: The significant effects on the solubility of 6 studied drugs, using GMO as a fatty acid derivative along with the other 4 Fa9SIF components.

The solubility of only two drugs out of six was statistically affected by the media components. The solubility of tadalafil was affected by all the five media components, with the greatest effect due to PL. The second drug was felodipine, which was significantly affected by the PL component with the BS component as a second statistically significant influence. Comparing this with Na oleate effects, the components' significant effects studied with GMO were found in half of the drugs which had significant effects with Na oleate, see Table 2.9. The reduction in the overall number of the drugs significantly affected by the media components refers to the higher overall lipophilicity introduced by GMO.

Table 2.9: A comparison of the significant components results of the Fa9SIF study using Na oleate versus glyceryl mono-oleate (GMO) as a fatty acid derivative.

Drug	Using Na oleate	Using GMO
Ibuprofen	NSF	NSF
Piroxicam	pH	NSF
Tadalafil	Phospholipid	Phospholipid, bile salt, pH, cholesterol, fatty acid
Felodipine	Phospholipid	Phospholipid, bile salt
Fenofibrate	Phospholipid	NSF
Griseofulvin	NSF	NSF

NSF: non-significant factor.

The solubility of piroxicam wasn't significantly affected by pH, as pH didn't affect GMO ionization, as did with Na oleate presence. On the other hand, the usage of GMO increased the micelles inclusion of specific drugs, which their solubility was only affected by PL, and became affected by all the media components, such as tadalafil (log P 1.7). This result could be confirmative if more basic drugs were analysed using GMO. The neutral drugs had variable results, the solubility of felodipine (log P 3.9) which was previously affected by PL only, is now affected by both PL and BS, whereas the solubility of fenofibrate (log P 5.3) which was affected by PL, wasn't affected by any component with the usage of GMO, which could be referred to its high lipophilicity.

d. Solubility Multiple

The solubility multiples for each of the 6 drugs analysed using GMO as a fatty acid derivative in this Fa9SIF study, versus Na oleate FA, were plotted in Figure 2.12. In 5 out of 6 drugs, the GMO solubility multiplier was lower than Na oleate's solubility multiplier. Only tadalafil was higher, which could be referred to having the lowest log P value among the analysed drugs (refer to Table 2.1).

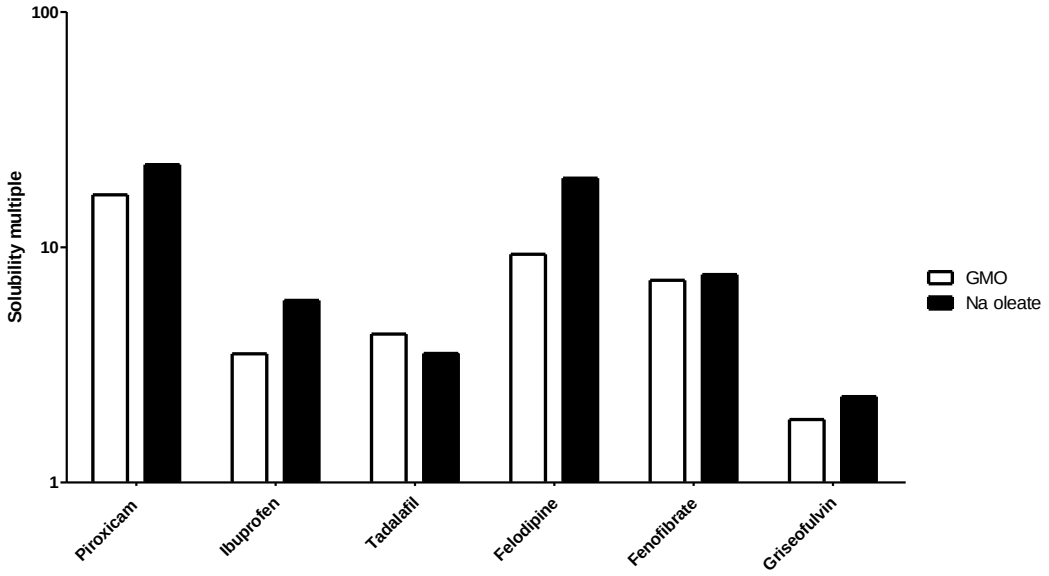


Figure 2.12: The solubility multiple of the Fa9SIF media with either GMO or Na oleate. Solubility multiple = highest solubility ÷ lowest solubility.

2.5. Conclusion

This study demonstrates that it is possible to assess the fasted intestinal equilibrium solubility distribution using a small number of Fa9SIF media recipes obtained from a multidimensional analysis of sampled fasted human intestinal fluid. The solubility distribution obtained is statistically equivalent to those determined using DoE studies, which indicates that this approach is examining the same solubility space. In addition, the data from this chapter in combination with the results from multiple DoE studies (Ainousah et al., 2017, Khadra et al., 2015, Madsen et al., 2018, McPherson et al., 2020), and other single point solubility measurements (Augustijns et al., 2014), indicate that the use of simulated media system, utilizing the same media factors and concentrations are likely to provide similar solubility distributions.

By creating a custom DoE using the Fa9SIF media recipe factor values, it is possible to calculate the factors significantly influencing drug solubility. This analysis showed the pH component to have the major significant effect on half of the acidic drugs' solubility, due to the ionization impact. While the PL component was the highest significant effect for half of the basic drugs, which is due to micelle-based solubilization. The solubility of one basic drug was significantly affected by both the

PL and the FA components. Lastly, only 2 out of 7 neutral drugs were affected by the PL component. However, the number of factors identified is reduced when compared to statistically designed small-scale studies (Ainousah et al., 2017, McPherson et al., 2020), which are again lower than the large-scale study (Khadra et al., 2015). From 11 available comparison cases of the acidic drugs, pH was found to have a significant effect in 10 cases (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020). The basic drugs had lower matched cases, of only 3 out of 9 cases having PL to be a significant effect, and for aprepitant, 2 out of 3 cases were having FA to be a significant effect. While for the neutral drugs, PL was found to be significant in 66 points DoE (Khadra et al., 2015), and 10 points DoE (Ainousah et al., 2017), but not in 9 points DoE (McPherson et al., 2020).

The solubility variability measured by this study is lower than the variability from the initial large-scale DoE (Khadra et al., 2015) study, or other DoE studies (Ainousah et al., 2017, McPherson et al., 2020). The studies are not directly comparable, and two factors could be responsible for this difference, the variable media composition and concentrations of each study, and that the Fa9SIF study didn't combine statistically driven measurement points, which linked a high value of one factor with another low value of another factor, as conducted in previous DoE studies (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020). However, based on the source for the media recipe compositions in this study, the lower solubility range measured is more likely to reflect the fasted intestinal solubility envelope than a DoE approach, and considered to be bioequivalent to *in vivo* behaviour. In addition, five drugs exhibit a very narrow solubility range, that has been revealed by the multi-point analysis, and which has not been previously picked up using a single point measurement. This might represent an interesting behaviour category for further biopharmaceutical consideration.

Finally, six drugs were tested by displacing the free FA component (Na oleate) with a FA derivative (GMO) and found a statistically different result in half of drugs components driven solubility effects. Also, the number of significant factors and the solubility variability were reduced. This is because the pH didn't affect GMO ionization which especially affected the ionizable drugs, such as piroxicam. Whereas, the solubility of some drugs was affected by more than one component, compared to

the Na oleate recipe, for example tadalafil, which could be referred to its lowest log P value. Overall, this proves the complexity of the GIT composition, and that using a less biorelevant component would result in variable solubility effects.

Chapter 3: Investigating the Structured Solubility Behavior in Bioequivalent Fasted Simulated Intestinal Fluids Along with Its Limits and Distributions

3.1. Introduction

The 9 fasted simulated intestinal fluid (Fa9SIF) media compositions (Pyper et al., 2020), along with a value in fasted simulated intestinal fluid version 1 (FaSSIF-v1) (McPherson et al., 2020), were applied to determine the fasted solubility of 21 drugs, 7 of them were assessed in the original Developability Classification System (DCS) paper (Butler and Dressman, 2010), as detailed in Chapter 2 of this thesis. As the nine media recipes provided a range of solubility values that, due to the derivation from sampled HIF, covered the fasted human intestinal fluid (FaHIF) range, therefore this can be considered bioequivalent.

This chapter will cover two parts: the first part, is the application of the equilibrium solubility values to the DCS grid and associated calculations which predict absorption, to provide the limits for likely *in vivo* solubility behavior. Also, determination of a solubility frequency distribution, to assess solubility behaviour across the population range, based on the twenty volunteers sampled in the original study (Riethorst et al., 2016). It should be noted that the frequency distribution represents the aggregated measured HIF compositions from all volunteers, and therefore intra- and inter-subject variability cannot be analysed using this approach.

The second part is to investigate the solubility behaviour of the Fa9SIF media recipes, to determine the consistency between each drug and its category. Consistent solubility behaviour might permit a further reduction or refinement of the number of media required to determine a FaHIF solubility range using fasted SIF media. Determination of an intestinal solubility range, with minimum addition of required media would be useful during early drug development, when active pharmaceutical ingredients (API) material is limited, but crucial decisions concerning for example API physical form and formulation need to be made (Bayliss et al., 2016, Di et al., 2012, Ding et al., 2012).

3.2. Aims and Objectives

- Predict the *in vivo* behaviour by investigating the developability classification system of oral drugs, extremes and ranges to reduce time, cost, and efforts for compounds screening and formulation.
- Study the solubility behaviour among a population range by determining a solubility frequency distribution.
- Reduce the media number used by investigating the solubility behaviour structure and consistency, to aid in drug discovery and development stages.

3.3. Methods

As detailed in Chapter 2, the equilibrium solubility of 21 drugs (physicochemical properties are detailed in Table 2.1) was measured in the 9 bioequivalent media recipes and the pre-prepared FaSSIF-v1 media (concentrations used are presented in Table 2.2). The solubility results were used in the following DCS calculations (using Excel®) and applied to the DCS grid.

All data analysis and Figures were conducted using Graph pad prism software version 5, using Windows 11. Spider plots were created by OriginPro® 2022 software.

3.4. Results and Discussion

3.4.1. Developability Classification System

3.4.1.1. Equilibrium Solubility Ranges

Using the measured bioequivalent maximum and minimum solubility values found in Chapter 2, the solubility multipliers ((Maximum Solubility) ÷ (Minimum Solubility)). were calculated, see Table 3.1 The values ranged from 1.15 for acyclovir to 40.0 for furosemide. Furthermore, using the centre point it is possible to calculate a skew value ((Maximum Solubility - Centre Point Solubility)) ÷ ((Centre Point Solubility - Minimum Solubility)) to determine the distribution symmetry, with values ranging from 0.445 (ibuprofen) to 23.9 (mefenamic acid). Generally, most of the drugs (67%) with the lowest solubility multiplier also have the lowest skew value, however, 33% of the drugs deviate from this trend. This variation indicates the individualistic drug behaviour in these complicated media systems (Dunn et al., 2019, Zhou et al., 2017b), and further results and discussion with respect to this issue are in the next section. This is the first experimental study that permits the calculation of these values, and a greater number of examples is required to assess the utility of this information.

At this stage, it could be surmised that for drugs with a low solubility multiplier and skew values, the *in vivo* bioavailability variability will not be influenced by intestinal solubility variability, and other factors such as permeability and/or metabolism will be more important. For drugs with high solubility multiplier and skew values, the intestinal solubility variability along with permeability and/or metabolism will contribute to *in vivo* bioavailability variability. Based on these results, it can be confirmed that the bioequivalent media system is detecting a relevant solubility range, and this range is dependent upon the drug's physicochemical properties, molecular structure, and media composition.

Table 3.1: Equilibrium solubility data and analysis.

Drug	**Minimum solubility (mg/mL)**	**Centre solubility (mg/mL)**	**Maximum solubility (mg/mL)**	**Solubility multiplier**	**Skew**	**P_{eff} *10^{-4} (cm/s)**	**Dose (mg)**
Furosemide	0.397	5.98	15.9	40.0	1.78	0.60[2]	80[1]
Ibuprofen	1.46	6.44	8.66	5.95	0.445	12[1]	400[1]
Indomethacin	0.144	0.614	2.71	18.8	4.47	7.5[7]	200[8]
Mefenamic acid	0.0134	0.0322	0.481	35.9	23.9	14[1]	250[1]
Naproxen	0.682	3.73	17.4	25.5	4.47	8.5[2]	1000[8]
Phenytoin	0.0240	0.0298	0.0579	2.41	4.86	8.4[7]	300[8]
Piroxicam	0.164	0.811	3.68	22.5	4.43	6.7[2]	20[8]
Zafirlukast	0.00152	0.00276	0.0229	15.1	16.2	6.4[4]	20[3]
Aprepitant	0.00456	0.0262	0.0641	14.1	1.75	7.1[5]	125[8]
Atazanavir	0.000990	0.00154	0.00424	4.27	4.88	*1.2[4]	300[8]
Carvedilol	0.0475	0.105	0.291	6.12	3.27	6.8[7]	25[8]
Dipyridamole	0.00813	0.0179	0.0608	7.48	4.40	1.5[1]	100[1]
Posaconazole	0.00229	0.00330	0.00631	2.76	2.97	7.1[6]	300[8]
Tadalafil	0.00351	0.00579	0.0124	3.54	2.91	7.3[7]	20[3]
Acyclovir	2.67	2.82	3.07	1.15	1.55	0.25[1]	800[1]
Carbamazepine	0.154	0.192	0.247	1.60	1.45	4.3[2]	300[8]

Drug	Minimum solubility (mg/mL)	Centre solubility (mg/mL)	Maximum solubility (mg/mL)	Solubility multiplier	Skew	P_{eff} *10^{-4} (cm/s)	Dose (mg)
Felodipine	0.00800	0.0520	0.154	19.7	2.33	7.8[5]	10^{8}
Fenofibrate	0.00383	0.0138	0.0293	7.65	1.56	7.7[5]	160^{8}
Griseofulvin	0.0103	0.0141	0.0240	2.32	2.64	8.7[1]	500^{1}
Paracetamol	18.0	19.9	22.0	1.22	1.10	1.3[1]	500^{1}
Probucol	0.00130	0.00410	0.0104	8.05	2.27	6.5[7]	500^{3}

*Atazanavir rat permeability was converted by GastroPlus® software, to the human permeability value.

Solubility Multiplier = (Maximum Solubility) / (Minimum Solubility).

Skew = ((Maximum Solubility - Centre Point Solubility)) / ((Centre Point Solubility - Minimum Solubility)).

References: 1- (Butler and Dressman, 2010) 2- (Lennernäs, 2014) 3- (Benet et al., 2011) 4- (Kis et al., 2013) 5- (Sjögren et al., 2016) 6- (Hens and Bolger, 2019) 7- (Perrier, 2019) 8- British National Formula.

3.4.1.2. Developability Classification System Range

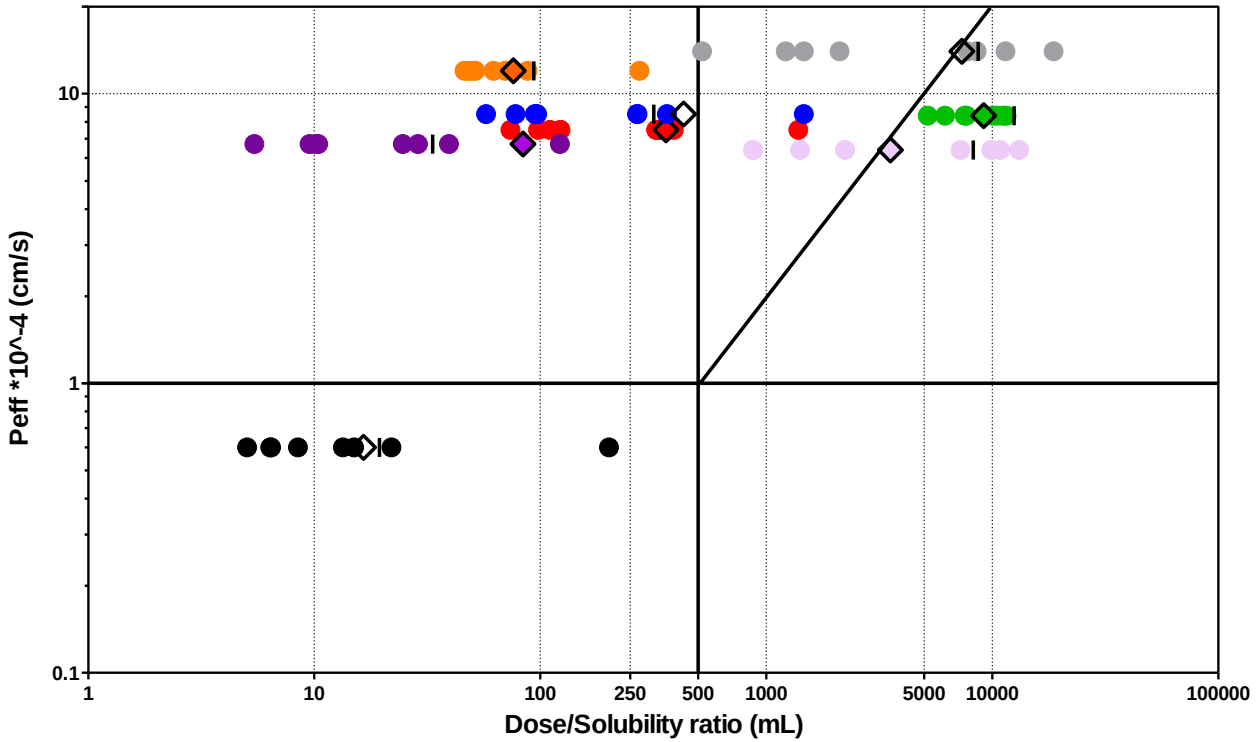
Using the drug's human intestinal permeability values along with the normal oral doses (Table 3.1), it is possible to plot the results on the DCS grid by calculating a dose/solubility ratio for each measurement point, as presented previously (Butler and Dressman, 2010). This is presented in Figure 3.1 (and summarized in Table 3.2) using FaSSIF-v1 and the nine bioequivalent media system measurements. The plot highlights the solubility range along with the multiplier and distribution issues discussed above.

The DCS drug classification for 5 out of 7 drugs categorized previously (Butler and Dressman, 2010) was the same with using bioequivalent solubility range values. Mefenamic acid crossed a DCS boundary, and acyclovir changed from category IV to III (enhanced solubility), which is determined using a range of solubility values compared to the previous single value (Butler and Dressman, 2010). Whereas for the other drugs with FaSSIF-v1 DCS categorization, seven drugs had no change in categorization with expanded solubility range, three drugs crossed a DCS boundary one acidic (zafirlukast), and two basic (aprepitant and tadalafil). Whereas the solubility range of four drugs (indomethacin, naproxen, carvedilol, and felodipine) expanded between the dissolution limited class (IIa) and class I. For the drugs which crossed a DCS boundary the centre point and/or FaSSIF-v1 value is located at or close to the boundary of 3 out of the 4 drugs (except aprepitant).

The additional range-based information arising from the multi-point measurement indicates that for drugs which crossed a DCS boundary, a worst-case formulation approach for the four drugs should be based on solubility limited performance rather than dissolution approaches. This demonstrates the utility of using a range over a single value measured either in FaHIF or SIF. Investigation of more drugs will reveal further candidates where different aspects of these scenarios are likely to arise.

Acidic drugs DCS classification

a



Furosemide 80 mg

Ibuprofen 400 mg

Indomethacin 200 mg

Mefenamic acid 250 mg

Naproxen 1000 mg

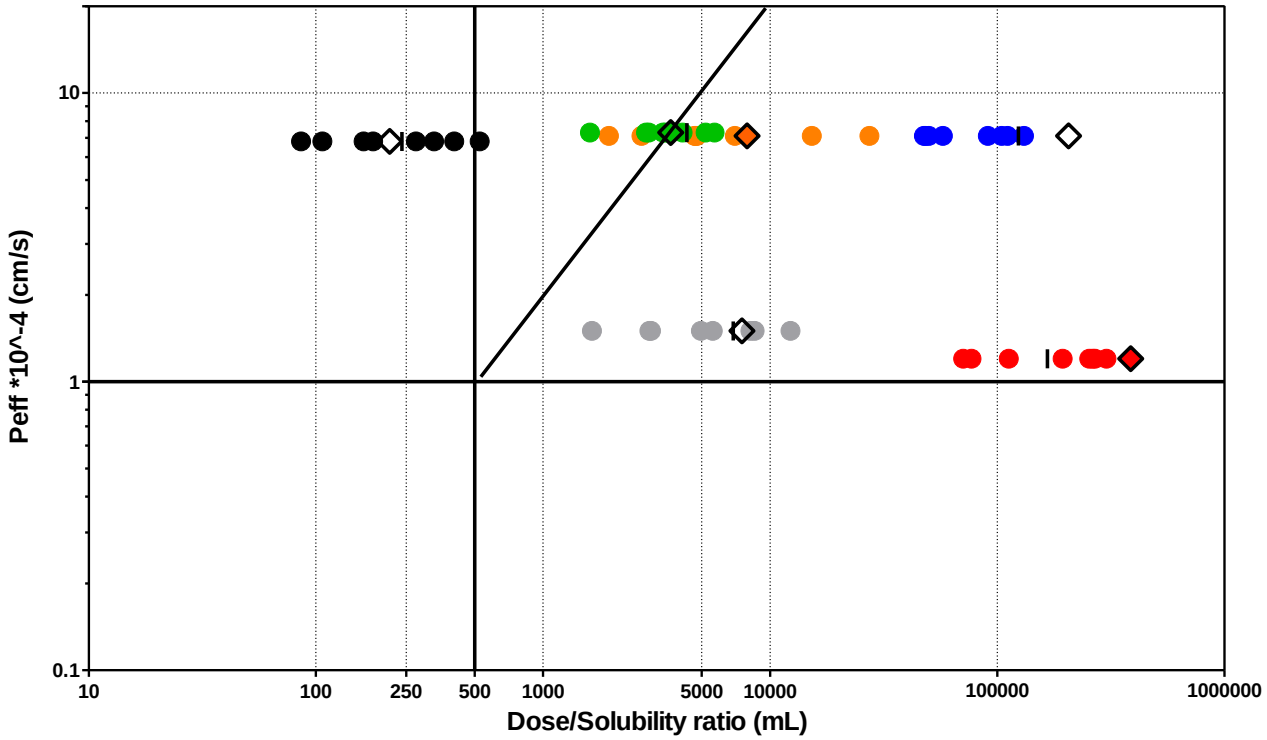
Phenytoin 300 mg

Piroxicam 20 mg

Zafirlukast 20 mg

Basic drugs DCS classification

b



Aprepitant 125 mg

Atazanavir 300 mg

Carvedilol 25 mg

Dipyridamole 100 mg

Posaconazole 300 mg

Tadalafil 20 mg

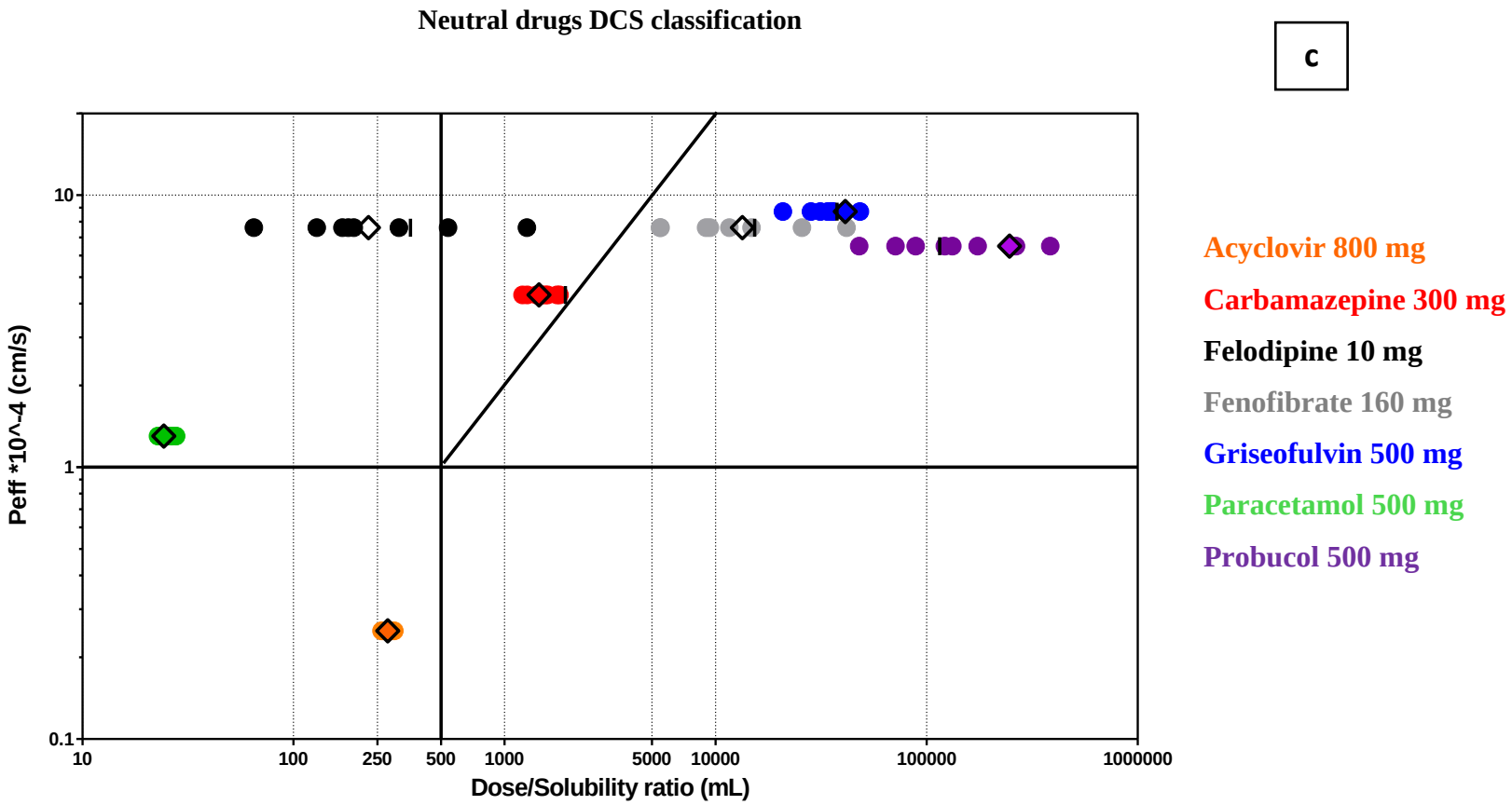
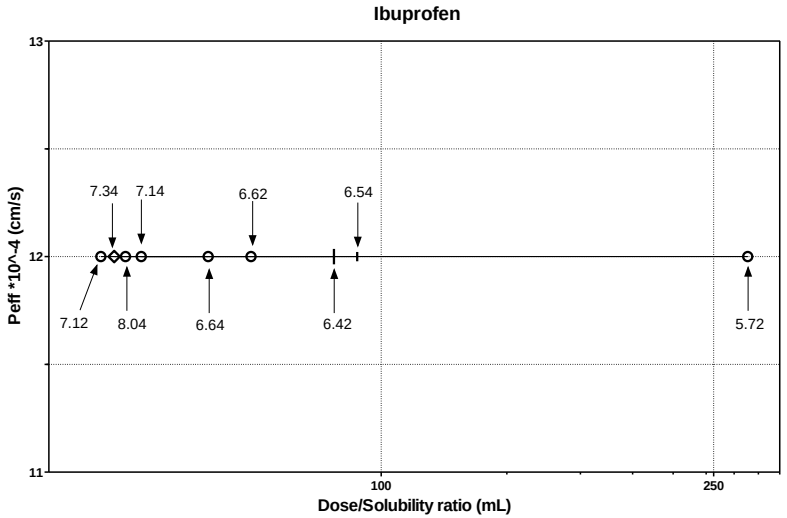
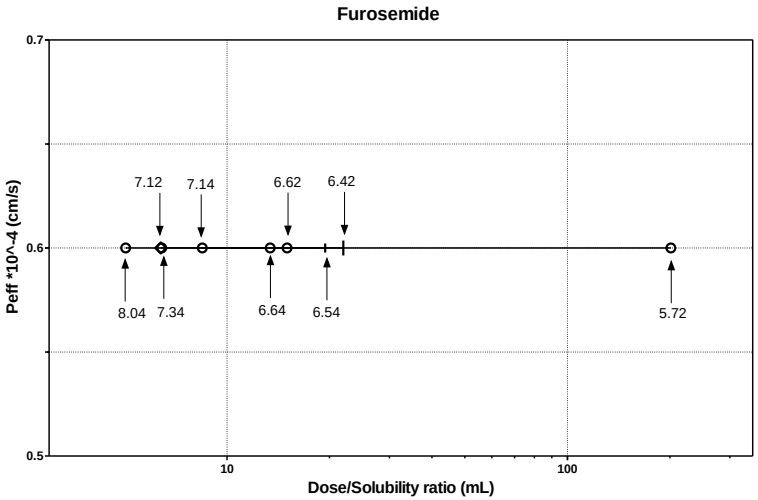


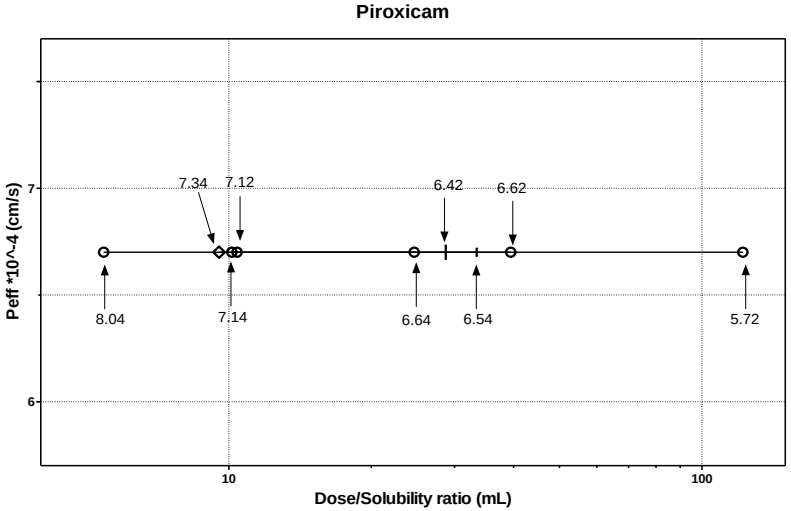
Figure 3.1: Bioequivalent systems on Developability Classification System grid of acidic (a), basic (b), and neutral (c) drugs, respectively. ◇FaSSIF-v1 (Fasted State Simulated Intestinal Fluid); ● Bioequivalent data points, ⎪ Bioequivalent centre point. Individual drugs and doses labelled as colour coded. Each point mean n = 3.

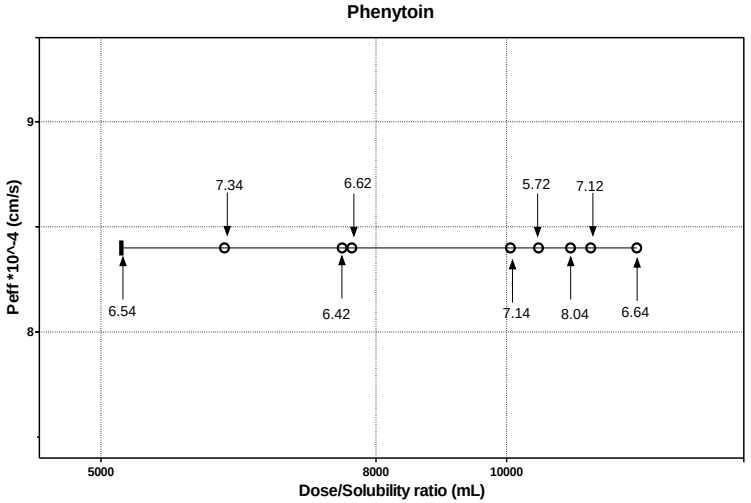
The behaviour of the acidic drugs with respect to measurement pH is illustrated in Figure 3.2. A predominant effect is that solubility increases (therefore dose/solubility volume decreases) with increasing pH, with minor variation due to the amphiphilic factors present in the media. This is consistent with the solubility drivers identified for acidic drugs in the original fasted DoE study (Khadra et al., 2015), and other related studies (Ainousah et al., 2017, McPherson et al., 2020, Perrier et al., 2018). This indicates that although the media component concentrations and ratios have been changed to provide equivalence to the measured HIF samples (Pyper et al., 2020), the system's solubility behaviour remains consistent with previous DoE studies.

The bioequivalent point compositions describe greater than ninety percent of the compositional variation present in the analysed HIF samples (Pyper et al., 2020). Therefore, it is reasonable to assume that the measured range for each drug in Figure 3.1 represents greater than ninety percent of a behaviour in the measured fasted intestinal space, and the calculated maximum drug's solubility and minimum values indicate a drug's intestinal fasted solubility range. The lowest solubility or largest dose/solubility volume ratio could be taken to represent a worst-case scenario, with greater than ninety percent of the distribution above this extreme limit exhibiting a higher solubility. Therefore, compound screening or formulation selection based on the lowest solubility point rather than a centre point or average fasted SIF point might be useful as a worst-case for a more cautious risk-based quality by design approach (Rosenberger et al., 2018). In addition, this eliminates the inherent risk associated with solubility range distributions if a centre point or average fasted SIF value is utilised, without any knowledge of the solubility range.


Ibuprofen
Peff *10^-4 (cm/s)
13
12
11
7.34
7.14
6.62
6.54
7.12
8.04
6.64
6.42
5.72
100
250
Dose/Solubility ratio (mL)


Furosemide
Peff *10^-4 (cm/s)
0.7
0.6
0.5
7.12
7.14
6.62
6.42
8.04
7.34
6.64
6.54
5.72
10
100
Dose/Solubility ratio (mL)


Piroxicam
Peff *10^-4 (cm/s)
7
6
7.34
7.12
6.42
6.62
8.04
7.14
6.64
6.54
5.72
10
100
Dose/Solubility ratio (mL)


Phenytoin
Peff *10^-4 (cm/s)
9
8
7.34
6.62
5.72
7.12
6.54
6.42
7.14
8.04
6.64
5000
8000
10000
Dose/Solubility ratio (mL)

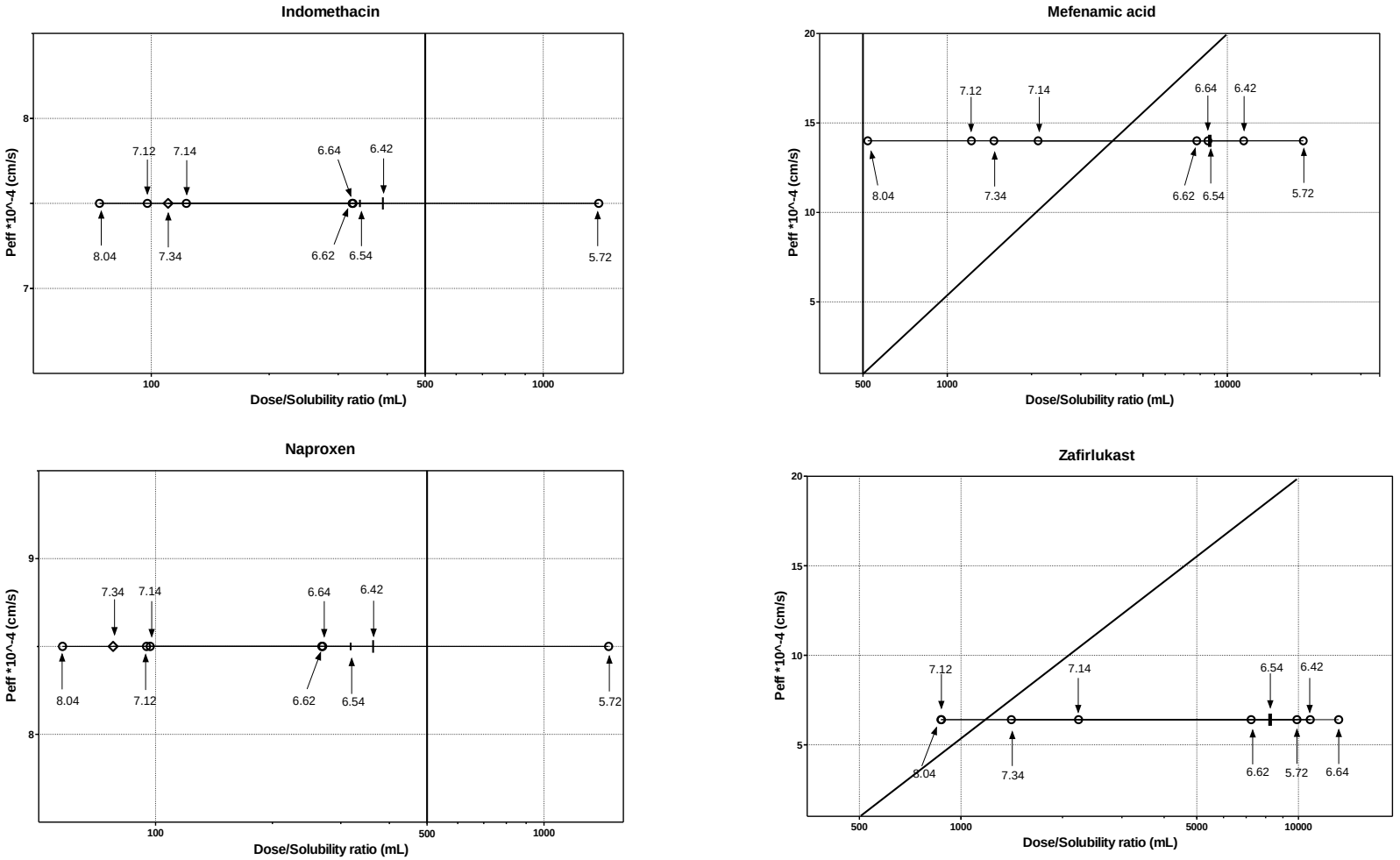


Figure 3.2: Acidic Drugs pH solubility behaviour. ○ Bioequivalent data points, ⎮ Bioequivalent centre point. Measurement pH values as labelled. Each point mean n = 3.

Table 3.2: Summary of the resulting DCS categories of the analysed drugs.

Drug	**DCS categorization**		**Bioequivalent media impact***
	Literature	**FaSSIF-v1**	
Furosemide	III	-	No change, expanded solubility range
Ibuprofen	I	-	
Phenytoin	-	IIb	
Piroxicam	-	I	
Atazanavir	-	IIb	
Dipyridamole	IIb	-	
Posaconazole	-	IIb	
Carbamazepine	-	IIa	
Fenofibrate	-	IIb	
Griseofulvin	IIb	-	
Paracetamol	I	-	
Probucol	-	IIb	
Mefenamic acid	IIa	-	Crossed a DCS boundary
Zafirlukast	-	IIb	
Aprepitant	-	IIb	
Tadalafil	-	On IIb/IIa boundary	
Indomethacin	-	I	Expanded solubility range
Naproxen	-	I	
Carvedilol	-	I	
Felodipine	-	I	
Acyclovir	IV	-	Solubility enhanced

DCS: Developability Classification System. *: See text.

DCS categorization literature references is (Butler and Dressman, 2010), FaSSIF-v1 is based on the measured FaSSIF-v1 value from Chapter 2.

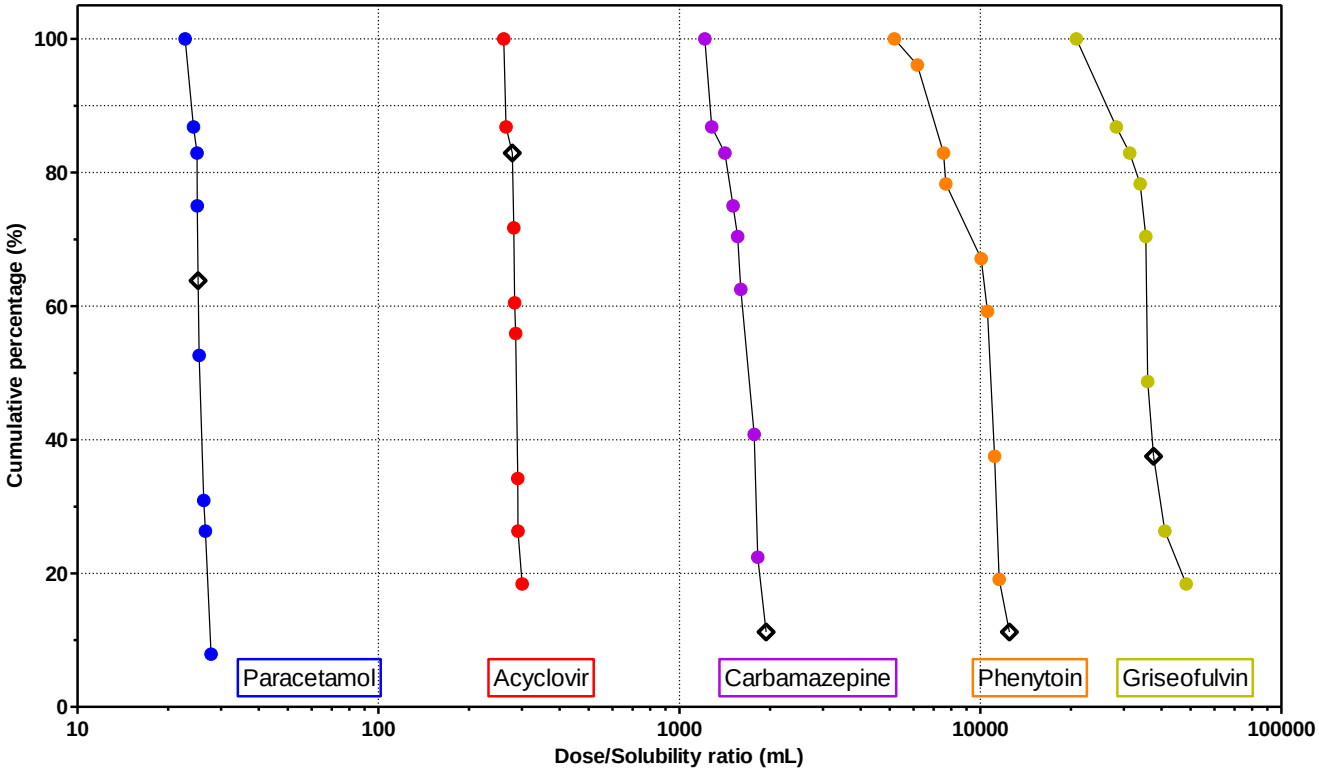
3.4.1.3. Fasted Solubility Frequency Distributions

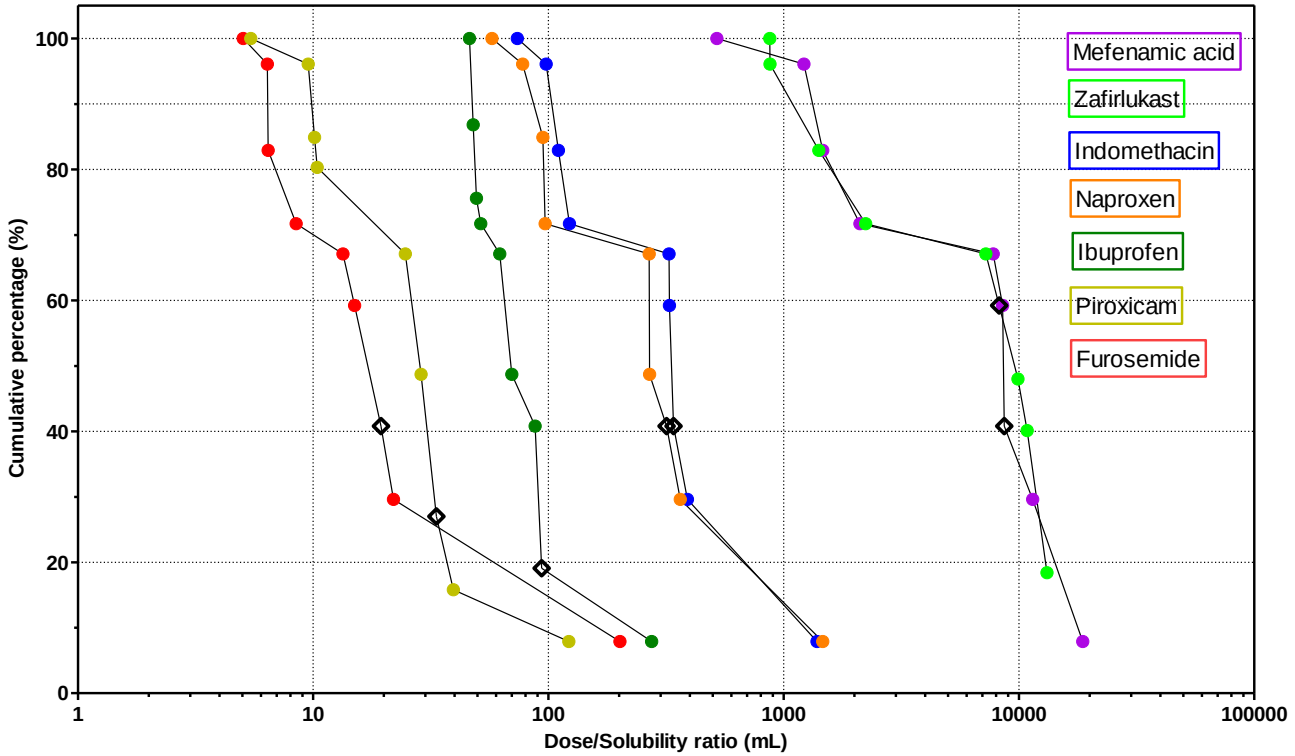
The bioequivalent point compositions were calculated to describe the compositional variation present in the 152 FaHIF samples within the analysed data set (Pyper et al., 2020). Through the application of 5-dimensional Euclidean space, it is possible to calculate the proximity of each data set point to an individual bioequivalent point composition to produce a frequency distribution based on the number of data set points closest to each bioequivalent point. Since the study has measured the equilibrium solubility of each bioequivalent point, this can then be converted to a dose/solubility volume frequency distribution, see Figure 3.3. a - d. It should be noted that this frequency distribution arises from the sampled FaHIF point compositions (Pyper et al., 2020, Riethorst et al., 2016), and cannot be related to measure *in vivo* pharmacokinetic variability at this stage (Sugihara et al., 2015). NB Drugs split between Figures on basis of presentation clarity.

In Figure 3.3.a the distribution for acyclovir, carbamazepine, griseofulvin, paracetamol, and phenytoin, is presented. Based on the presentation in Figure 3.1 and associated discussion in section 3.4.1.1, all the five drugs have very narrow frequency distributions with almost vertical cumulative lines, related to the very narrow solubility range (solubility multiple < 2.5) for these drugs. Drugs in Figure 3.3. b - d have a broader distribution range, and the points presented in Figure 3.3. a – d are not evenly distributed on the cumulative plot as centre point towards the lower end of the cumulative plot of 16 drugs out of 21. Only for paracetamol in Figure 3.3.a, and carvedilol in Figure 3.3.c does the centre point occur in the middle of the distribution. For the acidic drugs presented, the distribution was predominantly controlled by pH (see section 3.4.1.2 and Figure 3.2), but also display the same characteristics previously described with points not evenly distributed and centre point towards the lower end of the cumulative plot. The acidic drugs also exhibit an increased degree of structure (see Figure 3.3.b) in the cumulative plot with steps indicative of peaks in the distribution.

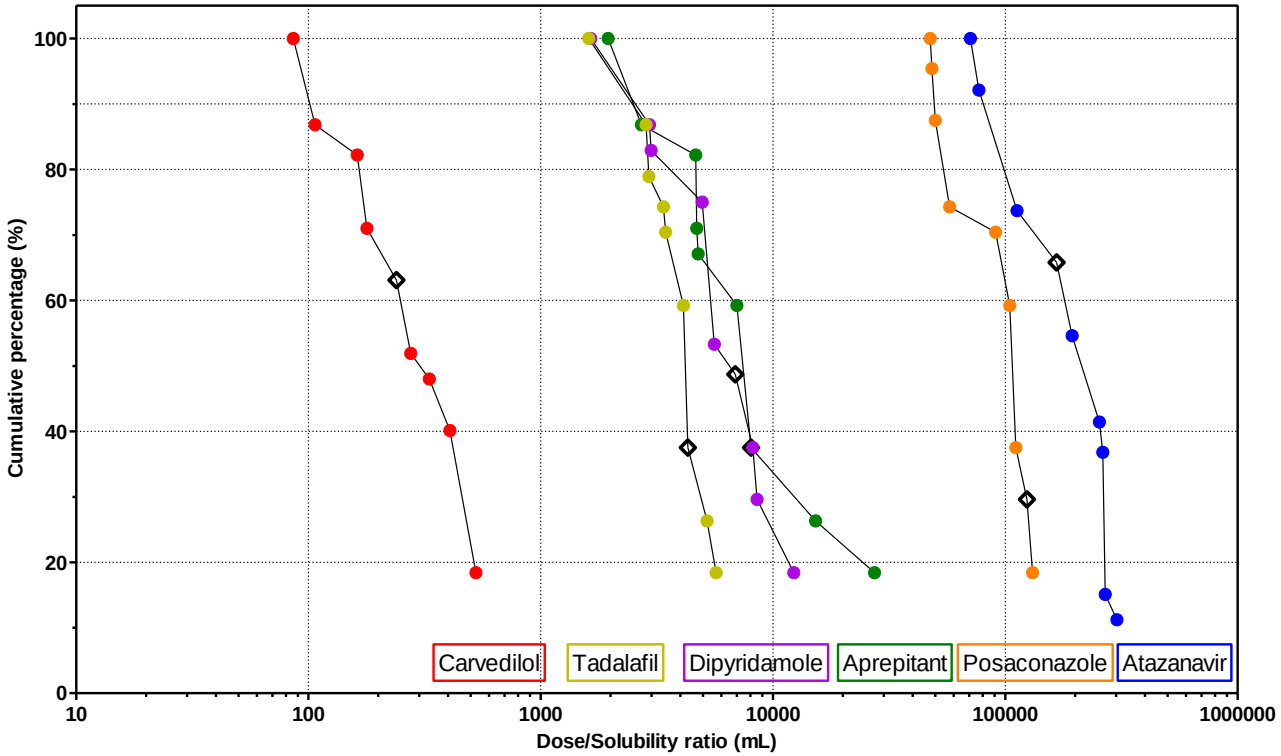
Statistical analysis of the distributions either for normal or log normal behaviour did not produce significant results. Previous statistical analysis of fasted SIF DoE solubility distributions (Ainousah et al., 2017, Perrier et al., 2018) highlighted that the distributions were not normal, also the FaHIF data points used to calculate the

bioequivalent points (Pyper et al., 2020) were not normally distributed. This result might reflect the well-known variability of these fluids (Elvang et al., 2018, Elvang et al., 2019), and the measurement of solubility in them (Clarysse et al., 2011, de la Cruz-Moreno et al., 2017, Zhou et al., 2017b). Within this bioequivalent system, and presumably HIF as well, the traverse from low to high solubility points is not a simple vector based on a single concentration of a media component, where a solubilisation relationship might be expected (Naylor et al., 1993, Pedersen et al., 2000b), but a five-dimensional (Dunn et al., 2019) (and in HIF more) transit through a complex compositional space. Therefore, the lack of an organised statistical distribution when traversing the solubility range based on individual discrete points is to be expected. This might represent an evolutionary aspect to HIF providing variability that maximises nutrient solubilisation, but also impacts administered drugs. This highlights why a single HIF aspirate will not be representative of the entire HIF space, and single measurements limited by a lack of knowledge of the sample's position in the space, which will be further complicated when drug properties are superimposed. This makes prediction difficult and points that knowledge of the solubility distribution via measurement is required with the information potentially useful for performing, as discussed, a risk analysis for the likely impact of solubility variability on absorption behaviour.


a
Cumulative percentage (%)
100
80
60
40
20
0
10
100
1000
10000
100000
Dose/Solubility ratio (mL)
Paracetamol
Acyclovir
Carbamazepine
Phenytoin
Griseofulvin


b
Cumulative percentage (%)
Dose/Solubility ratio (mL)
0
20
40
60
80
100
1
10
100
1000
10000
100000
Mefenamic acid
Zafirlukast
Indomethacin
Naproxen
Ibuprofen
Piroxicam
Furosemide

C


Cumulative percentage (%)
100
80
60
40
20
0
10
100
1000
10000
100000
1000000
Dose/Solubility ratio (mL)
Carvedilol
Tadalafil
Dipyridamole
Aprepitant
Posaconazole
Atazanavir

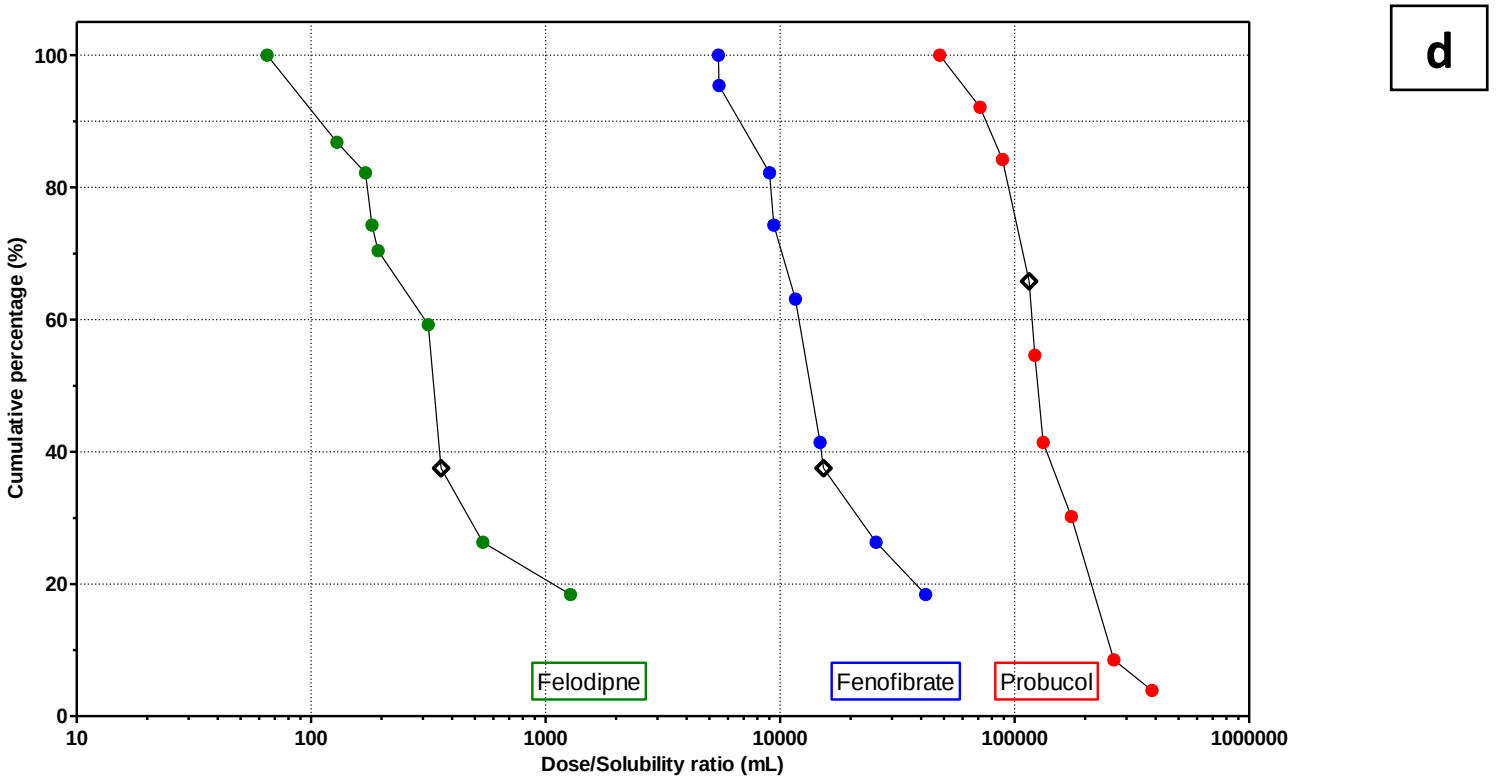


Figure 3.3: a – d, Cumulative percentage incidence of HIF data points. ● Bioequivalent data points, ◇ Bioequivalent centre point. Each point mean n = 3. Figure b is labelled by colour.

3.4.1.4. Solubility Limited Absorbable Dose Distribution

By applying the biopharmaceutical calculations detailed in Chapter 1, a solubility limited absorbable dose (SLAD) and a target particle size to avoid dissolution rate limiting issues were calculated as per literature (Butler and Dressman, 2010, Rosenberger et al., 2018) see Table 3.3. The calculation has been applied to the measured centre point and lowest solubility value as a worst-case situation, using the permeability values for each drug from the literature, as detailed in Table 3.1, and standard values for other properties. A comparison between the outputs arising from the centre point and lowest solubility measurements found that for the narrow solubility distribution drugs (acyclovir, carbamazepine, griseofulvin, paracetamol, and phenytoin) there is a minimal difference between the values, whilst for the other drugs the difference reflects the discussion in section 3.4.1.1. This hints that a narrow intestinal solubility range might be a useful drug development target, since the drug would then be intrinsically resistant to intestinal solubility variability. In this study, it is recognised that this interpretation might be an unrealistic target based on current medicinal chemistry structures.

For 9 out of 21 drugs (furosemide, ibuprofen, indomethacin, naproxen, piroxicam, carvedilol, carbamazepine, felodipine, and paracetamol), the calculated lowest SLAD is above the administered dose (Table 3.3), and therefore minimal solubility-based absorption issues are possible, reflective of their positions on the BCS/DCS grid. For the other drugs, the calculated lowest SLAD is below the dose, therefore solubility and dissolution rate limiting issues are likely to occur upon oral administration. For acidic and basic drugs, modifications could be applied to account for pH changes during transit through the gastric compartment and down the intestinal tract (Koziolek et al., 2015, Tsume et al., 2014). Investigation of intestinal tract pH indicates that this source of variation in the upper tract diminishes as material transits down the tract. Since the neutral drugs are not ionisable, a pH-based adaptation is not applicable. However, for 11 drugs (out of 12) even the SLAD centre point calculation (see Table 3.3) highlights a solubility issue with respect to the dose, tadalafil SLAD centre point was equal to the dose. By calculating the SLAD values for all bioequivalent points and linking to the cumulative percentage incidence (see section 3.4.1.3), it is feasible to determine

where solubility limitations no longer apply. This is presented in Figure 3.4 a – c for 12 drugs, drugs separated to aid graphical display.

The plots of 8 out of 12 drugs (Figure 3.4 b and c) do not reach the required oral dose value (Table 3.1). Whereas the plots of 4 out of 12 drugs, indicate that solubility limitations will only be resolved in approximately thirty percent for mefenamic acid, tadalafil, and zafirlukast, and in fifteen percent of aprepitant, of FaHIF compositions (see short vertical lines Figure 3.4 a). This information could be applied for a risk assessment-based approach to development and formulation. This represents a further advantage of solubility range knowledge and frequency distribution within the range to assess solubility associated biopharmaceutical issues, especially where the drug crosses a classification boundary. As above investigation of more drugs will reveal further candidates where this scenario is likely to arise, refine the compositional calculations for the bioequivalent points, and link *in vitro* solubility to *in vivo* pharmacokinetics.

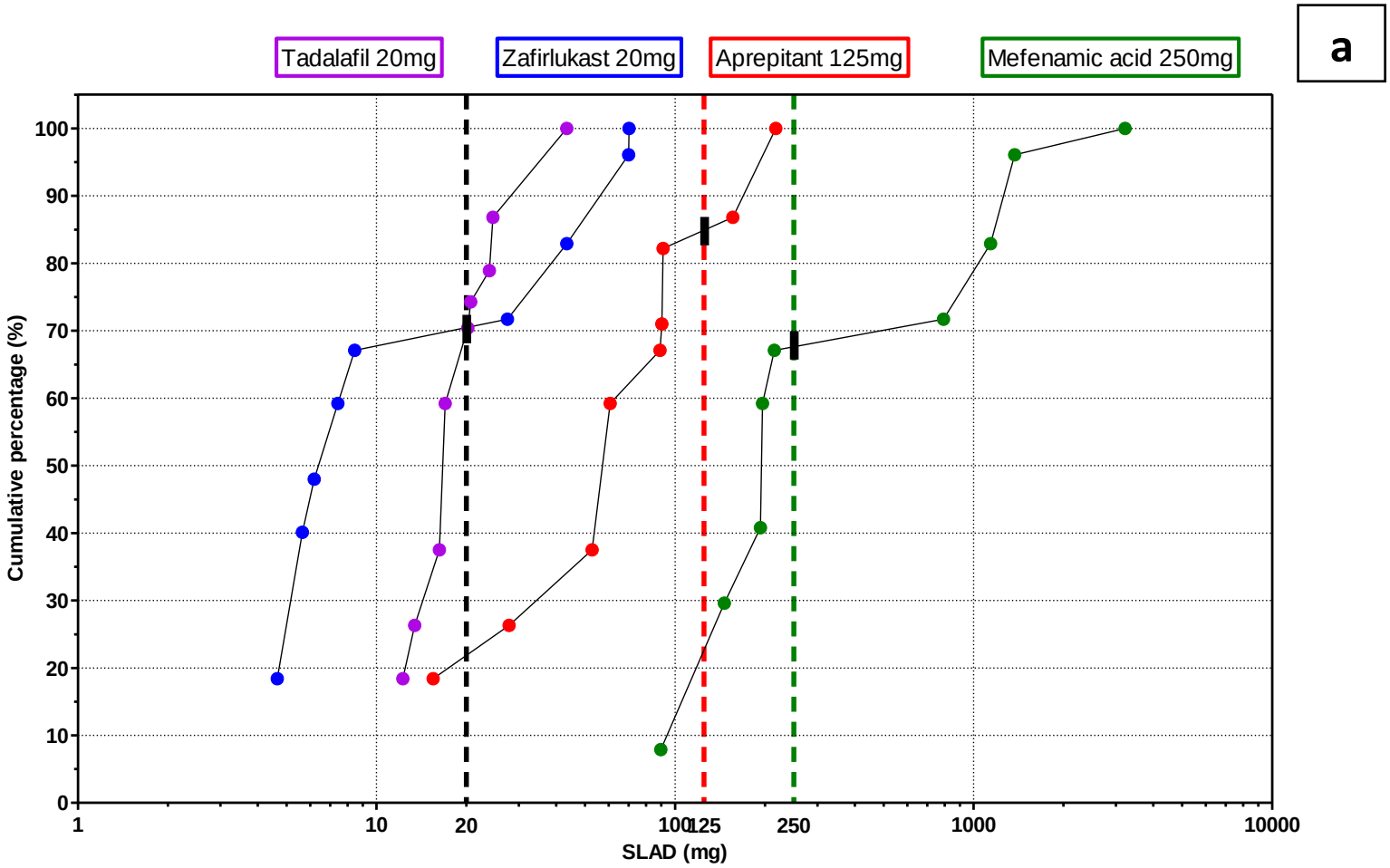
Table 3.3: Calculated biopharmaceutical data.

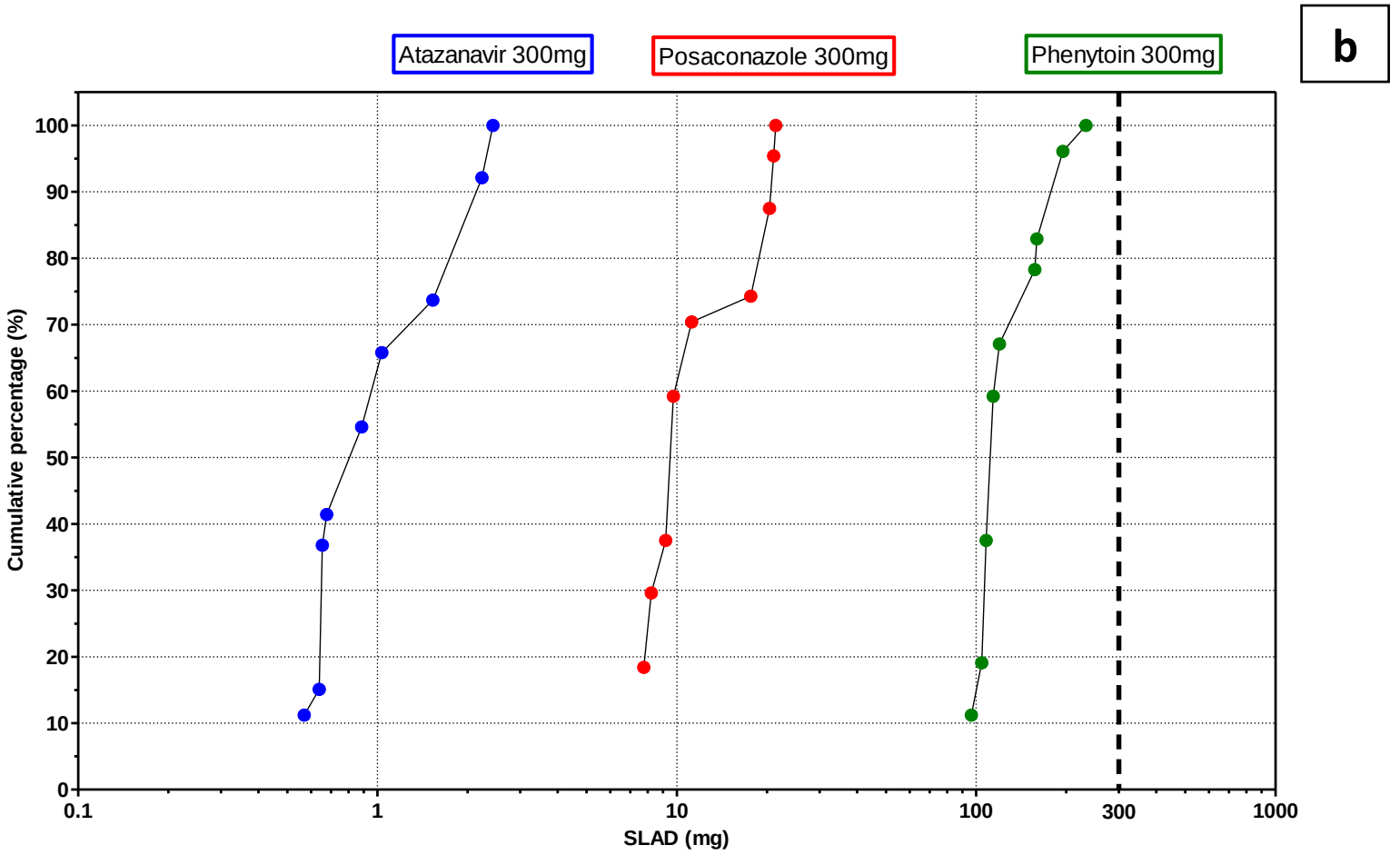
Drug	SLAD (mg)		Particle radius (μm)	
	Centre point solubility	**Minimum solubility**	**Centre point solubility**	**Minimum solubility**
Furosemide 80 mg	1714	114	299	77
Ibuprofen 400 mg	36969	8356	310	148
Indomethacin 200 mg	2202	518	96	46
Mefenamic acid 250 mg	215	90	22	14
Naproxen 1000 mg	15170	2771	236	101
Phenytoin 300 mg	120	96	21	19
Piroxicam 20 mg	2599	525	110	49
Zafirlukast 20 mg	8.5	4.7	6.4	4.8
Aprepitant 125 mg	89	15	20	8.3
Atazanavir 300 mg	0.89	0.57	4.8	3.9
Carvedilol 25 mg	340	154	40	27
Dipyridamole 100 mg	13	5.8	16	11
Posaconazole 300 mg	11	7.8	7.0	5.8
Tadalafil 20 mg	20	12	9.3	7.2

Drug	SLAD (mg)		Particle radius (μm)	
	Centre point solubility	**Minimum solubility**	**Centre point solubility**	**Minimum solubility**
Acyclovir 800 mg	337	319	205	200
Carbamazepine 300 mg	395	318	54	48
Felodipine 10 mg	193	29	28	11
Fenofibrate 160 mg	51	14	14	7.6
Griseofulvin 500 mg	59	43	15	12
Paracetamol 500 mg	12357	11183	545	518
Probucol 500 mg	13	4.0	7.8	4.4

Solubility Limited Absorbable Dose – SLAD = $S_{si} \times V \times An$, where S_{si} is the intestinal solubility (mg/mL) measurement as indicated in column header (see Table 3.1), V is the volume of intestinal fluid (500 mL), and An is the absorption number ($An = P_{eff} \times T_{si} / R$) where P_{eff} is the effective permeability of the intestine to the drug (see Table 3.1), T_{si} is the small intestinal transit time (3.32 hr) and R is the intestinal radius (1.25 cm).

Particle radius (r) $=\sqrt{(3D \times S_{si} \times T_{si}/(Dn \times \rho))}$, where D is the diffusion coefficient (typically at 5×10^{-6} cm/s), Dn is the dissolution number (set to 1) and ρ is the drug density (typically 1.2 $g.cm^{-3}$).


a
Tadalafil 20mg
Zafirlukast 20mg
Aprepitant 125mg
Mefenamic acid 250mg
Cumulative percentage (%)
SLAD (mg)
0
10
20
30
40
50
60
70
80
90
100
1
10
20
100
125
250
1000
10000


b
Atazanavir 300mg
Posaconazole 300mg
Phenytoin 300mg
Cumulative percentage (%)
SLAD (mg)
0
10
20
30
40
50
60
70
80
90
100
0.1
1
10
100
300
1000

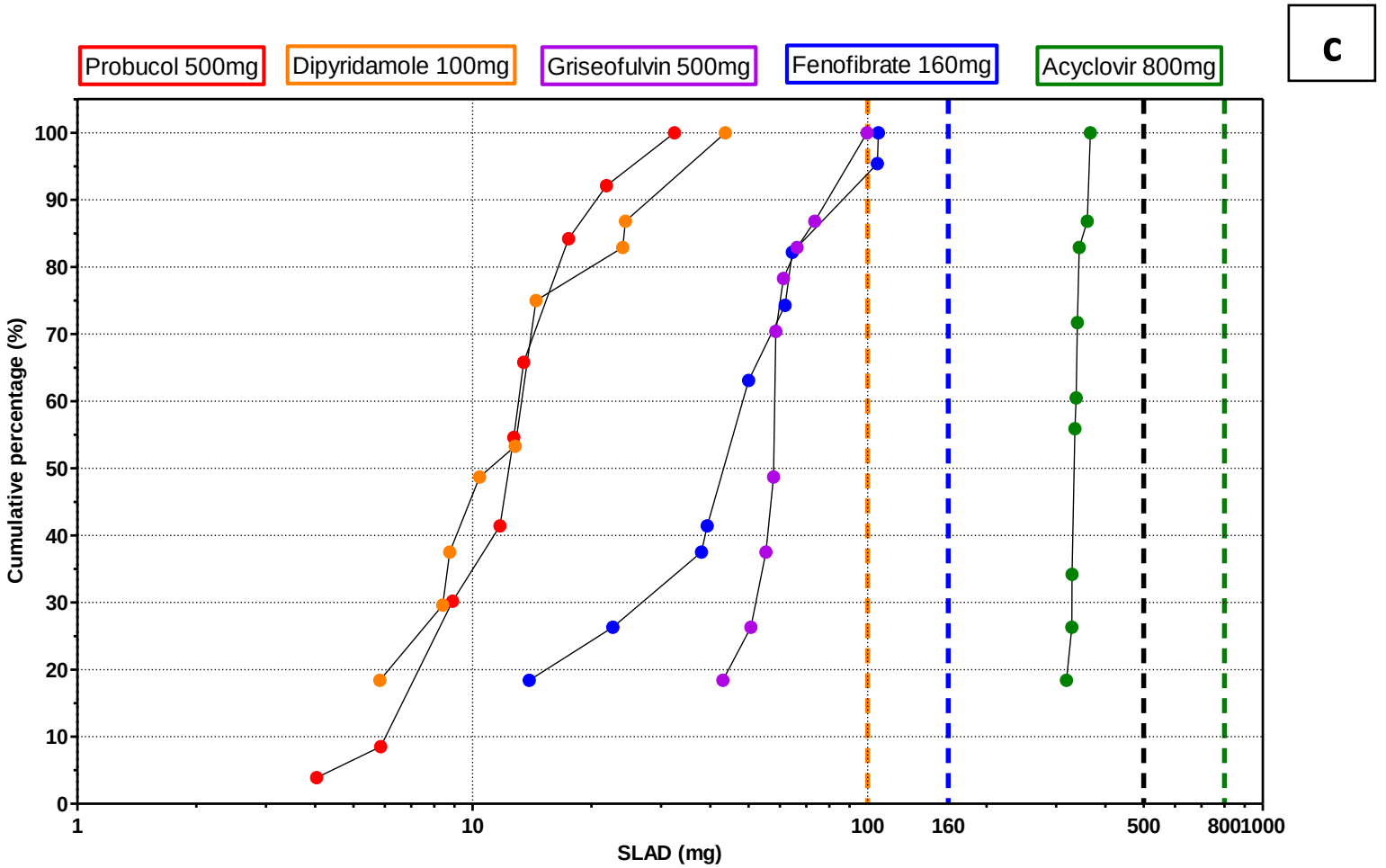


Figure 3.4: Cumulative percentage incidence of solubility limited absorbable dose. Drugs names are colour coded. Vertical line drug dose, value as indicated.

3.4.2. Structured Solubility Behaviour

3.4.2.1. Solubility Analysis

The multidimensional analysis of FaHIF composition included five factors pH, bile salt (BS), phospholipid (PL), fatty acid (FA) and cholesterol (Pyper et al., 2020), and in order to plot solubility data on x-y coordinates each media recipe has been reduced to a single value by either calculating the total amphiphile concentration (TAC) (mM) multiplied by the media pH value (Table 2.2), or using pH alone. The rationale for the former unusual data manipulation is based on three behaviour properties of these media systems. First, previous studies have used the TAC to correlate solubility, either individually (Pedersen et al., 2000b), or in combination (Ilardia-Arana et al., 2006). Second, the fasted DoE study (Khadra et al., 2015) indicated that for basic and neutral drugs the media components' (pH, FA, BS, and PL) standardised effect values on solubility were similar, and that the majority of significant 2-way interactions involved pH along with an ionisable amphiphile. Finally, a topographical analysis of solubilisation in simulated fluids, that employed a four component (BS, PL, FA and monoglyceride) mixture design with varying pH and TAC (Dunn et al., 2019), noted that solubility generally increased as both TAC and pH increased. The pH × TAC manipulation is not completely applicable to acidic drugs where in the fasted DoE, the standardised effect value for pH dominated solubility behaviour by a factor of twenty-fold greater than any of the amphiphilic media factors. Therefore, for acidic drugs a plot using media pH only is presented for the more soluble drugs and comparison, (pH and pH × TAC) plots for poorly soluble.

A representative plot of solubility against pH × TAC is presented in Figure 3.5, which illustrates the data structure of the media compositions induced by the multidimensional analysis. Media 1 and 2 are based on the major axis of the multidimensional ellipse that described the FaHIF data cloud (Pyper et al., 2020), and 3 and 4 on the minor axis. Media points 5 and 6 and 7 and 8 are based on further major and minor axes in other dimensions and the eight points cover 96.4% of the variability in the HIF samples. The statistical analysis means that media are arranged in approximately two columns of three, based on pH × TAC values (3, 5 and 7 and 4, 6 and 8) with the solubility measured in each media dependent upon the drug under investigation.

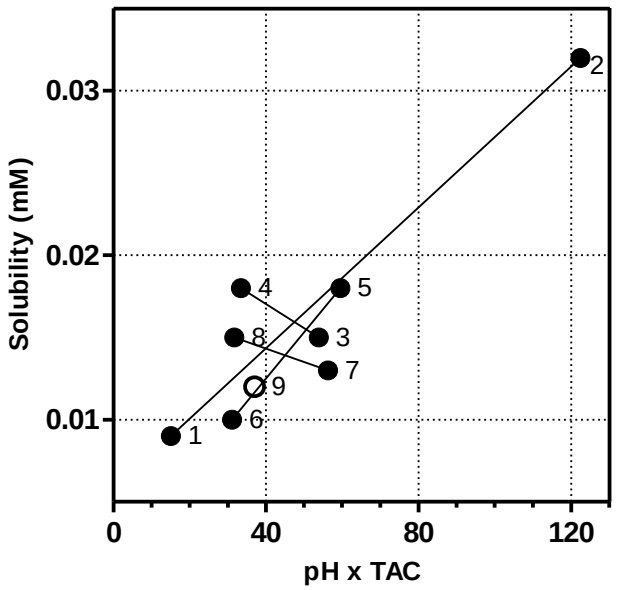


Figure 3.5: Representative plot of Solubility vs pH × TAC. Point label indicates media number (see Table 2.2) (Pyper et al., 2020).

3.4.2.2. Acidic Drugs

a. Solubility behaviour

The solubility plots for the acidic drugs are presented in Figure 3.6 a and b. Visual analysis indicates that there is a relationship of increasing solubility with media pH, and the drugs generally have a very similar behaviour with variation linked in the main to pKa but with minor influences from other amphiphilic media components. For example, the solubility ranking of media 2, 6 and 7 is not identical for all the drugs even although the pH values (7.12, 7.14 and 7.34 respectively) are very similar. For the majority of the drugs, the pKa value (Table 2.1) is below the pH of the lowest media (Table 2.2) and therefore, the measurement is of ionised drug solubility in the media. For piroxicam, the pKa value (6.4) is within the media range, and for phenytoin, the pKa (8.3) is greater than the highest media pH value. This pH dependent solubility behaviour is described as Category 1 in Table 3.4, and it's reinforced by the high correlation value of each drug, see Figure 3.6 a.

The solubility of phenytoin and zafirlukast in Figure 3.6 b is also presented based on the media pH × TAC value, since for both of these drugs, media 2 has the highest pH

× TAC value, which is providing a solubility greater than expected based simply on pH. This indicates that for these two drugs, solubilisation by the amphiphilic components is important, and this is reflected by the lower correlation values present in Figure 3.6 b. See Category 2 in Table 3.4.

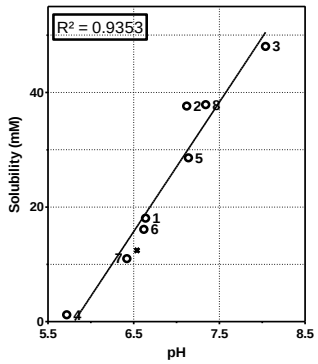
b. Solubility behaviour analysis

This solubility behaviour is consistent with previous literature for acidic drugs in intestinal media (Clarysse et al., 2009), and with the various DoE studies (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020, Perrier et al., 2018) in fasted simulated media that identify pH as the major media component driving solubility. The initial DoE study (Khadra et al., 2015) reported that for acidic drugs the average standardised effect value of pH on solubility was twenty times greater than any of the individual amphiphilic media components, an observation that is replicated by the behaviour in this study with these different simulated media recipes. The DoE also identified 2-way interactions between media components (Khadra et al., 2015), however, for acidic drugs the largest interaction was between pH and FA, but with a magnitude around a tenth of pH alone. Therefore, variations in concentrations and interactions between the amphiphilic media components are not interfering with the major solubility driver pH.

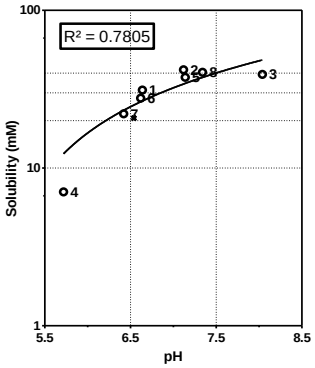
The initial data analysis performed by applying a DoE structure to the media, did detect pH as a significant factor for indomethacin, naproxen, and piroxicam (Chapter 2), but with only nine data points, the significance of other factors is, due to statistical limitations, not detected (McPherson et al., 2020). Furosemide, ibuprofen, and mefenamic acid, have not been assessed in the large sized DoE protocol, therefore it is not possible to analyse their solubility behaviour with respect to known standardised effect values for the media components. However, the consistent distribution of the points in Figure 3.6 a indicates that these drugs are behaving in a similar manner to the drugs that have been subjected to a DoE investigation. The point distribution may therefore represent a useful tool for determining major features of drug solubility behaviour without conducting a DoE.

Acidic drugs (a)

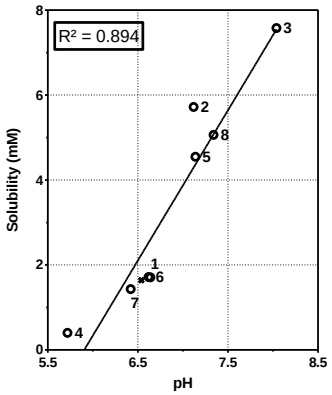
Furosemide, solubility ratio 39.95



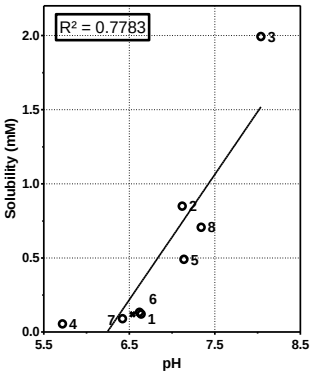
Ibuprofen, solubility ratio 5.93



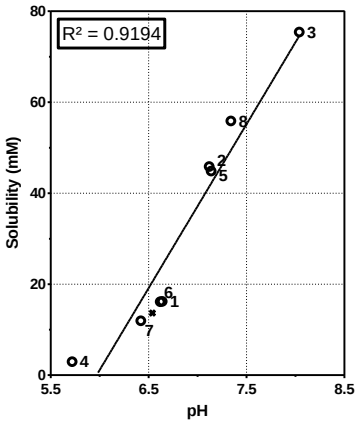
Indomethacin, solubility ratio 18.79



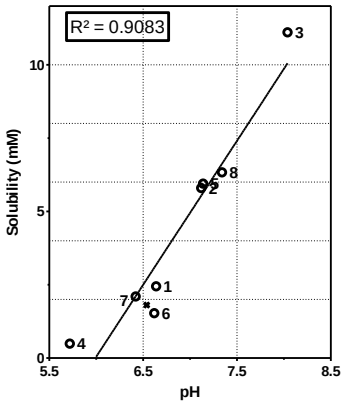
Mefenamic acid, solubility ratio 38.91



Naproxen, solubility ratio 25.46



Piroxicam, solubility ratio 22.49



Acidic drugs (b)

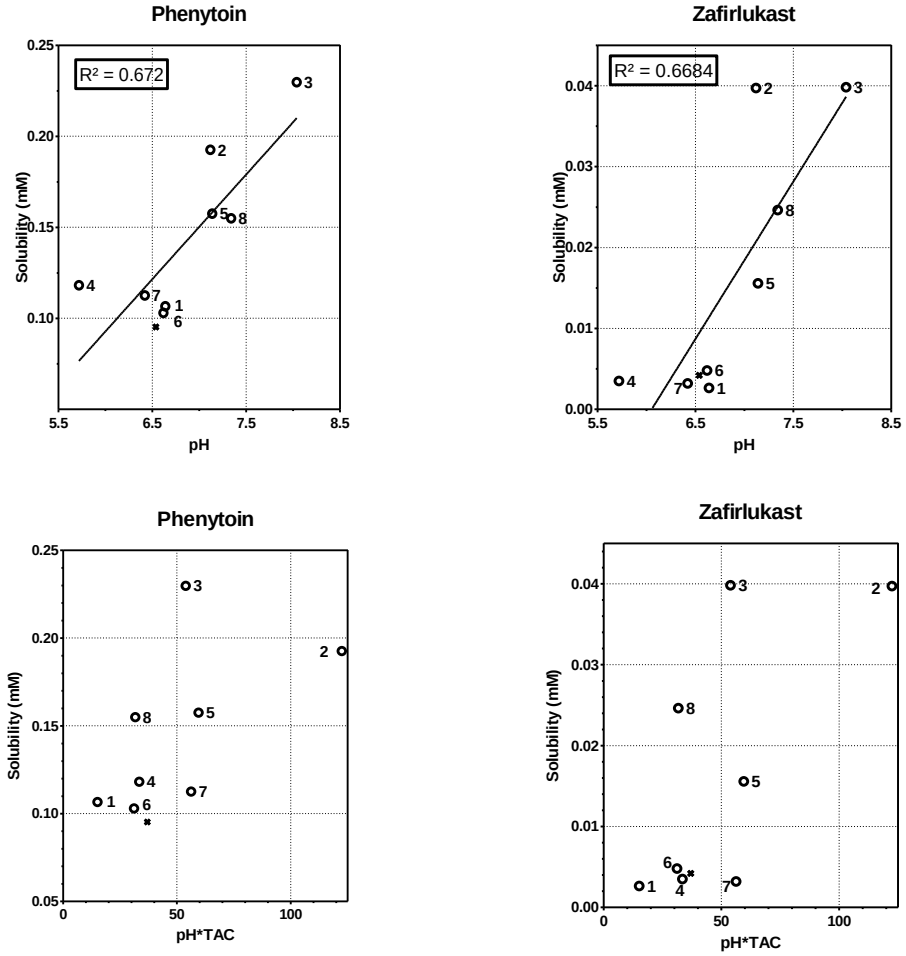


Figure 3.6: a and b. Acidic drugs – solubility plot. Point label indicates media number (see Table 2.2); x centre point - media 9. Each mean solubility point n = 3. Calculated solubility ratio (highest solubility/lowest solubility) value in text. R^2 = correlation coefficient of linear straight line.

c. Media frequency analysis

In Figure 3.7 the frequency of each media recipe as either the highest or lowest solubility value for an acidic drug is presented. In 7 out of 8 cases (88%), media number 3 provides the highest solubility value and is not surprisingly the media with the highest pH value (Table 2.2). The one exception is ibuprofen, where media 2 provides the highest solubility, examination of the data indicates that there is minimal solubility difference (media 2 = 42 mM, media 3 = 39 mM) between the media and for ibuprofen media numbers 3, 5, 8 and 2 are very similar. In 6 out of 8 cases (75%) media number 4 provides the lowest solubility value, and again this is not surprising since this media has the lowest pH value. The exceptions are phenytoin and zafirlukast, where the lowest solubility media are number 9 (centre point) and 1 respectively. For phenytoin, due to its pKa (8.3) this represents the interaction of the unionized molecule with the media components, and overall is an unusual result since media 9 or the centre point usually is located within the point cloud, see other Figures. However, phenytoin is a class of drug that has a very narrow solubility distribution (solubility ratio = highest solubility/lowest solubility) in FaSSIF systems (Khadra et al., 2015), and the solubility values for media systems 1, 4, 6 and 7 are very similar, see category 3 in Table 3.4. Zafirlukast is acidic but is known to be very poorly soluble in aqueous systems (Dekhuijzen and Koopmans, 2002), therefore, the lowest solubility in media 1 even although it's pH is higher than the pKa (pH 6.6 vs pKa 4.1), can be rationalised due to the low pH × TAC value of the media, see next section. In a similar manner to phenytoin, for zafirlukast the solubility in media systems 1, 4, 6 and 7 are very similar, therefore even if the lowest pH value media (number 4) was applied, a low solubility value would be determined.

Table 3.4: Biorelevant fasted simulated intestinal fluids – solubility behaviours.

Category	**1- pH - controlled (TAC minimal variation)**	**2- pH × TAC controlled**	**3- Minimal pH × TAC controlled**	**4- non-categorised solubility behaviour (Drug controlled)**
Solubility Behaviour	Solubility increases with increasing pH, minimal impact from amphiphilic media components	Gross solubility increases with increasing pH and total amphiphile content, solubility granularity controlled by individual drug interactions with media components	Minimal impact of media components on solubility	No gross solubility relationship between pH and total amphiphile content, drug dependent behaviour, increasing pH and total amphiphile content might reduce solubility
Description	Acidic drugs pKa < 6.4[A]	Basic, neutral drugs, and weak acidic drugs pKa > 8[B]	Neutral drugs[C]	Basic and neutral drugs – categorisation based on solubility behaviour
Drugs	Furosemide, Ibuprofen, indomethacin, mefenamic acid, naproxen, piroxicam, and zafirlukast	Aprepitant, carvedilol, dipyridamole, posaconazole tadalafil, felodipine, fenofibrate, griseofulvin, and phenytoin	Acyclovir, carbamazepine, griseofulvin[D], paracetamol, and phenytoin[D]	Atazanavir, and probucol
Comment	Five out of seven examples from non-steroidal anti-inflammatory therapeutic category, expansion into other therapeutic modalities required	Examples varied physicochemical properties; increased drug examples required	Increased drug examples required	Insufficient data for conclusive analysis, increased drug examples required

Category	**1- pH - controlled (TAC minimal variation)**	**2- pH × TAC controlled**	**3- Minimal pH × TAC controlled**	**4- non-categorised solubility behaviour (Drug controlled)**
Lowest Solubility Media[E] number and Frequency	4 86% 6 out of 7 examples	1 89% 8 out of 9 examples	1 40% 2 out of 5 examples	Not assigned
Highest Solubility Media[E] Number and Frequency	3 86% 6 out of 7 examples	2 78% 7 out of 9 examples	2 80% 4 out of 5 examples	Not assigned
Mean Solubility Ratio	23.4 ± 11.8/34.1 (n = 7)	7.34 ± 5.97/17.4 (n = 9)	1.74 ± 0.597/1.26 (n = 5)	-

TAC: Total Amphiphile Concentration; A: Based on highest pKa of acidic drugs measured – piroxicam; B: Based on the single example of phenytoin. C: Category could include acidic and basic drugs that have pKa values outside of the media pH ranges – see drugs section; D: Added to category based on solubility ratio < 2.5 – phenytoin and griseofulvin therefore in category 2 and 3; E: Values are not equal to Figure 3.8, consult drugs list for values included in each category.
Mean Solubility Ratio = (Highest/Lowest) ± Standard Deviation/Ratio Range.

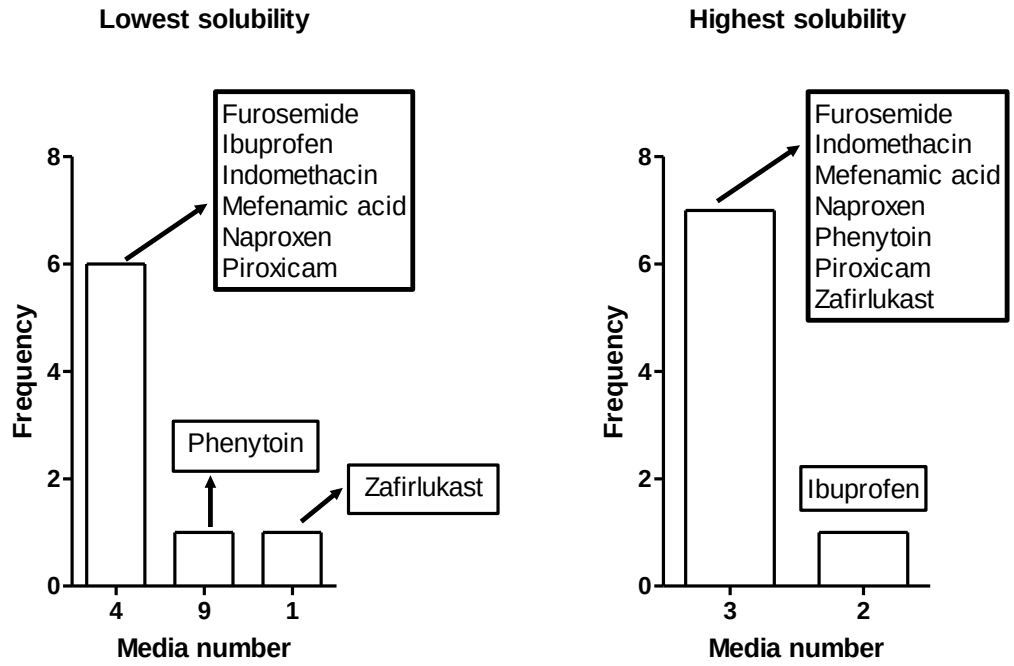


Figure 3.7: Acidic drugs – lowest and highest solubility media frequency of lowest and highest solubility media for drugs in Figure 3.6 a and b. Drugs as listed in boxes.

3.4.2.3. Basic and Neutral Drugs

a. Solubility behaviour

The solubility plots for the basic drugs are presented in Figure 3.8 a and b, with the neutral drugs in Figure 3.9 a and b. Visual analysis indicates that there is a general structure with the lowest solubility measured in media 1, the highest in media 2, and spatial arrangement of the intermediate media similar, although unlike, the acids, this is not identical or consistent between drugs. There are also noticeable exceptions in atazanavir and probucol. The data has been transformed into a spider or polar plot in Figures 3.10 and 3.11, where solubility is normalised to the highest value (set to 100), and arranged in a clockwise order around the plot starting at 12 o'clock with the lowest pH × TAC media value (media 1, see Table 2.2) and running to the highest (media 2). This also highlights further noticeable exceptions in addition to atazanavir and probucol, with acyclovir, carbamazepine, and paracetamol, displaying an almost circular polar plot. A universal solubility behaviour is not evident, but three categories can be identified.

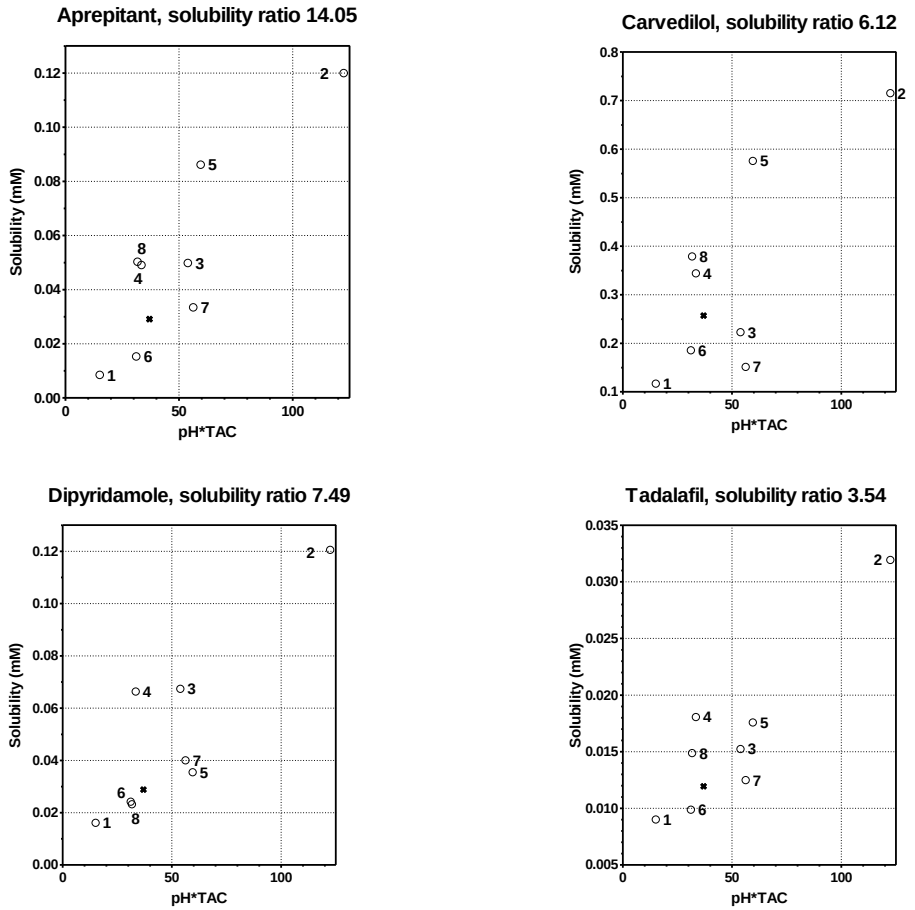
For aprepitant, carvedilol, dipyridamole, felodipine, fenofibrate, griseofulvin, posaconazole, and tadalafil, there is a general increase in solubility from media 1 around the plot to media 2, but the increase is not smooth and there are variations in the profile. This variation is evident in Figure 3.8 and 3.9, where for example in Figure 3.8 a, the solubility rank (highest to lowest) for aprepitant, carvedilol and tadalafil is of media 5, 3 and 7, but for dipyridamole is 3, 7 then 5. A similar variation analysis can be applied to acyclovir, carbamazepine, felodipine, fenofibrate, griseofulvin, paracetamol, and posaconazole, and the other media numbers 4, 6, and 8. This solubility behaviour pH × TAC dependent, is described in Category 2 in Table 3.4.

The circular polar plots for acyclovir, carbamazepine, and paracetamol, indicate that there is minimal variation in solubility with changing media (see solubility range values in Figure 3.9 b compared to other similar Figures. 3.6 and 3.8), a feature that has been previously highlighted for griseofulvin and phenytoin. This behaviour was also evident in the original DoE (Khadra et al., 2015), and a comparison drug (see Figures 3.6 a, 3.8 a and 3.9 a) also indicates a small solubility range is measured. As discussed previously, the identification of this solubility property is a feature of measurement using multiple media, and there is no direct literature comparison available. However, the reported standard deviation for fenofibrate solubility in multiple pooled FaHIF samples is one hundred and thirty two percent (Clarysse et al., 2011), whilst for griseofulvin it is twenty nine percent (Annaert et al., 2010). An arbitrary ratio cut off at phenytoin (solubility ratio 2.41) has been applied and this solubility behaviour, which is a subset of category 2, with a low solubility ratio is described in Category 3 in Table 3.4. This subset categorisation might be excessive, since it is based on a limited number of examples.

For probucol media 1 has a higher solubility than media 2, and the polar plot shape is unique amongst the neutral drugs with a flat top (between media 1 and 6) and a point out to media 4. Atazanavir has a very similar shape and although it is basic, has a pKa value (4.52) below the pH of the lowest pH media, and therefore the solubility measurements are on the neutral molecule. This behaviour is counter intuitive, probucol has the highest log P (11.3), and solubility would be expected to increase with TAC, see next section. The solubility is therefore not linked to pH × TAC, and is

described in Category 4 in Table 3.4. With only two examples, further research and examples are required.

Basic drugs (a)



Basic drugs (b)

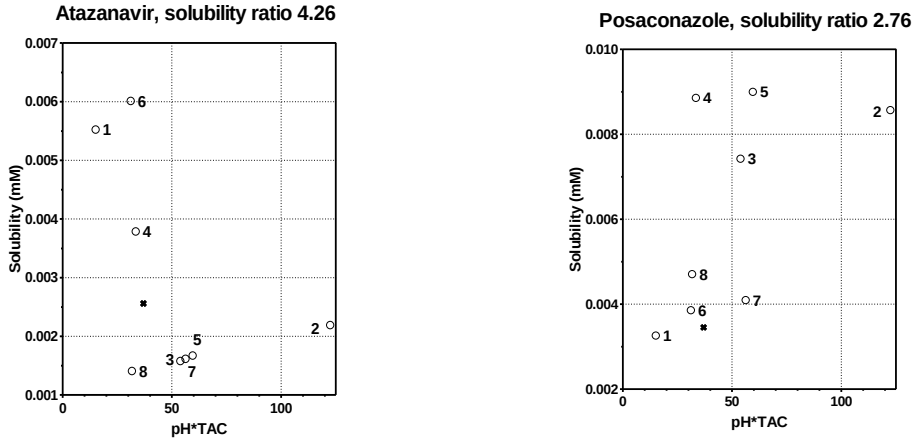
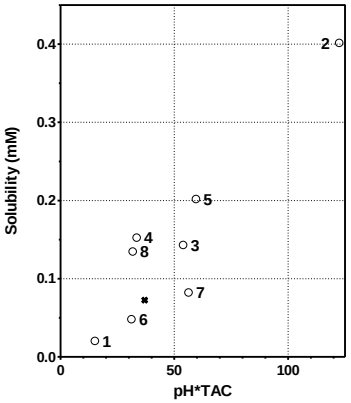


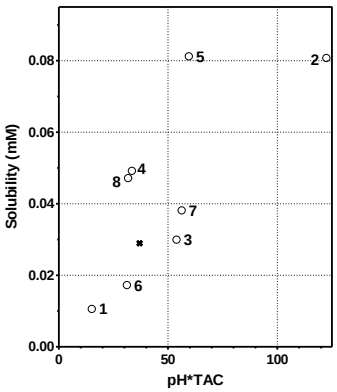
Figure 3.8: a and b. Basic drugs – solubility plot. Point label indicates media number (see Table 2.2); x centre point - media 9. Calculated solubility ratio (highest solubility/lowest solubility) value in text.

Neutral drugs (a)

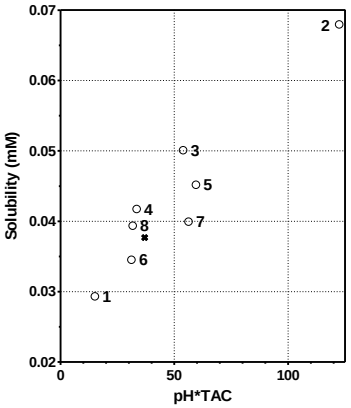
Felodipine, solubility ratio 19.69



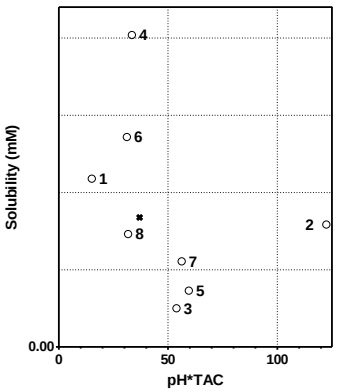
Fenofibrate, solubility ratio 7.65



Griseofulvin, solubility ratio 2.32



Probucol, solubility ratio 8.05



Neutral drugs (b)

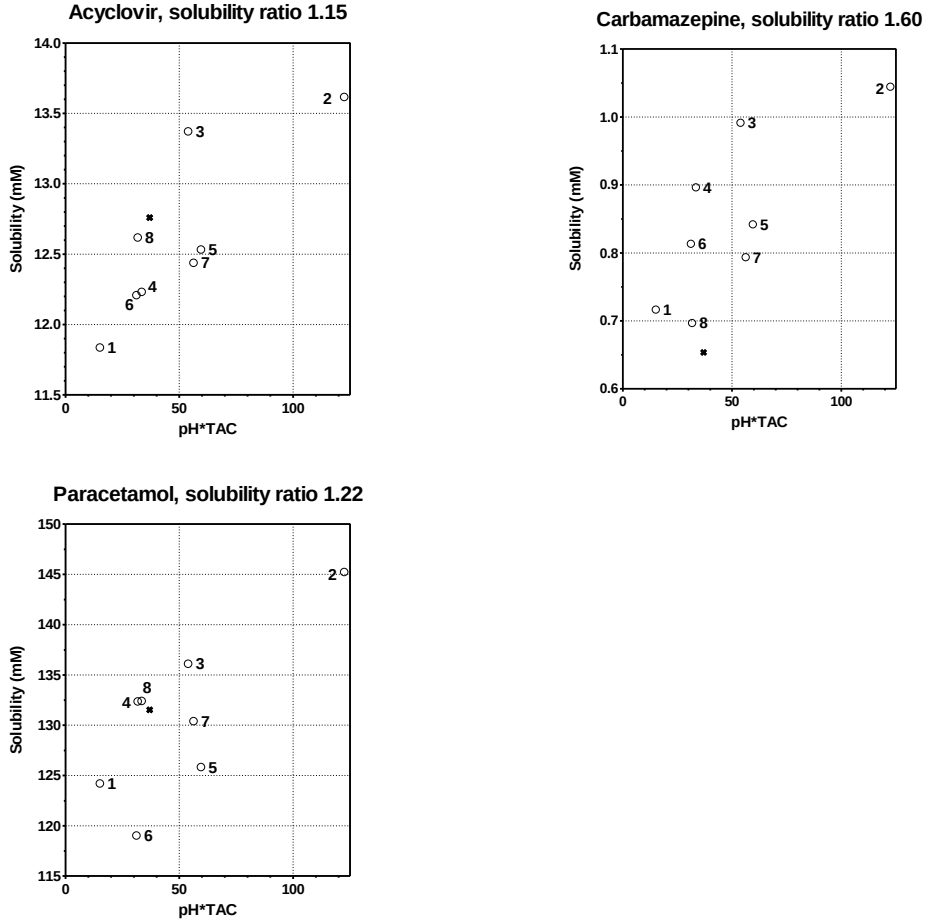


Figure 3.9: a and b. Neutral drugs – solubility plot. Point label indicates media number (see Table 2.2); x centre point - media 9. Calculated solubility ratio (highest solubility/lowest solubility) value in text.

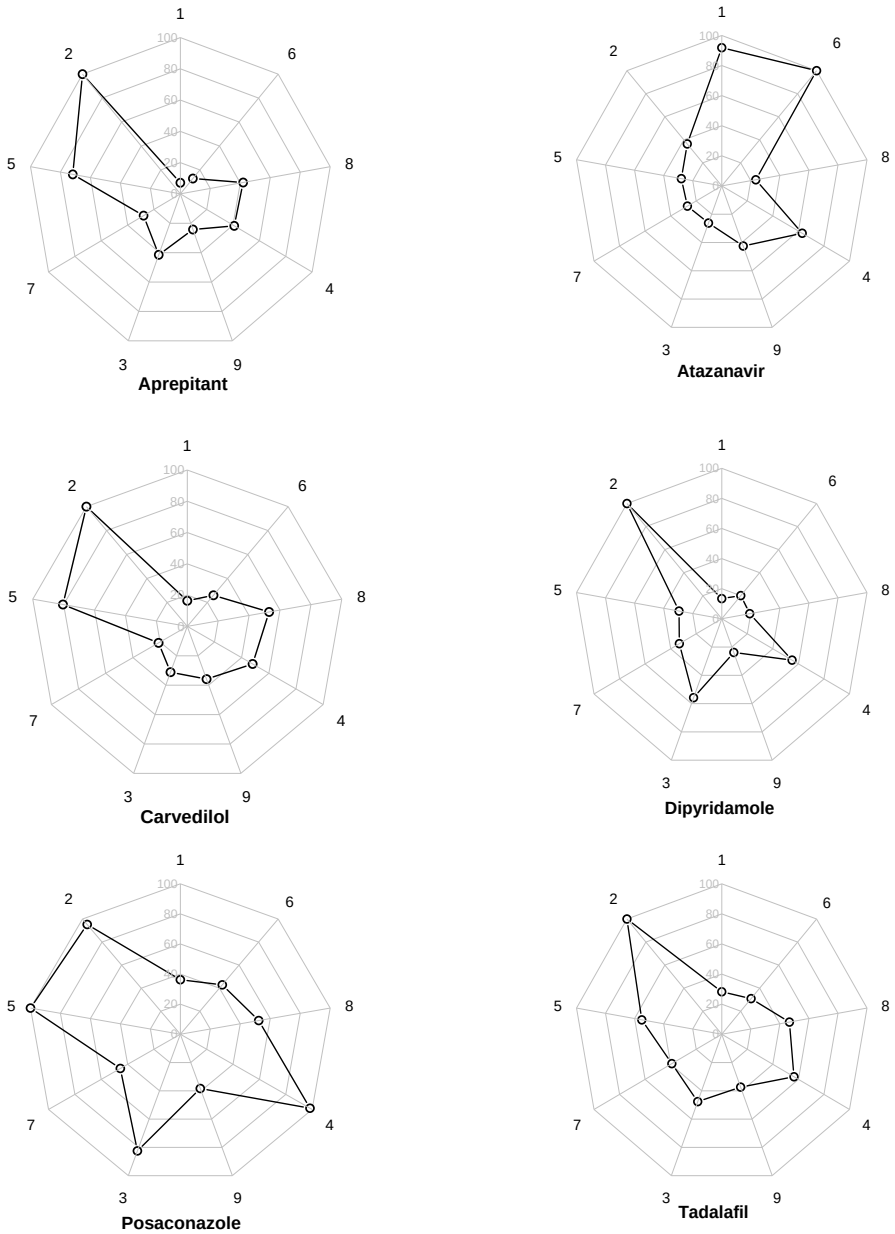
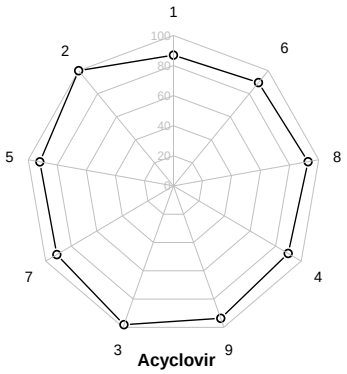
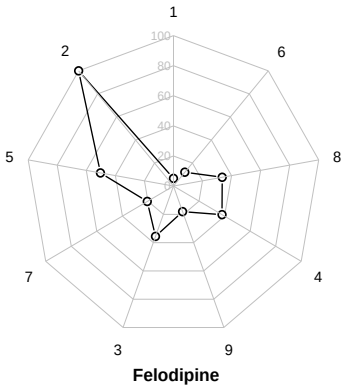


Figure 3.10: Basic drugs – spider plot. Highest solubility value normalised to 100; point label indicates media number (see Table 2.2) arranged in a clockwise order of increasing pH × TAC – lowest pH at 12 o'clock.



Acyclovir

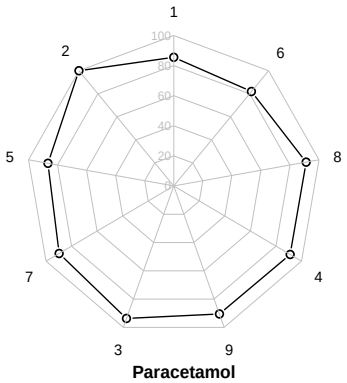
Carbamazepine



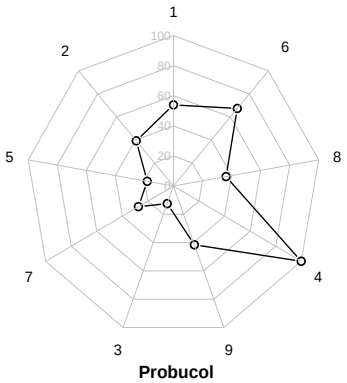
Felodipine

Fenofibrate

Griseofulvin



Paracetamol



Probucol

Figure 3.11: Neutral drugs – spider plot. Highest solubility value normalised to 100; point label indicates media number (see Table 2.2) arranged in a clockwise order of increasing pH × TAC – lowest pH at 12o'clock.

b. Solubility behaviour analysis

For basic and neutral drugs, the average standardised effect values from the original DoE studies for pH, FA, BS and PL[3] were equal, indicating that these media components all impact solubility (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020). For the basic drugs (aprepitant, carvedilol, and tadalafil) the standardised effect fingerprint is variable, pH and FA were generally not as significant as BS and PL with only aprepitant displaying a positive value for the four factors (Khadra et al., 2015). For felodipine, fenofibrate, and griseofulvin, the standardised effect values for pH, FA, BS and PL were positive (Khadra et al., 2015). Therefore, there is a generally increasing solubility with increasing pH × TAC. The variation of standardised effect value for media components for each drug is the reason behind the individual drug changes in media number solubility behaviour discussed above, within the background of an overall solubility increase related to increasing pH × TAC (Dunn et al., 2019).

Therefore, there will be a gross solubility trend of increasing solubility with increasing pH × TAC for the media, hence why point 1 universally has a lower solubility than point 2, atazanavir and probucol excepted. This average behaviour will be modified by each drug's individual solubility ranks previously mentioned. This behaviour is consistent with the original DoE (Khadra et al., 2015), and topographical analysis (Dunn et al., 2019). The original DoE also identified for basic and neutral drugs that 2-way interactions between media components were equivalent contributors to solubility as the components acting alone. For example, for both drug categories pH with FA was the third, and BS with FA the sixth most significant solubility drivers along with FA, pH, BS, and PL. These interactions will influence the analysis presented above and highlight that the media (Table 2.2), due to the method of calculation, are not optimised for a DoE, therefore only gross single component effects can be determined.

Dipyridamole and posaconazole, have not been measured in any of the fasted DoE protocols (Ainousah et al., 2017, Khadra et al., 2015, McPherson et al., 2020), therefore analysis with respect to media component standardised effect values is not possible. However, dipyridamole and posaconazole have a similar shape to the other

[3] NB Cholesterol was not examined as a media factor in the original DoE.

basic drugs (aprepitant, carvedilol and tadalafil) and therefore indicates that these drugs are behaving in a comparable manner to the DoE drugs.

The behaviour of acyclovir, carbamazepine, and paracetamol with essentially circular polar plots represent a category that has been previously recognised for griseofulvin and phenytoin, and for these drugs media variation has very limited solubility impact. Acyclovir, carbamazepine, and paracetamol have not been measured in the fasted DoE, and with only 5 examples, it is difficult to determine which parameters are involved in the property. However, it is interesting that these drugs have the simplest molecular structures amongst all the drugs in the study (Table 2.1), and are obviously different to atazanavir and probucol described below. Possibly indicating that molecular structure somehow has to be considered as a property over and above the total molecule physicochemical measurements of melting point, intrinsic solubility, pKa, and log P.

For probucol in the DoE (Khadra et al., 2015), BS and PL had no significant standardised effect value on solubility, and FA and pH were only just significant. This maybe the reason behind the unusual and paradoxical solubility behaviour (Figures 3.9 a or 3.11), with no correlation between solubility and pH × TAC, media 1 (lowest pH × TAC) has a higher solubility than media 2. This behaviour is also present for atazanavir, which as discussed will be behaving as a neutral molecule. With only two examples it is very difficult to rationalise this solubility behaviour, but it does indicate for these drugs a complex solubility behaviour across the fasted intestinal media space. It is also interesting that the solubility ratio for atazanavir is relatively low (4.27), which in a similar manner to category 3 is indicating that it is not interacting with the media components but has a complex molecular structure.

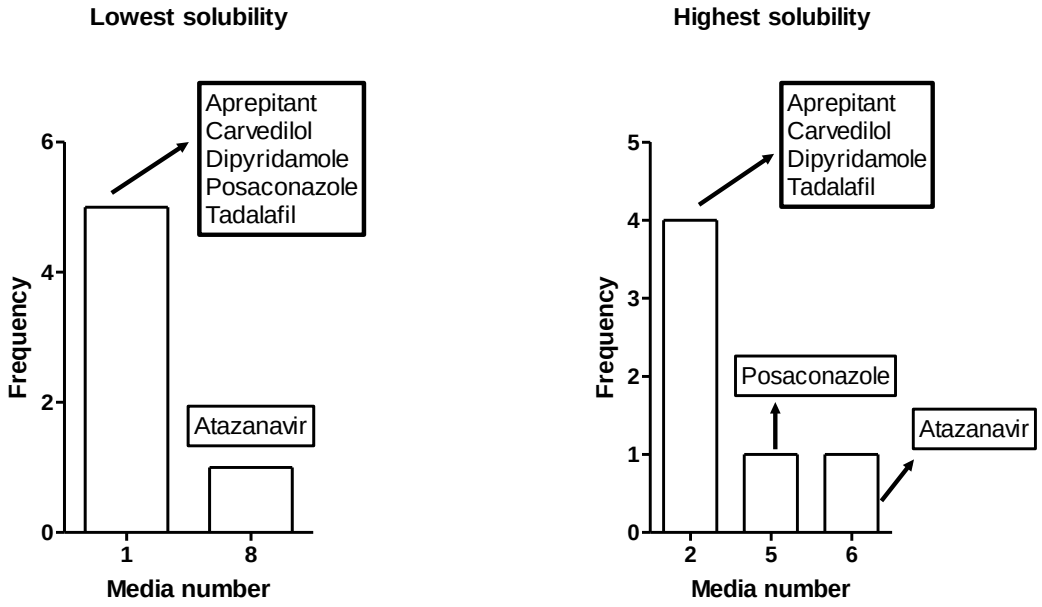
For poorly soluble drugs the use of micellar surfactant solutions as a surrogate for intestinal fluids has been suggested with BS one of the initial systems examined (Bou-Chacra et al., 2017, Mithani et al., 1996). For a range of drugs solubility in D-α-tocopheryl polyethylene glycol 1000 succinate increased with increasing amphiphile concentration (Clarysse et al., 2011). A recent paper has published a similar relationship with atazanavir (Indulkar et al., 2017) using sodium dodecyl sulphate. However, the result for atazanavir in Figure 3.8 b, indicates that in the multicomponent bioequivalent media systems, the relationship between solubility and TAC is not

applicable. This is only a single example but potentially indicates that for some drugs, the use of single surfactant systems could provide misleading results, a situation that can only be discovered if a multiple FaSSIF media measurement is conducted.

c. Media frequency analysis

In Figure 3.12 the frequency of each media recipe as either the highest or lowest solubility value for the basic and neutral drugs is presented. For the basic drugs media number 2 provides the highest solubility in 4 out of the 6 (67%) drugs analysed with posaconazole registering media number 5 and atazanavir number 6. With posaconazole the highest measured solubility in media 5 was very close to the value of media 2 (see Figure 3.8 b) indicating that the difference is minor. For the neutral drugs media number 2 provides the highest solubility in 6 out of 7 (86%) cases, with probucol registering media number 4. For the basic drugs the lowest solubility is measured in media number 1 in 5 out of 6 (83%) drugs analysed with atazanavir registering in media number 8. For the neutral drugs the lowest solubility occurs in 4 out of 7 cases (57%) in media 1 with probucol registering media 3, paracetamol media 6, and carbamazepine media 9. Due to the low solubility variability exhibited by paracetamol and carbamazepine, the difference in solubility measurement between media 1 and the lowest solubility media will be low. Atazanavir and probucol are also two of the four drugs that do not register media number 1 for the lowest solubility, and, as discussed above, exhibit solubility behaviour that is very different from the other drugs.

Basic drugs



Neutral drugs

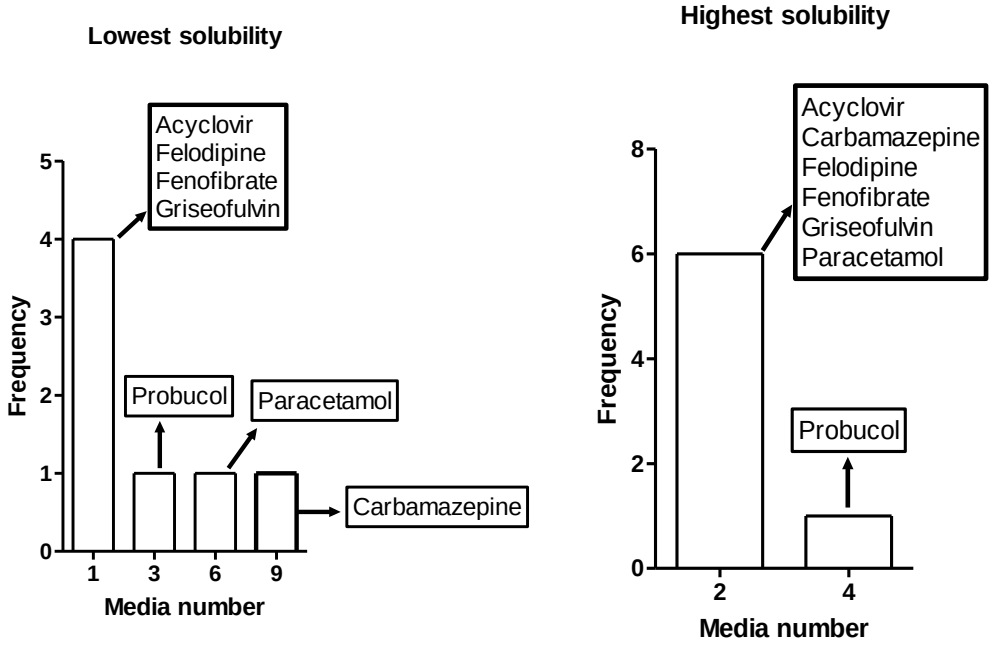


Figure 3.12: Basic and neutral drugs – lowest and highest solubility media frequency of lowest and highest solubility media for drugs in Figures 3.8 and 3.9. Drugs as listed in boxes.

3.4.2.4. Solubility Behaviour Categorisation

The grouping of the drugs analysed in this study, as discussed above, is based on a simple classification around ionisation for comparability with previous studies (Khadra et al., 2015). Based on the results in this study the categorisation can be modified to reflect the drugs solubility behaviour in the bioequivalent fasted simulated intestinal media, but also utilising the ionisation properties, see Table 3.4. This provides four categories of solubility behaviour, with two defined by physicochemical properties, a third as a subset based on behaviour in the bioequivalent fasted simulated intestinal media, with the final category based on drug dependent solubility behaviour alone.

The first category is acidic drugs with a pKa value < 6.4 (defined by the highest pKa of the ionised drug in the sample set), where pH is the main solubility driver, resulting in a consistent pH dependent solubility behaviour, and the lowest and highest solubility is measured in media number 4 and 3 respectively in over 86% of cases. This category also exhibits a high solubility ratio and range of solubilities, reflective of the impact of pH on ionisation and solubility. Further studies would be required to expand this set and refine the behaviour pattern, especially since the majority of examples are derived from the non-steroidal analgesic therapeutic category.

The second category includes, weakly acidic drugs with a pKa value greater than 8, and basic and neutral drugs where the main solubility driver is a combination of pH and TAC resulting in a general trend of increasing solubility with increasing pH × TAC. The solubility behaviour in the bioequivalent fasted simulated intestinal media will be drug dependent but the lowest and highest solubility is measured in media number 1 and 2 respectively in almost 80% of cases. With only 9 examples it would be sensible to expand the data set as for category 1. Based on physicochemical principles, an acidic drug with a pKa between 6.4 and 8 and with a low solubility (comparable to zafirlukast) would be likely to exhibit solubility behaviour that is associated with both categories.

Category 3 drugs have a very low solubility ratio, which is logical based on physicochemical properties, and present on polar plots as an almost circular distribution. An arbitrary (based on phenytoin) solubility ratio of < 2.5 has been applied for this category, which means that in this analysis it includes neutral and

acidic drugs. Solubility measurement in media number 2 would identify the highest solubility in 80% of cases, but the lowest solubility if media 1 was applied would only be identified in 40% of cases. However, the low solubility ratio would assist in identification of the categorisation and the low range would indicate that the low value solubility error is likely to be small.

In Category 4, drug solubility does not increase with pH and TAC and both drugs (atazanavir and probucol) in this category exhibit different solubility behaviour to all other measured drugs. With only two examples in this category, it is not possible to fully define properties and the categorisation could be an artefact of the inclusion of these two drugs in the study. However, if media numbers 1 and 2 were applied to measure solubility, the signature of lower solubility in media 2 than 1 would identify the behaviour.

This indicates that if a drug can be categorized as an acid or weak acid, base or neutral, then two simulated intestinal fluid media, either 4 and 3 (representing minimum and maximum pH values for acidic drugs), or 1 and 2 (representing minimum and maximum pH × TAC values for weak acid, basic and neutral drugs) can be used to determine *in vitro* the fasted intestinal solubility range (minimum to maximum). The measurement can then be applied to refine the categorization and relate solubility behaviour to previous DoE studied examples (Khadra et al., 2015). Coupled with a central point measure, three media can provide limits and information on the potential BCS or DCS classification and position with respect to the boundaries. If three media are too onerous, then the lowest solubility media can be applied as a worst-case scenario. This fasted intestinal solubility measurement will provide more information than a single FaSSIF value, and can be applied to assess the suitability during drug discovery (Di et al., 2012), and development strategies (Bayliss et al., 2016) for oral administration.

3.4.2.5. Drug solubility and Media Component Interactions

The solubility behaviour analysis above is predicated based on the results from the original fasted DoE study (Khadra et al., 2015), which applied a quarter fraction factorial design and required 66 experiments per drug. This measured a standardised

effect value for the impact of each media component, and 2-way interactions between components on solubility, although in the latter case, some of these were conflicted due to the reduced (quarter fraction) design. The DoE focus was to understand the importance of the media components on drug solubility, whereas the current study has applied bioequivalent media derived from a mathematical analysis of FaHIF samples to determine fasted intestinal fluid solubility limits. The two approaches are complementary, with the current less resource intensive than the DoE. Simple statistical issues around sample numbers indicate that this low media number approach (McPherson et al., 2020) will never provide the depth of information available from a fully structured DoE. Therefore, during drug development, a combination approach would be sensible, solubility screen with limited media numbers with exemplar candidates investigated by a DoE, to link the statistical with the bioequivalent to guide development and reduce the possibility for solubility surprises.

3.5. Conclusion

Application of the dose/solubility calculation to the bioequivalent points allows a DCS range to be plotted, which represents greater than ninety percent of the drug's intestinal solubility based on the derivation of bioequivalent points. The calculated range provides greater information than single point measurements, and the lowest solubility value represents a worst-case scenario that could be helpful during drug screening, development, and formulation.

The bioequivalent points can be linked to the original HIF data set to provide a frequency distribution for the measured solubility value. The solubility distributions do not follow a normal or log normal pattern, which it can now be concluded in part due to the measurement points being distributed in multidimensional space. Therefore, the traverse from low to high solubility points is not a simple vector based on a single concentration or property. In addition, the distribution can be used to refine quality by design risk assessments, since it provides a population value for solubility behaviour.

Overall, the results indicate that the small scale fasted bioequivalent study provides greater information than single point measurements in either FaHIF or SIF, by determining a fasted intestinal solubility range, with a population frequency

distribution (based on the original population (Riethorst et al., 2016) and analysis (Pyper et al., 2020)) that can be applied to biopharmaceutical calculations.

For the structured solubility behaviour findings, this was the first study that examined the solubility behaviour of a range of drugs in a structured set of bioequivalent fasted intestinal fluid media, and no consistent solubility behaviour that covers all the drugs tested is evident (Augustijns et al., 2014, Bou-Chacra et al., 2017). However, the measured solubility behaviour can be categorized into two types using physicochemical properties, and two further categories based on media solubility behaviour.

For acidic drugs (pKa < 6.4) (Category 1, Table 3.4) equilibrium solubility is directly linked to media pH, an identical solubility behaviour is present for all drugs and the lowest and highest solubility can be determined in the lowest (media 4) and highest (media 3) pH media with a greater than 86% frequency. For weakly acidic (pKa > 8), basic and neutral drugs (Category 2, Table 3.4) equilibrium solubility is correlated to increasing media pH × TAC, but the solubility behaviour is not consistent between drugs. The lowest and highest solubility can be determined in the lowest (media 1) and highest (media 2) pH × TAC media with almost 80% frequency. Category 3 is a subset of category 2 including neutral or drugs not ionized within the media pH range, and characterized by solubility behaviour that is not sensitive to media composition, leading to a very narrow solubility range. The lowest and highest solubility can be determined in the lowest (media 1) with a 40% certainty and highest (media 2) with an 80% certainty. Any possible error in the low solubility measurement would be easily spotted by the narrow solubility ratio and likely to be minimal due to the narrow range. The final category, with only two drug examples, is not well defined but would be detected if media numbers 1 and 2 were applied, since the solubility in media 1 would be higher than 2, which is opposite to categories 2 and 3. This category requires further examples to fully define, and for all categories increased example numbers and analysis would be prudent.

Chapter 4: Simulated Fasted Intestinal Fluids, *in vitro in vivo* Solubility Correlation

4.1. Introduction

Fasted state human intestinal fluid (FaHIF) (Rosenberger et al., 2018) is the "gold standard" solubility medium but is difficult to obtain (Bergström et al., 2014), is only available in small volumes (de la Cruz-Moreno et al., 2017), is highly variable (Riethorst et al., 2016), and population and disease dependent (Vinarov et al., 2021). Measured drug solubilities therefore are highly variable and difficult to correlate *in vitro* to *in vivo* performance. To mitigate availability issues, fasted state simulated intestinal fluids (FaSSIF) were introduced as *in vitro* biorelevant surrogates, based around average compositions of pH, BS, and PL (Dressman et al., 1998). Different fixed composition FaSSIF versions are available (Bou-Chacra et al., 2017), and there is solubility variation between them (Fuchs et al., 2015). Since FaHIF (Fuchs and Dressman, 2014) is variable and composition influences solubility (Clarysse et al., 2009, Khadra et al., 2015, Pedersen et al., 2000a, Zhou et al., 2017b), there is no consensus on the optimal FaSSIF media to represent FaHIF solubility or range.

In order to capture the inherent variability in FaHIF physicochemical composition (Fuchs and Dressman, 2014, Vinarov et al., 2021), a recent study (Pyper et al., 2020) reported a five-dimensional analysis of 152 FaHIF samples (Riethorst et al., 2016), with eight points plus a centre point. Due to their derivation, the nine media could be considered bioequivalent, rather than simply biorelevant, with the potential to determine an *in vitro* FaHIF solubility range covering 96% of possible solubility values (Augustijns et al., 2014). To test this, Fa9SIF equilibrium solubility data for seventeen drugs (out of 21 drugs studied in Chapter 2) were compared to published FaHIF solubility values.

Establishing an *in vitro in vivo* solubility correlation will introduce a transformational change throughout drug discovery, development, and formulation. This correlation will permit the application of Quality by Design principles with performance boundaries and population distribution information, which is especially important for poorly soluble drugs.

4.2. Aims and Objective

- Reduce the need to perform *in vivo* studies by studying the statistical differences between current Fa9SIF bioequivalent solubility data to published FaHIF solubility values, and thus reduce time, cost, and efforts.
- Assess the suitability during drug discovery, development, and formulation strategies by examining possible correlations between the *in vitro* measurements and the *in vivo* data and finding the solubility boundaries.

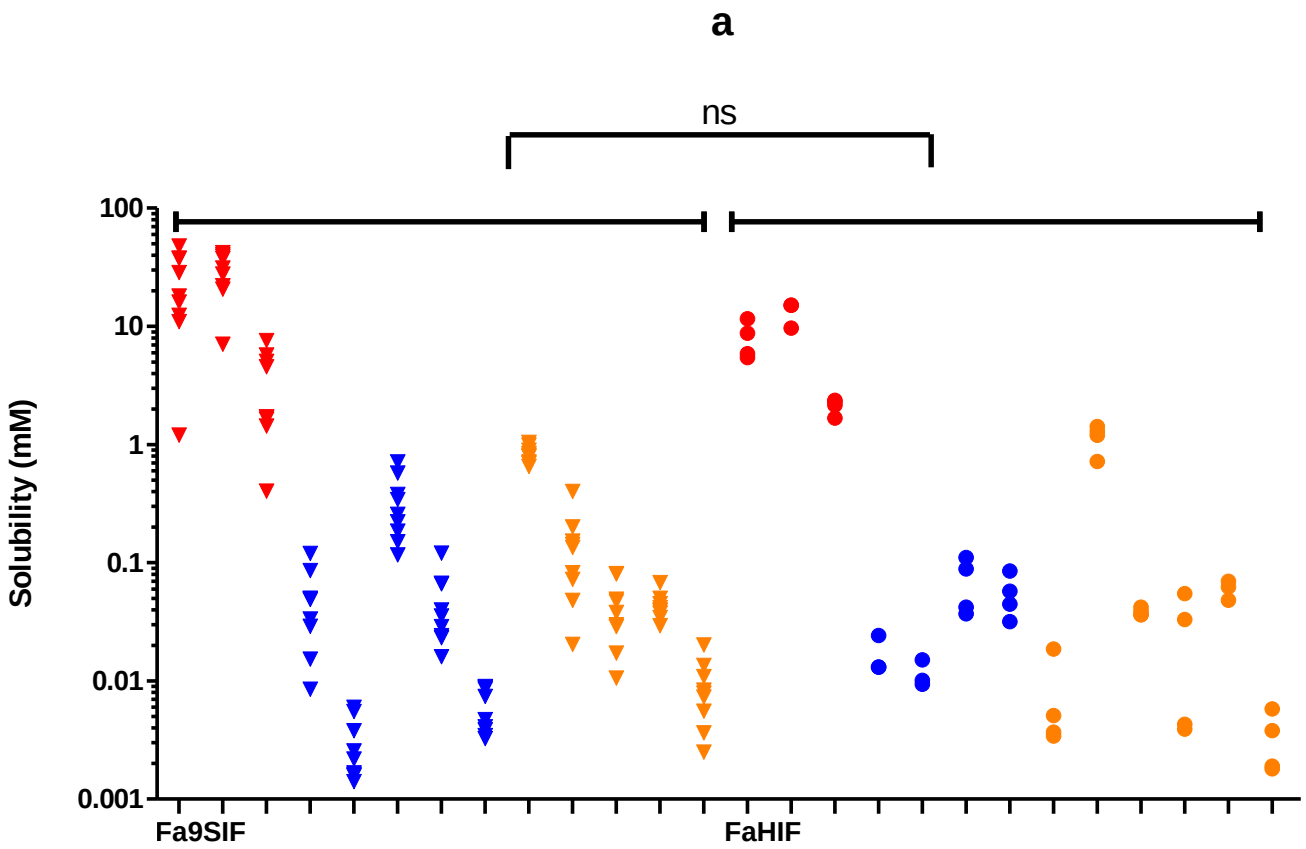
4.3. Methods

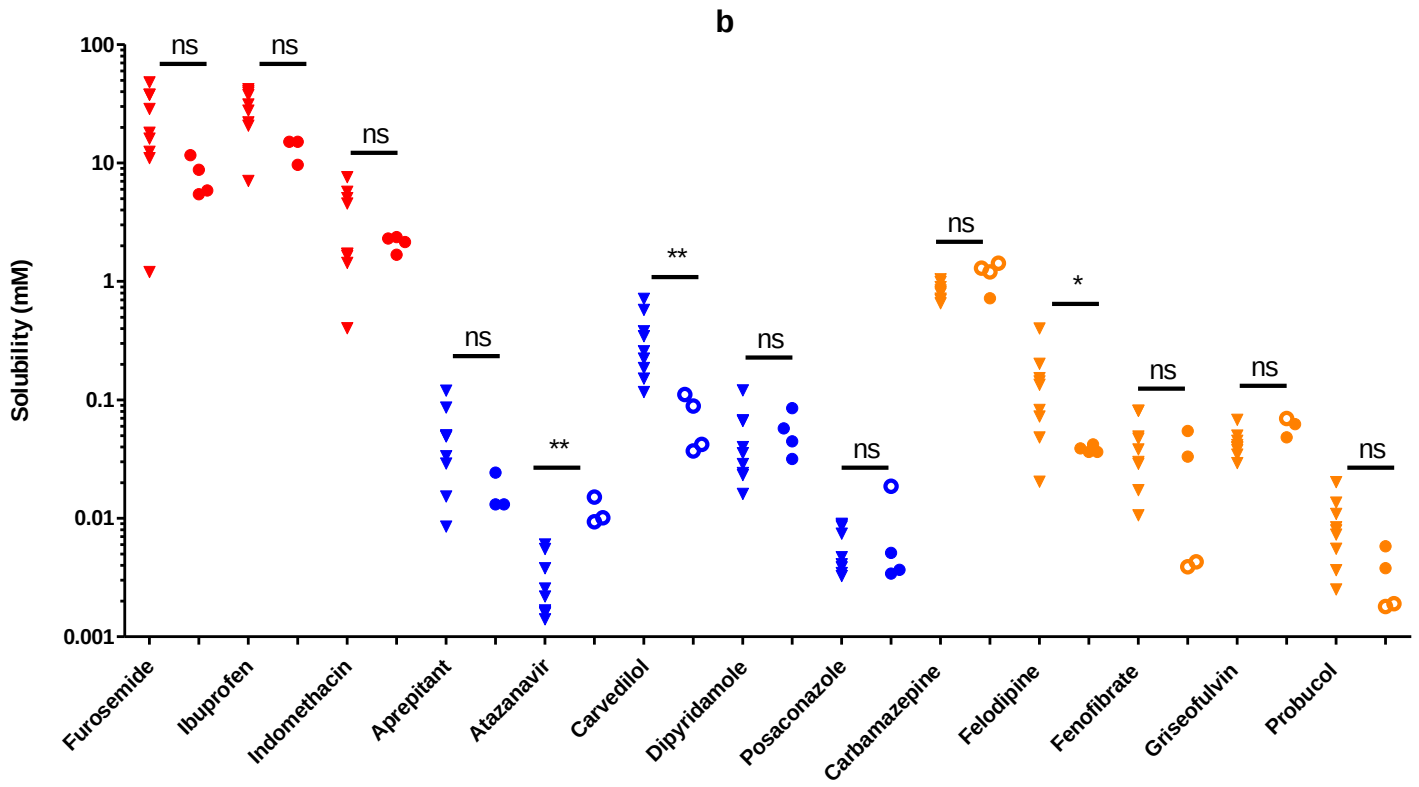
4.3.1. Statistical tests

Wilcoxon matched pairs signed rank test, $P < 0.05$ (Two Tailed), and Mann-Whitney, two tailed test. Previous papers have highlighted that the simulated data sets (Perrier et al., 2018) and FaHIF chemical compositions (Pyper et al., 2020) do not follow a normal distribution and therefore a non-parametric statistical comparison is appropriate.

4.3.2. Equilibrium Solubility Data Sets

Fifty-six FaHIF literature equilibrium solubility values for seventeen drugs (Table 4.1) are plotted in Figure 4.1. The data are from sixteen studies and span from a single value for a single drug, to a maximum of seven values from five studies for a single drug. These drugs have been assessed using the Fa9SIF system (Figure 4.1), and the results were presented previously in comparison to DoE studies (Chapter 2). The data sets are not balanced (FaHIF 56 vs Fa9SIF 153 (17 x 9) values) and reflect issues associated with FaHIF availability, study specific drug choices, and the lack of uniformity between previous studies (Clarysse et al., 2011, Khadra et al., 2015).


a
ns
100
10
1
0.1
0.01
0.001
Solubility (mM)
Fa9SIF
FaHIF


b
Solubility (mM)
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Furosemide
Ibuprofen
Indomethacin
Aprepitant
Atazanavir
Carvedilol
Dipyridamole
Posaconazole
Carbamazepine
Felodipine
Fenofibrate
Griseofulvin
Probucol

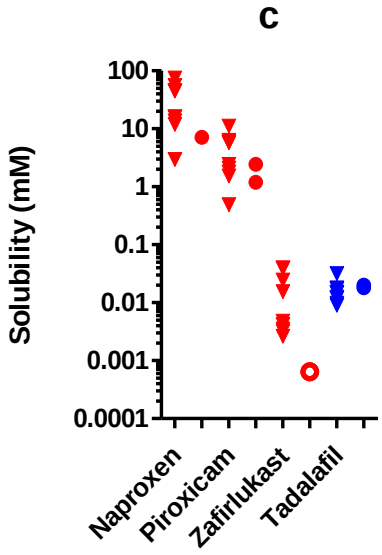

Figure 4.1: Comparison plots of equilibrium solubility values 9 media (Fa9SIF) and FaHIF. ▼ 9 media (Fa9SIF), ● FaHIF; red – acidic, blue – basic, orange – neutral; ns – no significant difference between media. In a and b; closed symbols value lies within Fa9SIF solubility range, open symbols value lies outside range.

a: Drugs with 3 or more FaHIF solubility values. Wilcoxon matched pairs signed rank test, $P < 0.05$ (Two Tailed) (Pairing significantly effective $P < 0.0001$ (One Tailed) Spearman value = 0.9273); drug order as per Figure 4.1 b.

b: Drugs with 3 or more FaHIF solubility values. Mann-Whitney test, $P < 0.05$ (Two Tailed); * P = 0.0336; ** P = 0.0028 - 0.0091.

c: Drugs with less than 3 FaHIF solubility values.

Table 4.1: Sampled fasted HIF equilibrium solubility value analysis.

Drug	Solubility (mM) (SD)	Time (hrs)	Temp (°C)	Solid/ Volume	Separation	Sample location	Number	Age (yrs)	Pooled	Reference
Furosemide	5.862 (1.71)	24	37	1mg/0.3mL	Centrifugation	Duodenum	6	20-29	N	[7]
	11.62	30	23	NA/0.25mL	Centrifugation	Duodenum	5	24-39	Y	[1] from [2]
	8.76 (0.15)	24	37	NA/0.2mL	Centrifugation	Jejunum	10	NA	Y	[8]
	5.44 (4.84)	24	37	NA/0.2mL	Centrifugation	Ileum	10	NA	N	[8]
	2.95	3	37	2mg/mL	Centrifugation	Duodenum	5	22-35	N	[9]
	2.52	3	37	2mg/mL	Centrifugation	Duodenum	5	22-35	N	[9]
	5.49	3	37	2mg/mL	Centrifugation	Duodenum	5	22-35	N	[9]
	5.13	3	37	2mg/mL	Centrifugation	Jejunum	5	22-35	N	[9]
Ibuprofen	9.65	30	23	NA/0.25mL	Centrifugation	Duodenum	5	24-49	Y	[1] from [2]
	15.1	24	37	2mg/1mL	Filtration	Duodenum	16	18-45	Y	[3]
	15.1	24	37	2mg/1mL	Filtration	Duodenum	16	18-45	Y	[3]
Indomethacin	1.677	NA	NA	NA	NA	NA	NA	NA	NA	[4]
	2.368 (1.88)	NA	37	NA/0.5mL	Centrifugation	Duodenum	5	21-37	N	[5]
	2.151 (0.42)	24	37	0.5mg/0.5mL	Centrifugation	Duodenum	8	18-25	N	[6]
	2.301 (1.09)	24	37	1mg/0.3mL	Centrifugation	Duodenum	6	20-29	N	[7]
	6.658	30	23	NA/0.25mL	Centrifugation	Duodenum	5	24-39	Y	[1] from [2]
Naproxen	7.148	30	23	NA/0.25mL	Centrifugation	Duodenum	5	24-39	Y	[1] from [2]
Piroxicam	1.198 (0.012)	24	37	1mg/1mL	Centrifugation	Jejunum	NA	NA	Y	[10]
	2.454	30	23	NA/0.5mL	Centrifugation	Duodenum	5	24-39	Y	[1] from [2]
Zafirlukast	$6.43x10^{-4}$	24	37	2mg/1mL	Filtration	Duodenum	16	18-45	Y	[3]
	$6.43x10^{-4}$	24	37	2mg/1mL	Filtration	Duodenum	16	18-45	Y	[3]
Aprepitant	0.0243 (0.005)	24	37	1mg/1mL	Centrifugation	Jejunum	NA	NA	Y	[10]
	0.0131	24	37	2mg/1mL	Filtration	Duodenum	16	18-45	Y	[3]

Drug	Solubility (mM) (SD)	Time (hrs)	Temp (°C)	Solid/ Volume	Separation	Sample location	Number	Age (yrs)	Pooled	Reference
	0.0131	24	37	2mg/1mL	Filtration	Duodenum	16	18-45	Y	[3]
Atazanavir	0.0151 (0.0013)	27	37	0.8mg/0.3mL	Centrifugation	Duodenum	4	24-27	Y	[17]
	0.0101 (0.0008)	48	37	NA/1mL	Centrifugation	Duodenum	20	NA	Y	[15]
	0.00936[c] (0.0004)	48	37	2mg/1mL	Centrifugation	Duodenum	20	18-31	Y	[16]
Carvedilol	0.0886 (0.001)	24	37	1mg/mL	Centrifugation	Jejunum	NA	NA	Y	[10]
	0.111	30	37	1mg/0.5mL	Centrifugation	Duodenum	11	NA	Y	[11]
	0.037	24	37	2mg/1mL	Filtration	Duodenum	16	18-45	Y	[3]
	0.042	24	37	2mg/1mL	Filtration	Duodenum	16	18-45	Y	[3]
Dipyridamole	0.0446 (0.004)	3	37	45mg/4.5mL	Centrifugation	Duodenum	12	NA	Y	[12]
	0.0575 (0.0008)	24	37	1mg/mL	Centrifugation	Jejunum	NA	NA	Y	[10]
	0.0317	24	37	NA/0.2mL	Centrifugation	Jejunum	10	NA	Y	[8]
	0.0851 (0.046)	24	37	NA/0.2mL	Centrifugation	Ileum	10	NA	N	[8]
Posaconazole	0.0051 (0.0002)	30	23	NA/0.5mL	Centrifugation	Duodenum	5	20-32	Y	from [2]
	0.00368 (0.00074)	48	37	NA/1mL	Centrifugation	Duodenum	20	18-31	Y	[15]
	0.00342[c] (0.0006)	48	37	2mg/1mL	Centrifugation	Duodenum	20	18-31	Y	[16]
	0.0186[a] (0.004)	48	37	2mg/1mL	Centrifugation	Duodenum	20	18-31	Y	[16]
Tadalafil	0.018	24	37	2mg/1mL	Filtration	Duodenum	16	18-45	Y	[3]
	0.02	24	37	2mg/1mL	Filtration	Duodenum	16	18-45	Y	[3]
Carbamazepine	1.422 (0.076)	24	37	0.5mg/0.5mL	Centrifugation	Duodenum	8	18-25	N	[6]
	1.2 (0.024)	24	37	1mg/1mL	Centrifugation	Jejunum	NA	NA	Y	[10]
	1.294 (0.29)	24	37	1mg/0.3mL	Centrifugation	Duodenum	6	20-29	N	[7]
	0.72	30	23	NA/0.25mL	Centrifugation	Duodenum	5	24-39	Y	[1] from [2]
	0.644	3	37	2mg/1mL	Filtration	Duodenum	5	22-35	N	[9]
	1.01	3	37	2mg/1mL	Filtration	Jejunum	5	22-35	N	[9]

Drug	Solubility (mM) (SD)	Time (hrs)	Temp (°C)	Solid/ Volume	Separation	Sample location	Number	Age (yrs)	Pooled	Reference
	0.767	3	37	2mg/1mL	Filtration	Duodenum	5	22-35	N	[9]
Felodipine	0.0364	24	37	1.2mg/1.2mL	Centrifugation	Jejunum	12	24-40	Y	[13]
	0.0364 (0.0001)	24	37	1mg/1mL	Centrifugation	Jejunum	NA	NA	Y	[10]
	0.00343	3	37	1mg/mL	Centrifugation	Duodenum	5	22-35	N	[9]
	< 0.0006	3	37	1mg/mL	Centrifugation	Jejunum	5	22-35	N	[9]
	0.042	24	37	2mg/mL	Filtration	Duodenum	16	18-45	Y	[3]
	0.039	24	37	2mg/mL	Filtration	Duodenum	16	18-45	Y	[3]
Fenofibrate	0.0546 (0.072)	24	37	1mg/0.3mL	Centrifugation	Duodenum	6	20-29	N	[7]
	0.0331 (0.0017)	24	37	2mg/0.5mL	Centrifugation	Duodenum	4	19-35	Y	[14]
	0.0043	24	37	2mg/1mL	Filtration	Duodenum	16	18-45	Y	[3]
	0.0039	24	37	2mg/mL	Filtration	Duodenum	16	18-45	Y	[3]
Griseofulvin	0.0623	24	37	1.2mg/1.2mL	Centrifugation	Jejunum	12	24-40	Y	[13]
	0.0697 (0.0071)	24	37	0.5mg/0.5mL	Centrifugation	Duodenum	8	18-25	N	[6]
	0.0482 (0.0038)	24	37	1mg/1mL	Centrifugation	Jejunum	NA	NA	Y	[10]
Phenytoin	0.00721	3	37	2mg/mL	Centrifugation	Jejunum	5	22-35	N	[9]
	0.0719	3	37	2mg/mL	Centrifugation	Duodenum	5	22-35	N	[9]
	0.0125	3	37	2mg/mL	Centrifugation	Duodenum	5	22-35	N	[9]
Probucol	0.0019	24	37	1.2mg/1.2mL	Centrifugation	Jejunum	12	24-40	Y	[13]
	0.0018 (0.0009)	24	37	1mg/1mL	Centrifugation	Jejunum	NA	NA	Y	[10]
	0.0058	24	37	2mg/1mL	Filtration	Duodenum	16	18-45	Y	[3]
	0.0038	24	37	2mg/1mL	Filtration	Duodenum	16	18-45	Y	[3]

SD: standard deviation, NA: not available, Y: yes, N: no, a: amorphous solid form; c: crystalline solid form.

References: 1- (Heikkilä et al., 2011) 2- (Augustijns et al., 2014) 3- (Dahlgren et al., 2021) 4- (McGinnity et al., 2007) 5- (Clarysse et al., 2009) 6- (Annaert et al., 2010) 7- (Clarysse et al., 2011) 8- (Rabbie et al., 2015) 9- (de la Cruz-Moreno et al.,

2017) 10- (Söderlind et al., 2010) 11- (Stappaerts et al., 2014) 12- (Kalantzi et al., 2006) 13- (Persson et al., 2005) 14- (Bevernage et al., 2011) 15- (Elkhabaz et al., 2019) 16- (Elkhabaz et al., 2021) 17- (Wuyts et al., 2013).

4.3.3. Fasted Human Intestinal Fluid Solubility Protocols

The FaHIF protocols sample from the duodenum or jejunum, but one utilizes ileum samples (Rabbie et al., 2015). The limited FaHIF composition data available indicates a high variability but minimal differences between the duodenum and jejunum (Bergström et al., 2014), the ileum samples exhibit similar variability, and therefore have been included. Subjects' ages range from 18 to 45 years, with the age range per study variable from three (Wuyts et al., 2013) to twenty seven years (Dahlgren et al., 2021). One early study (Annaert et al., 2010) investigated the impact of age (18 - 25 years vs 62 - 72 years) on solubility in FaHIF and concluded that although the samples exhibited a high interindividual variability, specific age-dependency did not impact solubility. The subject number ranges from four to twenty, and the inter and intra individual differences, mentioned with the previous study and with FaHIF in general (Dahlgren et al., 2021, Vinarov et al., 2021), will impact the sampled FaHIF's composition, which will in turn influence the measured solubility (Khadra et al., 2015). However, the greatest impact however, is that in the majority of protocols (11 out of 16) the sampled FaHIF is pooled before solubility measurement, and therefore the reported solubility value is based on aggregated behavior. In addition, the majority of pooled FaHIF have an unknown composition and therefore cannot be directly related to the un-pooled FaHIF samples used to determine the nine media (Riethorst et al., 2016). In protocols where samples are not pooled there will possibly be a larger variation in composition between samples due to inter and intra individual variability and therefore reported solubilities see Pyper (Pyper et al., 2020) for visualization. Therefore, this variation in composition and therefore solubility means that the Fa9SIF media's 96% coverage is not transferable, especially for pooled FaHIF, but insufficient data is available to recalculate possible coverage.

Solubility measurement protocols although not identical, are in agreement that 37°C is required, an equilibration time of greater than or equal to 24 hours, and separation of undissolved drug prior to analysis. In one case, room temperature was applied (Heikkilä et al., 2011), which might have a minor impact on solubility (Bates et al., 1966). In one study, a 3 hour incubation time (de la Cruz-Moreno et al., 2017) was applied to mimic gastrointestinal transit time, previous studies indicate that to attain equilibrium solubility for poorly soluble drugs twelve hours is required, therefore,

these results have been excluded (Khadra et al., 2015). Drug solid form has not been uniformly assessed, which could impact solubility, for atazanavir for example, only the crystalline equilibrium solubility values have been utilized (Elkhabaz et al., 2019, Elkhabaz et al., 2021). Overall, the FaHIF samples and solubility protocols are broadly similar permitting comparison with the Fa9SIF media, and due to the issues above, realistically all that is available.

4.4. Results and Discussion

4.4.1. Comparison of Solubility Data Sets

Thirteen drugs have three or more FaHIF values, and there is no statistically significant difference between FaHIF or Fa9SIF media system for measuring equilibrium solubility (Figure 4.1a) when tested using a Wilcoxon matched (by drugs) pairs test. For ten of the thirteen drugs when compared individually, using a Mann-Whitney test, there was no statistically significant difference (Figure 4.1b). The felodipine difference is due to the narrow FaHIF solubility distribution, but within the Fa9SIF media solubility range, and not considered significant based on the aim of this comparison. This result may be due to the pooling of large numbers of FaHIF samples (see Table 4.1), see discussion below. Therefore, for eleven drugs (85%) there is no statistically significant solubility difference between FaHIF or the Fa9SIF media system. Comparison of the fifty-six individual FaHIF solubility values with the Fa9SIF media range (Figures 4.1 b and c) indicates that 69% are within the boundaries, and acidic drugs provide a higher agreement than either basic or neutral. There are no comparable published studies however, one correlated ten poorly soluble drugs in three different FaSSIF media, and where a comparison to a FaHIF range is presented, 48% were within the range (Fuchs et al., 2015). The level of agreement in the current study is higher, although the difference between the studies in approach and drugs examined impacts the utility of this comparison.

The results indicate that increasing the number of measured FaHIF values for comparison, by targeting additional drugs with multiple (≥ 3) FaHIF solubility measurements, includes a greater proportion of possible FaHIF compositions and solubility variability, which provides a greater chance of agreement with the Fa9SIF

media range. For example, carbamazepine, fenofibrate, griseofulvin, posaconazole, and probucol all have FaHIF solubility values that lie outside the range, but this is not statistically significant. This highlights that the single value comparison is a stringent test, and multiple value comparisons provide greater coverage of behaviour.

For acidic drugs, only zafirlukast FaHIF values are outside the Fa9SIF media range (Dahlgren et al., 2021), and for basic and neutral drugs, atazanavir, carvedilol, carbamazepine, fenofibrate, griseofulvin, posaconazole, and probucol have extraneous values with the study by Dahlgren et al., (Dahlgren et al., 2021) providing three examples. Dahlgren utilized pooled FaHIF, and analysed for pH, BS and PL concentrations, which can be plotted as three dimensions from the multidimensional study (Pyper et al., 2020) (Figure 4.2), and indicate that BS and PL concentrations are outside the Fa9SIF media points, although pH is within. This is a possible explanation for the low zafirlukast solubility, since although acidic, its solubility is driven by the amphiphilic media components, and this may also impact carvedilol and fenofibrate solubilities but not the higher aprepitant. However, aprepitant's solubility is known to be positively influenced by free fatty acid (Khadra et al., 2015), which is one of the dimensions where no FaHIF information is available. This discrepancy highlights the issue associated with single and pooled FaHIF samples, and the potential for compositions outside the Fa9SIF data cloud, leading to differences in equilibrium solubility values.

The higher solubility agreement for acidic drugs can be explained by behaviour in DoE simulated media systems (Ainousah et al., 2017, Dunn et al., 2019, Khadra et al., 2015, Perrier et al., 2018), where solubility is predominantly pH controlled with minimal influence from amphiphilic media components. Therefore, only one dimension (pH) is operational, and the results indicate that the methods and analysis are adequate to achieve equivalence. This exemplifies the application of simpler media systems (Markopoulos et al., 2015), and the requirement for increasing media complexity associated with other categories. Basic and neutral drug solubility is controlled by multiple media components; therefore, all dimensions are important. The individual solubility value comparisons indicate that this multiple dependency reduces the level of agreement. This possibly implies that a more extensive FaHIF data set is required for a revised multidimensional analysis, using either more and/or different dimensions

(Pyper et al., 2020), and that FaHIF solubility determination should ideally be linked to the chemical composition (Dahlgren et al., 2021) (Figure 4.2). This latter modification would permit a systematic comparison of solubility values based on fluid composition.

The nine media system solubility of carbamazepine and griseofulvin is minimally influenced by media component variation, leading to a very narrow solubility range (Fuchs et al., 2015, Khoshakhlagh et al., 2015), which is also present in the FaHIF values. Further indicating the similarity of solubility performance for the two approaches. Although points lie outside the Fa9SIF media range, the distributions are not statistically different (Figure 4.1b), possibly indicating that this relates to variability in the solubility experimental protocols rather than media effects (Vertzoni et al., 2022).

The study drugs are predominantly BCS Class II, reflecting the depth of research interest in this class (Lennernäs, 2014). The results therefore are applicable to the other low solubility quadrant, Class IV, and further examples from BCS class I and III are required to fully assess performance in this sector. Regulatory *in vivo* bioequivalence limits are 80 - 125% of the comparator product. The Wilcoxon test statistical equivalence of the media and Mann-Whitney individual drug agreement of 85%, indicates that the Fa9SIF media system will provide an *in vitro* equilibrium solubility range bioequivalent to *in vivo* derived FaHIF measurements.

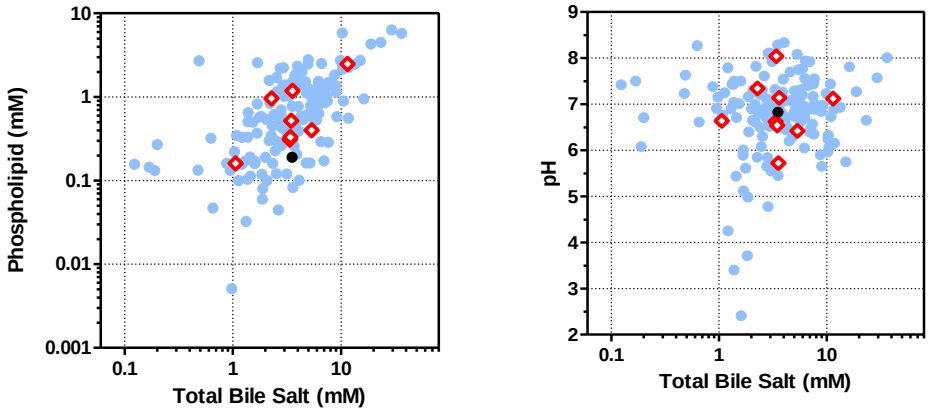

Figure 4.2: Compositional comparison. ● Bile salt, phospholipid and pH individual sample values from Pyper (Pyper et al., 2020). ◇ Fa9SIF media points; ● Dahlgren pooled FaHIF values (Dahlgren et al., 2021).

4.4.2. Solubility Correlation Boundary

If the Fa9SIF media system is equivalent to FaHIF, the solubility range, minimum to maximum, should be applicable in FaHIF. To determine potential correlation boundaries within which all drug solubility values will reside, the minimum and maximum solubility values (X_{min}, Y_{max}; X_{max}, Y_{min} where min or max represents the 9 media solubilities) have been plotted graphically (Figure 4.3), and an upper and lower correlation line or boundary calculated. The correlation lines for the acidic and basic drugs are statistically significant, but not for the neutral drugs, this is not critical since the relationship defines a best fitting boundary. The boundary's span is approximately equal to the average solubility range, and reflects the drugs examined in the Fa9SIF media system and solubility behaviour properties previously presented.

A literature (Augustijns et al., 2014) FaHIF solubility data set (replicate drugs removed) consisting of forty-seven values from thirty drugs has been plotted on top of the Fa9SIF media correlation (Figure 4.4) and 94% are within the boundaries. This is a first exploration of this relationship, and the result reinforces the statistical conclusion that the Fa9SIF media system is examining an *in vitro in vivo* equilibrium solubility correlation but should be treated with caution. A wide enough boundary will accommodate any data, especially if centred on the equivalence line around which a correlation is unavoidable. The boundary is based on the drugs measured to date in the Fa9SIF media system and may not be a representative sample. The boundary's differing shapes indicate that the correlation is linked to drug physicochemical properties, in itself an interesting and new finding. This observation is worthy of further investigation to fully explore the relationship and examine its potential biopharmaceutical implications and applications.

The conclusion that the equilibrium Fa9SIF media solubility range is bioequivalent to FaHIF solubility can be applied in novel ways and adapted to fit requirements. If solubility screening is required (Di et al., 2012), a small number of selected media can be utilized to determine either the minimum, or the worst case solubility, or the range extremes.

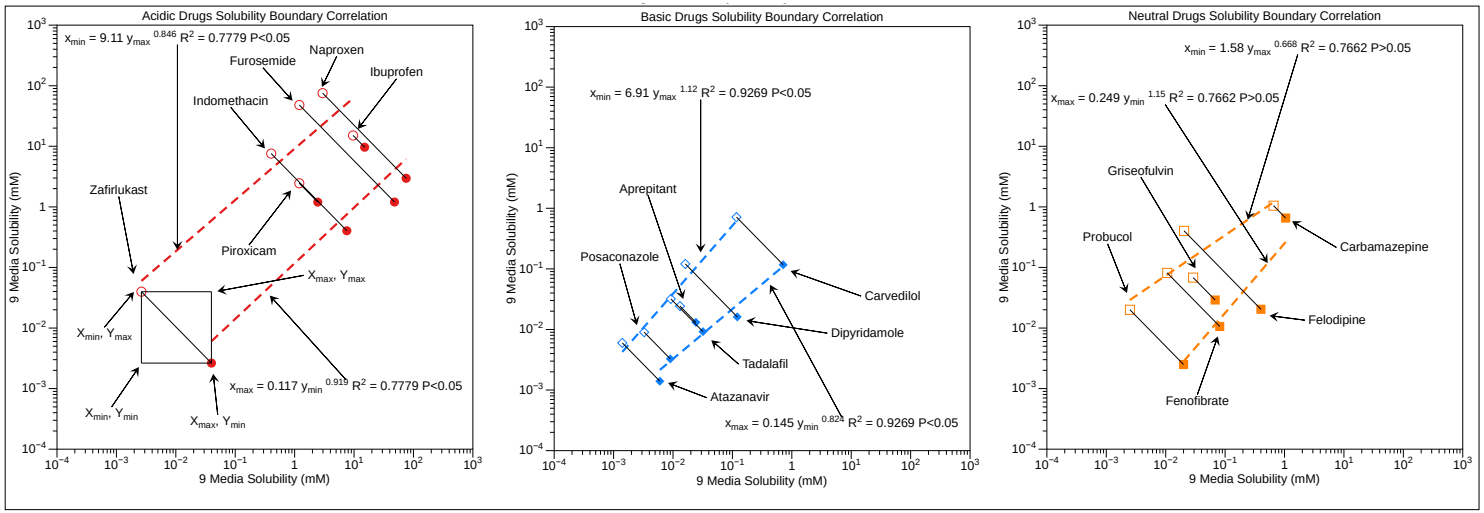

Figure 4.3: Solubility boundary correlation. Acidic, basic and neutral correlation boundaries based on the minimum and maximum solubility for each drug plotted as X_{min}, Y_{max} (open symbol); X_{max}, Y_{min} (closed symbol); drugs as listed on Figures; solubility window drawn for zafirlukast; ---- best fitting power correlation line, values as indicated.

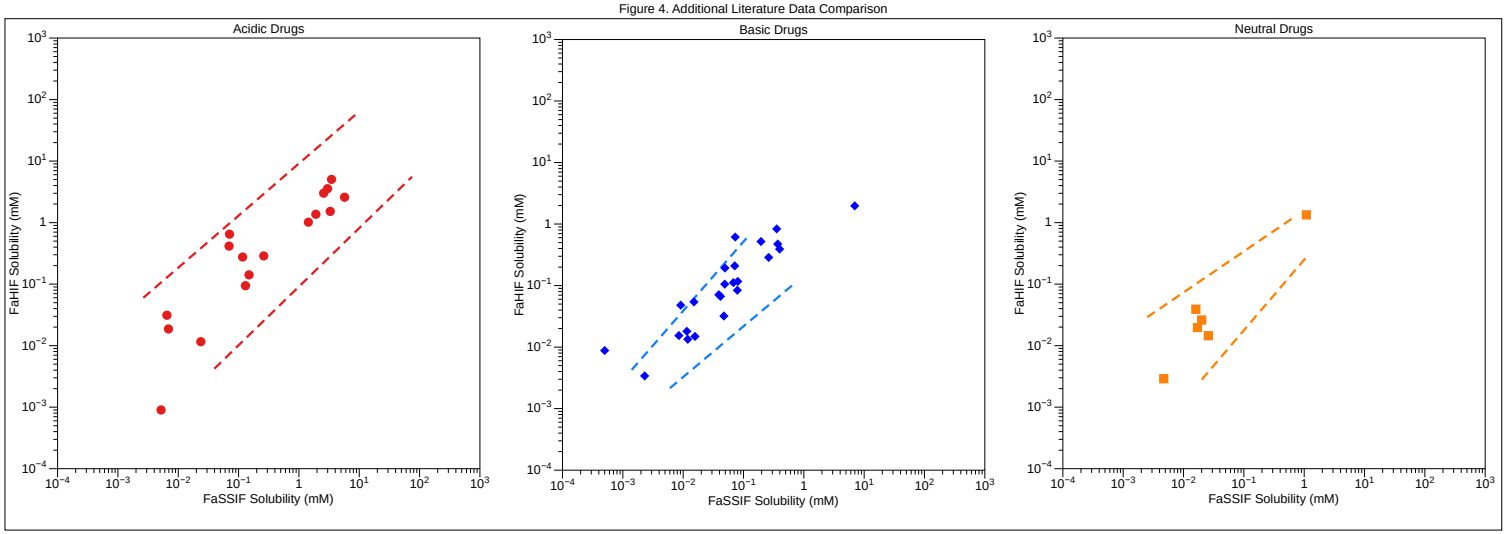

Figure 4.4: Solubility boundary correlation - additional literature data comparison, see Figure 4.3 for details, additional solubility data from (Augustijns et al., 2014).

4.5. Conclusion

Solubility comparison is, in principle simple but, confounded by the requirement to utilize FaHIF values from multiple studies, and limited by the available data. Seventeen drugs is not a large sample, and arises due to the drug set previously utilized (Khadra et al., 2015, McPherson et al., 2020) and the coverage in sampled FaHIF studies (Augustijns et al., 2014).

The Wilcoxon test indicates that for thirteen of the seventeen drugs, the Fa9SIF media system is equivalent to FaHIF, and when these drugs are examined individually, the agreement is 85%. A correlation that is within standard regulatory oral bioequivalence criteria. An individual point-based comparison reduces the correlation level and highlights that solubility agreement is related to drug ionization properties. Acidic drugs provide the best correlation, and this can be linked to drug behaviour in fasted simulated media DoE studies, and the influence of media components on solubility. Although the individual point-based comparison is a stringent test, it possibly indicates media factor refinement, or the usage of a larger FaHIF sample data set, to generate the factors and concentrations which might improve correlation.

A novel investigation, based on the bioequivalent solubility range, is to determine possible Fa9SIF vs FaHIF correlation boundaries. The boundaries encompass thirty different drugs from a separate study with the 94% fit additional correlation validation, and worthy of further investigation as a possible general system property.

If placement on the Biopharmaceutics / Developability Classification Systems (BCS/DCS) (Butler and Dressman, 2010, Rosenberger et al., 2018) is required along with possible population behaviour, then the Fa9SIF media set can be applied and linked to various biopharmaceutical performance predictors. The Fa9SIF media system is therefore worthy of further investigation with linkage of system results to *in vivo* performance, a key next stage and may represent a methodology applicable to other multicomponent biological fluids, where no single component is responsible for performance.

Chapter 5: Supersaturation Study of Poorly Soluble Drugs Using Fasted Bioequivalent Simulated Intestina Media

5.1. Introduction

The poor solubility of oral drugs has been one of the major problems for the drug absorption through the human gastrointestinal tract (GIT) (Plum et al., 2020a). One way to overcome this problem was by using a supersaturated form of such drugs, which will enable a higher absorption rate at the absorption site (Brouwers et al., 2009). The supersaturation (SS) state can be determined when the assay solution, the simulated intestinal fluid (SIF) in this study, exceeds its equilibrium solubility (Taylor and Zhang, 2016), and move to a thermodynamically metastable state with high energy properties. The SS state can be induced by different methods, but in this study the solvent shift method was used, due to the simple procedure, and the usage of small injection volumes (Plum et al., 2020a). The solvent shift method involves dissolving the drug of interest into an organic solvent which is highly soluble, for example di-methyl sulfoxide (DMSO), and then inject small volumes of this drug-DMSO mixture into the SIF medium, until SS is induced (Palmelund et al., 2016). Each drug has its own degree of supersaturation (DS), which is represented by the ratio between the drug's SS concentration and its equilibrium solubility (Palmelund et al., 2016). The main disadvantage of this method, is the inconsistent methods to choose the SS concentrations, which imposes difficulty in comparing literature results (Plum et al., 2020a). In previous SS studies, the supersaturation concentration (Css), was investigated using four different concentrations: at Css 100%; where the maximum concentration is reached before it causes immediate precipitation of the drug, 87.5%, 75%, and 50% of the Css 100% (Palmelund et al., 2016). In this study, an automated instrument to measure SS and, where possible, precipitation was used, which is the InForm®.

Different terminologies are used in SS assays, starting from the SS concentration, which is thermodynamically meta stable, and is the maximum concentration needed for the drug to stay in-solution before it destabilizes and precipitates. Whereas the kinetic solubility is the highest concentration of the fastest precipitating particles (Sou and Bergström, 2018) which is higher than the SS concentration, and where it starts to

form precipitation. Lastly, the crystalline (equilibrium) solubility, is the most thermodynamically stable concentration, as it's formed by the equilibrium between solid (undissolved) and liquid (dissolved) phases after the precipitation process (Sou and Bergström, 2018). In this chapter, the term 'crystalline solubility' will be used to present this SS study results, whereas the term 'equilibrium solubility' will be used to present the Fa9SIF results from Chapter 2.

5.2. Aims and Objectives

- Measure a more reliable SS concentration, time needed to induce SS, and where possible, the precipitation rate, of 10 drugs, using the inForm® instrument.
- Help to decide on best formulation strategy by proving the importance of studying the SS concentration by different SIF media recipes.
- Increase the drug development accuracy using the bioequivalent SS concentration by a statistical comparison to available literature findings.

5.3. Materials and Methods

5.3.1. Materials

Refer to section 2.3.1 for details.

5.3.2. Methods

5.3.2.1. Bioequivalent SIF Media Preparation

Various SIF media were tested (1, 2, and 8), for concentrations see Table 2.1, depending on Chapter 2 results of the lowest, middle, and/or highest solubility media results, except for phenytoin and probucol, see Table 5.2. SIF medium 1 was analysed for phenytoin and probucol, which is the closest to their middle solubility media results, but medium 1 (the lowest components concentrations) was attempted to be tested for all the drugs for its simplicity to prepare. The drugs were tested by single, double, or/and triple SIF media, due to limited time and difficulty to determine the DS of each drug.

To prepare 1 L of each media recipe, a concentrated lipid stock was prepared. The required weight of bile salt (BS) (sodium taurocholate), phospholipid (PL) (soybean lecithin) and fatty acid (FA) (sodium oleate) for each media recipe was dissolved in chloroform – stock A. The required weight of cholesterol for each media recipe was dissolved in chloroform – stock B. An aliquot of stock B was added to stock A to reach the desired concentration (Table 2.2), mixed, and stock A with chloroform was evaporated under a stream of dry nitrogen gas. The dry lipid film was resuspended in water, quantitatively transferred to a volumetric flask, and made up to volume with water. Stock aqueous solutions of buffer ($NaH_2PO_4.H_2O$; 28.4 mM) and salt (NaCl; 105.9 mM) were prepared in water. The method was based on multiple previous papers (Khadra et al., 2015, McPherson et al., 2020). Into a Duran bottle (I L) was added aliquots of the lipid, buffer and salt stock solutions, and water to make a final aqueous system volume of 1 L with the desired concentrations. pH was adjusted to the required value (Table 2.2, target value ± 0.05) using KOH or HCl (1 M) as required. 50 mL of the SIF media were transferred into the InForm® assay vials for further analysis.

Table 5.2: The SIF media number of the drug's analysed lowest, middle, and/or highest solubility result from Chapter 2.

Drug	Lowest SIF media	Middle SIF media	Highest SIF media
Aprepitant	1	-	2
Carvedilol	1	-	2
Felodipine	1	8	2
Fenofibrate	1	-	-
Griseofulvin	1	-	-
Phenytoin	-	1	-
Piroxicam	-	1	-
Probucol	-	1	-
Tadalafil	1	8	2
Zafirlukast	1	-	-

5.3.2.2. Samples Preparation

Each study drug was dissolved with DMSO solvent, in 5 mL volumetric flasks, to form a concentration of either 50, 100, or 200 mM. 1 mL of each solution was transferred into HPLC vial for the following assays. Those DMSO/drug stock solutions were chosen to be as concentrated as possible, taking into consideration adding DMSO less than 2% of the assay vials total volume.

5.3.2.3. InForm® Instrument Assays

All the assays were conducted by the inForm® instrument under the license of the Pion company. Two clean up assays were run before and after each assay, along with calibrating the pH electrode and the UV spectrometer daily, to ensure both are within the normal ranges. Blank readings were taken daily after the preparation of new SIF medium.

All the assays were performed at 37°C, a UV probe path length of 5 or 10 mm, and using the physicochemical properties mentioned in Table 2.1 for 10 drugs. The wavelength used was between 260 – 390 nm, with a simple baseline correction.

The drug analysis used two main assays:

1- UV-metric pKa assay

This assay was performed to get the MEC value needed for the later refinement and quantitation of the controlled SS assay. This is done automatically by the InForm® system. When importing the resulting MEC into the SS assay of the studied drug, the inForm® converts the UV absorbance data, into a concentration data by using Beer-Lambert's law (Wypych, 2020):

$A = \varepsilon . C . L$

Where A is the absorbance value, ε is the MEC value, C is the final desired concentration, and L is the UV probe path length.

The UV-metric pKa assay was performed by manually adding 50 mL of the prepared SIF media into one of the 20-position vial rack, with five subsequent aliquots of the DMSO/drug stock solution, chosen by the user, and automatically dispensed from the HPLC excipient rack by the vial arm's needle. The assay takes around 15 minutes to be completed before being refined to get the MEC spectrum needed for the SS quantitation results.

In order to correctly collect the useful information of the SS assays, the molar absorption profiles for the added aliquots must be parallel and identical. As the molar absorption value of a specific path length and concentration shouldn't differ unless the drug transformed to another physical state, for example from dissolved state to precipitated particles, or by different SIF media content, as proved previously (Madsen et al., 2016). The change from dissolved state to precipitated particles causes a difference of the actual drug quantity, as the amount of drug in-solution is not changing as it should be relative to the amount the inForm® has recorded, so when the inForm® attempts to calculate the MEC it does not produce overlaying profiles. This is indicative by the sloped shape extending to lower wavelengths from the visible wavelength region, and as the studied drugs don't absorb at those wavelengths, so this means that the drug is precipitating, and the MEC wouldn't be reliable in further SS determination.

2- The controlled supersaturation by UV

This assay is parametrized following the same properties set for the UV-metric pKa assay. An advanced manual addition of 50 mL SIF media, with a single automatic addition of a defined concentration of the DMSO/drug stock solution were added into the assay vial. Different concentrations were studied, as the drugs have different propensities to supersaturate, to find their SS concentration, induction time, and where possible, the precipitation rate. The induction time is the time spent for a drug at a specific concentration, before it precipitates (Palmelund et al., 2016).

The SS results were evaluated depending on two criteria:

1- If the drug resulted in a constant concentration profile, this would mean that its either below its equilibrium solubility, or between the equilibrium and the kinetic solubility, but still didn't reach SS. In such cases, if the analysed vial developed a precipitate, this would indicate that the initial added concentration is between the equilibrium and the kinetic solubility, and it needs a further time to destabilize and precipitate.

2- An immediate precipitation is indicative of either the initial drug concentration is higher than the kinetic solubility, or that the drug is incapable of supersaturating (its kinetic solubility is equal to the equilibrium solubility).

5.4. Results and Discussion

The concentrations for 8 out of the 10 drugs tested in this chapter were higher than their equilibrium solubility results of Chapter 2. The exception was carvedilol and piroxicam, which both showed in-solution behavior during the entire assay time. All the drugs which formed precipitation, where having a crystalline concentration higher than the studied equilibrium solubility results found in Chapter 2. The latter result is comparable with literature results (Edueng, 2019, Palmelund et al., 2016). NB all the results of the 10 drugs analysed in this chapter are summarized in Table 5.3 and 5.4.

5.4.1. Felodipine

5.4.1.1. SIF Medium 1 – Lowest Solubility

The path length of the UV probe used was either 5 or 10 mm.

a- Path length 10mm:

The SS assays tested were ranging between 0.1 - 0.3 mM, concentrations lower than 0.16 mM gave an in-solution results for 1 hr duration. Whereas the 0.3 mM assay resulted in an immediate concentration drop at the beginning of the assay, detected by the elevated absorbance at the visible light wavelength range, followed by a plateau at 0.22 mM concentration, see Figure 5.1. This concentration drop corresponding to an immediate precipitation, is indicative that the drug exceeded its kinetic solubility.

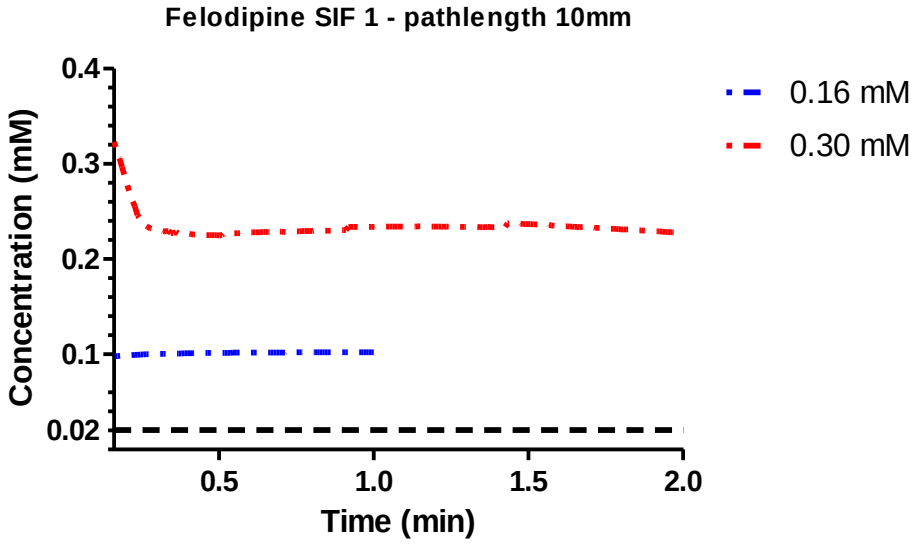

Figure 5.1: Felodipine SS assays in SIF medium 1, at pathlength 10mm, the black line is representative of the drug's equilibrium solubility.

b- Path length 5mm:

The SS assays were analysed in a range between 0.04 - 0.4 mM, and the concentrations below 0.16 mM were in-solution for the whole assay time, 2 hours (same as pathlength 10mm). Whereas at a concentration of 0.2 mM, 4 hours assay duration was run, and it resulted in a precipitation event at 7 minutes after the third hour (induction time). As seen in Figure 5.2, there is quite a long-lived metastable state before the precipitation event started, which means that the 0.2 mM concentration lies between the drug's kinetic and crystalline solubilities. The SS assays of concentrations greater than 0.2 mM, were also plateauing at the 0.2 mM concentration, followed by another plateauing event at around 0.05 mM (crystalline solubility), where the drug should have precipitated to its most stable state. For the concentration assays of 0.24 – 0.4 mM, an initial concentration drop at the beginning of the assay was observed. This concentration drop is potentially indicative of a temporary liquid-liquid phase separation (LLPS) (Raina et al., 2015), followed by conversion to an amorphous metastable state, with a subsequent crystalline event. LLPS only forms when the kinetic solubility is exceeded, so any concentration tested above 0.24 mM started above the maximum achievable level of supersaturation for felodipine in this medium.

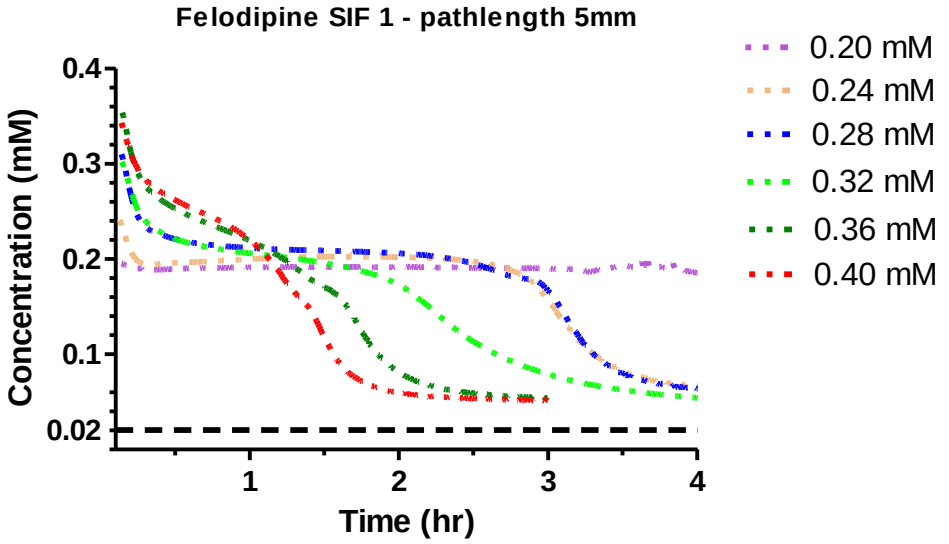

Figure 5.2: Felodipine SS assay in SIF medium 1, at path length 5mm, of different concentration, the black line is representative of the drug's equilibrium solubility.

Also, from the 0.28 mM concentration and above, the data points which forms a shoulder shape just after the first plateau, suggests that the sample is forming crystalline particles, as the scattering which is seen in the spectrum is losing its sloped profile. Finally, the inconsistent concentration drops in the assays between 0.32 – 0.4 mM, is caused by the high precipitation event, and by the inconsistent conversion of liquid-phase separated sample converting to the solid form.

The measured precipitation rate of felodipine using 2 path lengths was different. For concentrations 0.3 and 0.32 mM of path lengths 10- and 5- mm respectively, the measured precipitation rate of path length 5 mm was 12 times lower than the 10 mm. This is referred to the lower sensitivity detecting by the instrument.

Furthermore, the measured precipitation rate of aprepitant and zafirlukast (results will be shown in following sections) was less affected by path length change, compared to felodipine. This result is explained by the reported 'borderline' SS stability of felodipine, compared to aprepitant's 'high' SS stability status (no data were available for the SS stability of zafirlukast), which accounted for a lower effect on detector sensitivity (Skolnik et al., 2018).

5.4.1.2. SIF Medium 2 – Highest Solubility, 5mm Path Length Only

There was a dramatic increase in the solubility of felodipine in this medium compared to medium 1, because of the higher SIF media components concentrations. This result is correlated to a previous literature (Madsen et al., 2016). The SS assays with concentrations range between 0.7 - 1.4 mM seem to observe a complete dissolution of the whole drug, for up to 6 hours. Whereas the assay with a concentration of 2 mM observed a metastable SS state at 1.9 mM, followed by a precipitation event. See Figure 5.3.

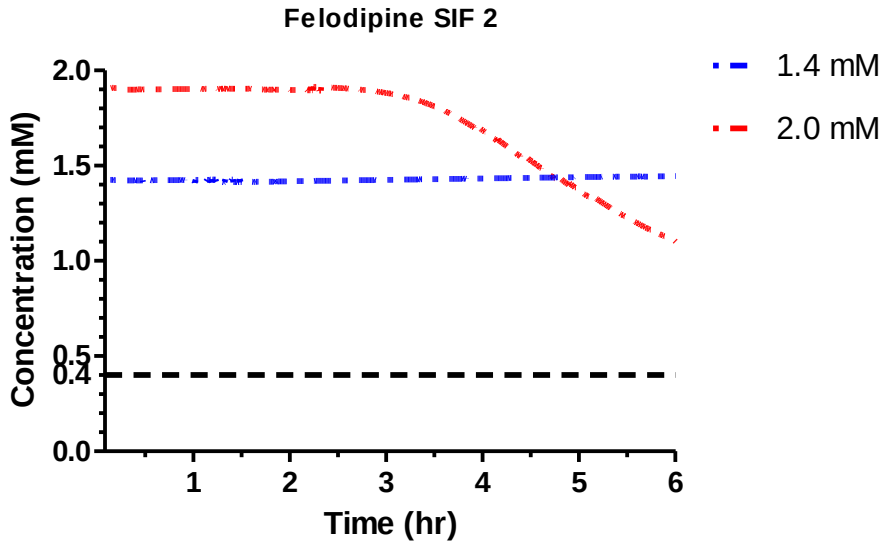

Figure 5.3: Felodipine SIF medium 2, at concentrations 1.4 and 2 mM respectively, the black line is representative of the drug's equilibrium solubility.

The 2 mM assay precipitated to a concentration lower than the concentration observed in the 1.4 mM run, so the drug in the 1.4 mM run is supersaturated too, and just hasn't precipitated over the duration of the 6 hours run. The concentration levels are much higher than in the previous SIF medium 1, as these medium components are having a large effect on the compound's behaviour. Also, there is no evidence of LLPS as observed in the SIF 1 runs, so the kinetic solubility could be much higher in this medium.

5.4.1.3. SIF Medium 8 – Middle Solubility, 5mm Path Length Only

The behaviour of the drug in this medium also seems to suggest the maximum SS concentration is higher in this medium compared to medium 1, but lower than medium 2. The SS assays ranged between 0.16 - 0.79 mM. The drug was in-solution between concentrations 0.16 - 0.4 mM, over 3 hours run time. Whereas between 0.66 – 0.79 mM, the drug started to supersaturate with different induction times, but all precipitated to 0.27 mM (crystalline solubility), see Figure 5.4.

The measured precipitation rate ranged between 4.8 – 10 µM/min, by the concentration increase between 0.66 – 0.79 mM. SIF medium 1 of concentration 0.4 mM had the same measured precipitation rate found in SIF medium 8 of concentration 0.7 mM, which is explained by the higher SIF media component concentrations present in SIF medium 8, which increased the drug stability.

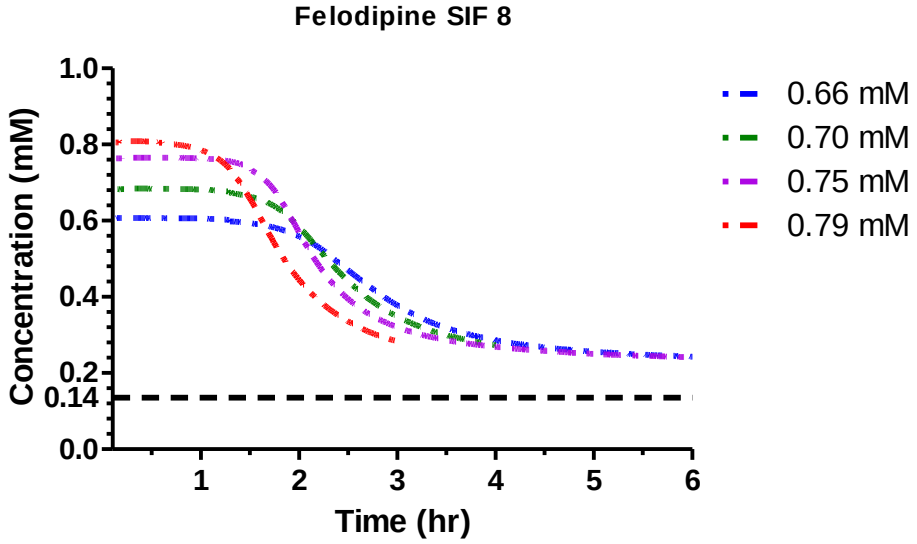

Figure 5.4: Felodipine SS assays of different concentrations tested in SIF medium 8, the black line is representative of the drug's equilibrium solubility.

In a previous study, the 50 – 100% SS concentrations found for felodipine of pathlength 5mm was between 0.26 - 0.5 mM, which lies within the SS concentrations range found in the three media of this study, 0.2 – 2 Mm (Palmelund et al., 2016). The induction times were slightly different, as it was reported to be less than 0.5 hr (Palmelund et al., 2016), and in this study ranged between 0.6 – 3.1 hr. Finally, the literature crystalline solubility reached to as low as 0.16 mM, which is also in the same

range found in this study, 0.05 – 0.27 mM. The reported literature equilibrium solubility, 0.09 mM (Palmelund et al., 2016), was also within the 3 SIF media equilibrium solubilities of this study, 0.02 – 0.4 mM.

5.4.2. Tadalafil

5.4.2.1. SIF Medium 1 – Lowest Solubility

One SS assay of a concentration of 0.4 mM of each 5- and 10- mm path lengths, resulted in an instant precipitation, exceeding the kinetic solubility. The tell is that the concentration drops immediately, and baseline elevation rise from the very first point, indicating the sample is crashing.

5.4.2.2. SIF Medium 8 – Middle Solubility

Path length 5 mm only

SS assays ranged between 0.04 – 0.36 mM. The 0.04 mM assay was in-solution for the whole 3 hours run time, whereas at 0.08 mM tadalafil started to precipitate after 2 hours and 10 minutes after having a stable concentration of 0.08 mM. Higher concentrations of 0.12 and 0.14 mM, for 5 and 3 hours respectively, were carried out, the initial concentration was stabilized for 0.8 and 0.4 hr respectively, before precipitating to around 0.026 mM, see Figure 5.5. The measured precipitation rate was 6 µM/min in the 0.12 mM concentration assay, and was faster in the higher concentration, for around 10 µM/min. Both of those concentrations were following an inconsistent precipitation pattern, which is correlated to the heavy precipitation action that affected the sensitivity to detect the measured precipitation rate. Also, the upward slope was also found by the $Css_{100\%}$ of a previous literature, at a concentration of 0.26 mM, but not the lower concentrations 0.13 – 0.22 mM ($Css_{50\text{-}87.5\%}$) (Palmelund et al., 2016). This discrepancy is related to the different protocols used compared to this study, as the literature equilibrium solubility of Palmelund et al. was reported to be 0.15 mM (Palmelund et al., 2016), which is 10 times higher than the equilibrium solubility found in this Fa9SSIF study.

Two further concentrations were measured at 0.26 and 0.36 mM, but both were highly concentrated.

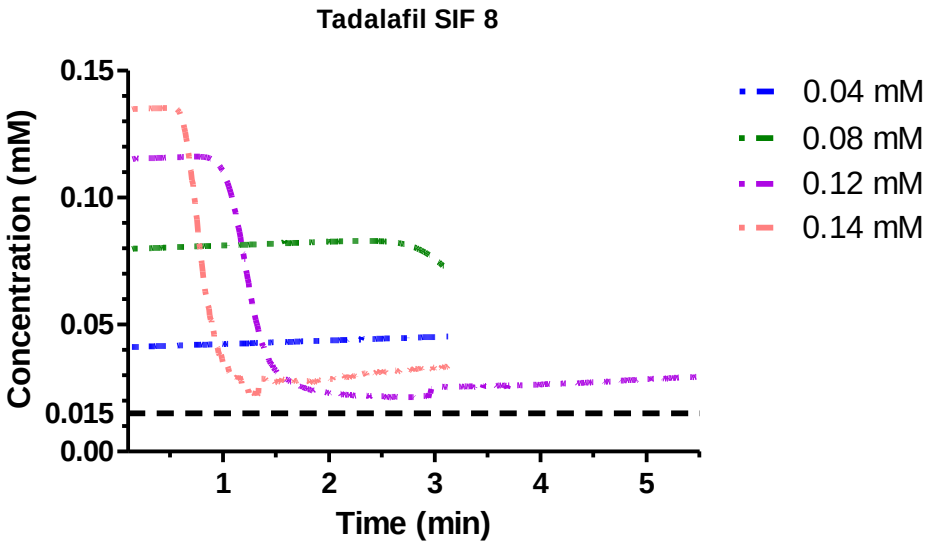

Figure 5.5: Tadalafil SS assays of different concentration of SIF medium 8, the black line is representative of the drug's equilibrium solubility.

5.4.2.3. SIF Medium 2 – Highest Solubility

Path length 5 mm only

SS assays were carried out between 0.26 – 0.79 mM concentration range. The 0.26 mM assay plateaued for 2.5 hr, before it precipitated with a 6.5 µM/min measured rate, to 0.06 mM (crystalline solubility). On the other hand, the 0.4 mM assay immediately precipitated with a 16 µM/min measured rate, to the same concentration 0.06, see Figure 5.6.

The relatively doubled concentration of this medium (0.26 mM) compared to SIF medium 1 concentration (0.12 mM), showed a relevant measured precipitation rate, due to the higher media components concentrations, which increased the solution stability.

The concentrations of 0.6 and 0.79 showed an odd result, as both corresponded to a high and a stable concentration of 3.5 and 19 mM respectively. The behavior of those two higher concentrations was difficult to explain, since the highest $Css_{100\%}$ found for tadalafil was 0.26 mM (Palmelund et al., 2016) which is the same SS concentration resulting in this study.

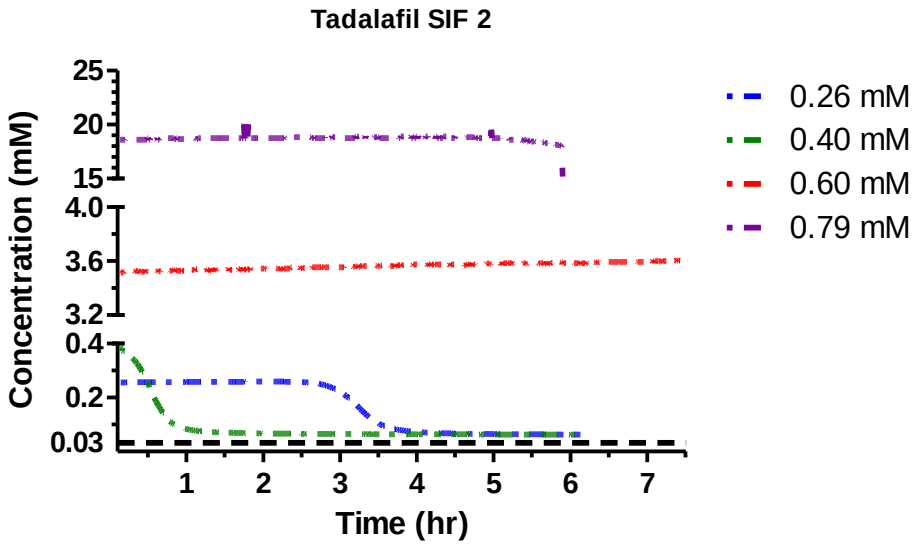

Figure 5.6: Tadalafil SS assays of different concentration in SIF medium 2, the black line is representative of the drug's equilibrium solubility, Y-scale is segmented.

In a previous study (Palmelund et al., 2016), the 50 – 100% SS concentrations found for tadalafil of pathlength 2mm was between 0.13 - 0.26 mM, which is within the SS concentrations range found in media 2 and 8 of this study, 0.08 – 0.4 mM. The induction times were also different, as previously it was less than 0.75 hr (Palmelund et al., 2016), and in this study ranged between 2.5 – 3.2 hr. Finally, the literature crystalline solubility reached to 0.05 mM, which is within the value found in this study, 0.026 – 0.06 mM, as the equilibrium solubility was measured as 0.15 mM (Palmelund et al., 2016), which is 5 times higher than the highest solubility found in medium 2 of this study.

5.4.3. Aprepitant

5.4.3.1. SIF 1 Medium - Lowest Solubility Properties

The path lengths of the UV probe used were 5 and 10mm.

a- Path length 10mm

The only SS assay analysed was 0.79 mM concentration, which showed an immediate drop in concentration from the start of the assay and plateaued at around 0.2 mM

corresponding that the analysed concentration is higher than the drug's kinetic solubility, see Figure 5.7.

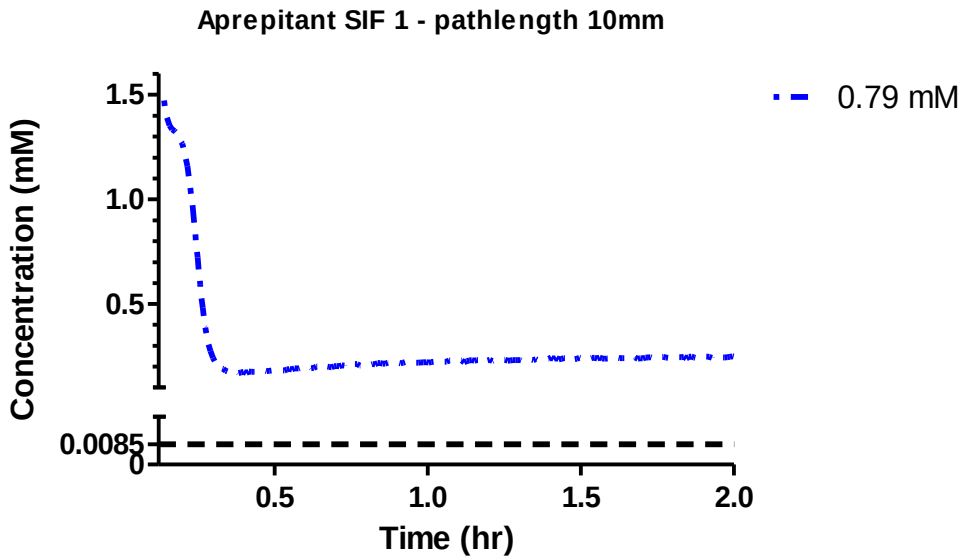

Figure 5.7: SS assay of Aprepitant SIF medium 1, with path length 10mm, and 0.79 mM concertation, the black line is representative of the drug's equilibrium solubility, Y-scale is segmented.

b- Path length 5mm:

The SS assays analysed using this path length was ranging between 0.1 - 1.38 mM concentration, with variable results. All the assays had a short induction time before it started to precipitate, lower than 10 minutes. Whereas the time needed to reach equilibrium ranged between 0.5 – 1 hour. Concentrations of 0.1 - 0.4 mM were precipitating around 0.1 mM, whereas concentrations between 0.7 – 1.38 mM all plateaued at around 0.16 mM, but with a rising slope due to the high precipitation, see Figure 5.8.

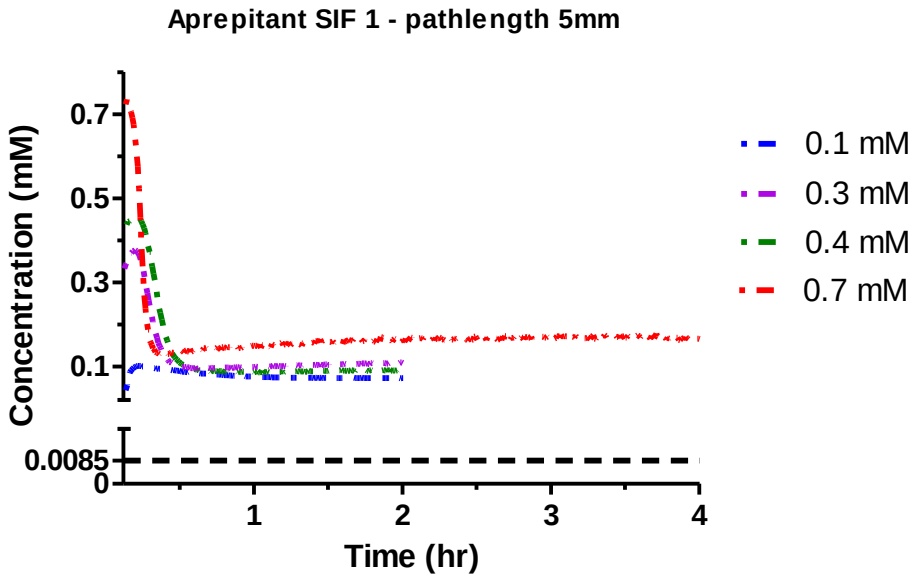

Figure 5.8: Aprepitant SS assays in SIF medium 1, path length 5mm of different concentrations, the black line is representative of the drug's equilibrium solubility, Y-scale is segmented.

5.4.3.2. SIF 2 Medium - Highest Solubility Properties

Path length 5mm only

Noticeably, SIF 2 medium (with higher intestinal components concentrations) had a massive effect on the solubility of aprepitant. As seen from Figure 5.9, in SIF medium 2, despite the higher concentrations used, the baseline is significantly flatter as opposed to SIF 1. This indicates that the spectra collected in SIF medium 2 are in-solution, as it doesn't possess visible wavelength scattering, which could imply a presence of small precipitated particles as in SIF medium 1. Considering the different concentration between these assays, the change is immense. SIF medium 2 seems to be in-solution up to 1.59 mM concentration, whereas in SIF medium 1 aprepitant is already precipitating in its first spectrum at 0.24 mM concentration.

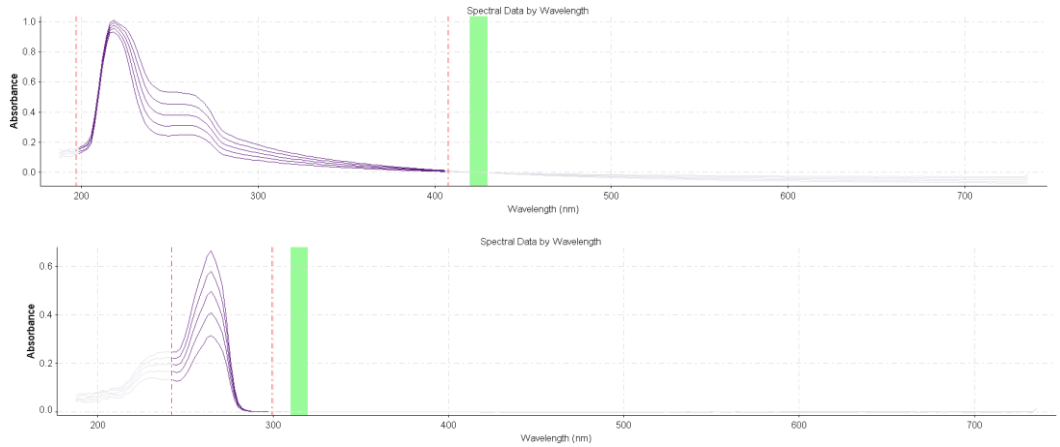

Figure 5.9: Aprepitant MEC assays in SIF medium 1 at concentration 0.24 - 0.4 mM, and SIF medium 2 at concentration 0.8 - 1.59 mM, respectively.

Following onto the supersaturation assays, 3 concentrations were analysed, 0.2, 1.19, and 1.77 mM. As seen in Figure 5.10, the rising concentration for the lowest concentration assay, indicates that the drug is still dissolving. Whereas, for the higher concentrations, the supersaturated state generated seems to be very short-lived, as the samples started precipitating after less than 10 minutes and plateaued at around 0.25 mM.

The measured precipitation rate was proportionally increasing by the concentration increase in SIF medium 2, whereas, increasing the concertation by almost the double between SIF media 1 and 2, has tripled the precipitation rate. This result was not consistent with the doubled concentration found in felodipine and tadalafil, which resulted in constant precipitation rate, between media 1 to 8, and 8 to 2, respectively. The reason behind this inconsistency could be related to the higher SIF media components concentrations, as aprepitant was the only drug which precipitated in its lowest and highest media, compared to others which precipitated in their lowest and middle, or middle and highest media.

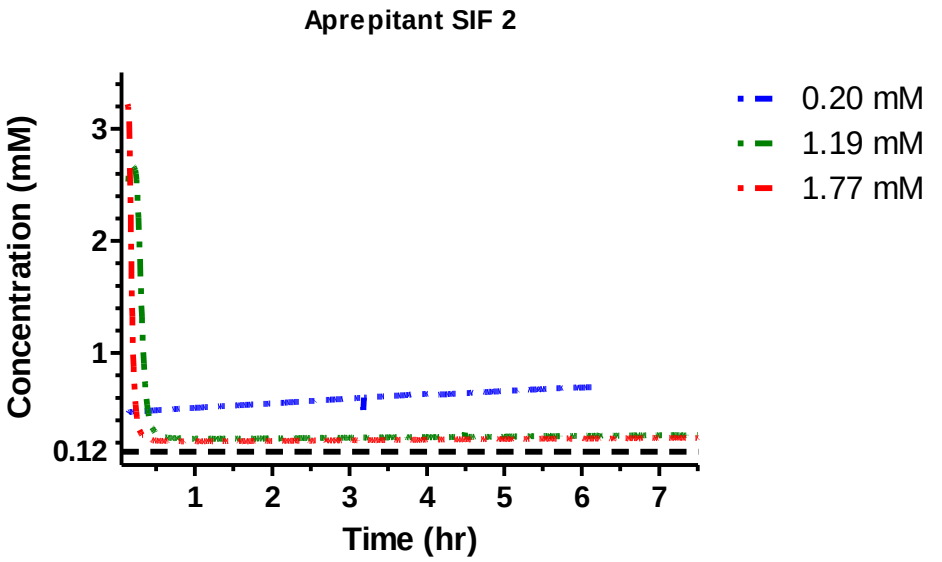

Figure 5.10: Aprepitant SS assays of SIF medium 2, the black line is representative of the drug's equilibrium solubility.

In a previous study (Palmelund et al., 2016), the 50 – 100% SS concentrations found for aprepitant of pathlength 20mm was between 0.17 - 0.34 mM, which lies within the SS concentrations range found in medium 1 of this study, 0.1 – 0.4 mM. The literature induction time for the concentrations above 0.26 mM were matching this study's results, less than 10 minutes. Finally, the literature crystalline solubility averaged about 0.076 mM, which is within the range found in this study, 0.07 – 0.25 mM. The literature equilibrium solubility, 0.09 mM, was within the two SIF media equilibrium solubilities of this study, 0.0085 – 0.12 mM.

5.4.4. Carvedilol

5.4.4.1. SIF Medium 1 – Lowest Solubility

No SS assay was carried out with this medium, but one MEC concentration was analysed, between 0.16 - 0.3 mM. This concentration range resulted in an appearance of absorbance at visible wavelengths, which means that there are precipitated particle scattering light, and resulting in such absorbance. The equilibrium solubility for this medium was found to be 0.12 mM, so the carvedilol SS range in this medium should be narrow, 0.12 – 0.16 mM.

5.4.4.2. SIF Medium 2 – Highest Solubility

A noticeable difference between media 1 and 2 solubility effects on carvedilol was observed, as by MEC assay of 0.16 mM concentration, a precipitation was observed in medium 1, whereas a concentration of 0.4 mM in SIF medium 2, was still dissolving. See Figure 5.11.

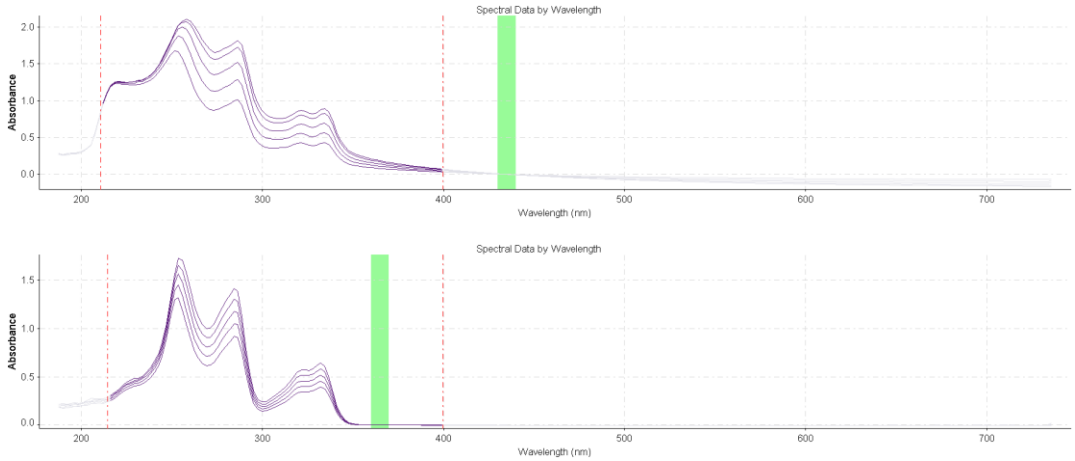

Figure 5.11: Carvedilol MEC assays in SIF medium 1 at 0.16 - 0.3 mM concentration, and SIF medium 2 at 0.24 - 0.4 mM concentration respectively.

Two SS assays were carried out, a concentration of 0.36 and 0.70 mM, for 6 hours run time. Both showed an in-solution profile for the whole assay, which is the expected result, as both concentrations were lower than the equilibrium solubility result found in Chapter 2, see Figure 5.12.

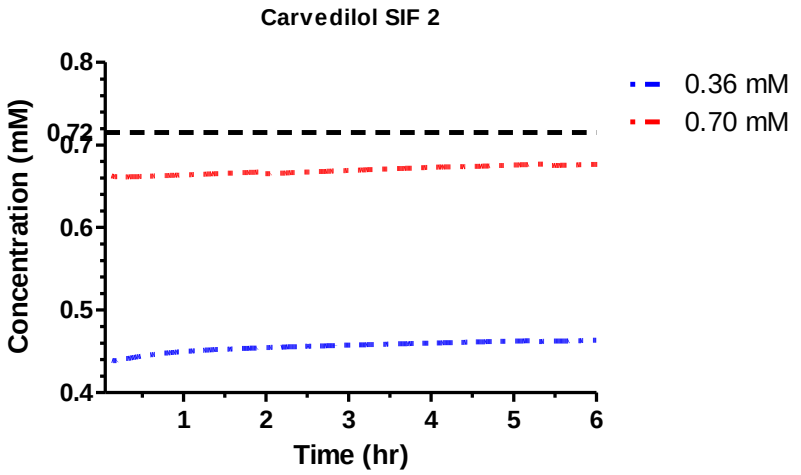

Figure 5.12: Carvedilol SS assays of SIF medium 2, the black line is representative of the drug's equilibrium solubility.

In a previous literature (Skolnik et al., 2018), the SS concentration was reported to be 0.26 mM, which is lower than the equilibrium solubility found for SIF medium 2. On the other hand, this value was within the SIF medium 1 MEC assay's range, which showed some elevation in the visible wavelength, indicating precipitation, which also confirms the narrow SS range of carvedilol.

5.4.5. Griseofulvin

SIF Medium 1 - The Lowest Solubility

a- Path length 10mm

The only SS assay tested was 0.5 mM concentration, see Figure 5.13. Based on the dissolution profile, 2 hours assay resulted in supersaturation and precipitation events. There is a period at the start of the assay where there is a stable sample concentration, which begins to precipitate around 15 minutes. The curve becomes a bit peculiar around 40 minutes in were it erroneously plateaus for a while, reaching a final concentration of 0.07 mM.

b- Path length 5mm

The only SS assay tested was 0.2 mM concentration, see Figure 5.13, and based on the dissolution profile, the sample was in-solution for the whole 1 hr assay run time.

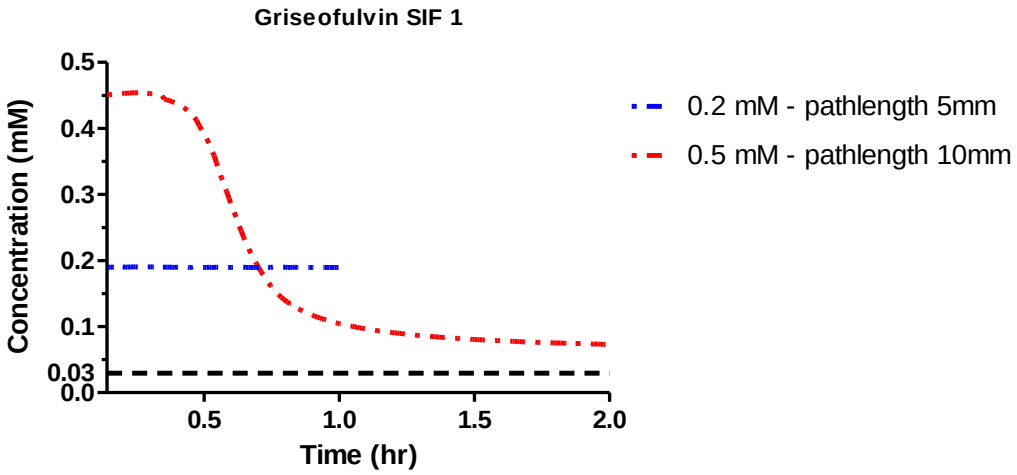

Figure 5.13: The concentration profile of the SS assays of griseofulvin with path length 5- and 10- mm.

The 0.5 mM (path length 10mm) assay precipitated to a concentration lower than 0.2 mM (path length 5mm), which indicates that the 0.2 mM assay is supersaturating but needs a longer time to precipitate to a more stable form. In a previous literature (Skolnik et al., 2018), the SS concertation was reported to be 0.27 mM, which is in the same range resulting in this study (0.2 – 0.5 mM).

5.4.6. Fenofibrate

SIF Medium 1 – The Lowest Solubility

Path length 5mm only

The SS assays ranged between concentrations 0.02 – 0.59 mM. The concentrations between 0.02 – 0.05 shows a little elevation in the visible wavelength, which could be correlated to either an initial precipitation event, or, more probably, just a change in the SIF medium transparency with the introduction of the fenofibrate/DMSO sample. All the concentrations above that were precipitating, a range between 0.1 – 0.2 mM, included light scattering, and the MEC fitting progressively worsened, with the further sample precipitation. This is why no stable supersaturated concentration was able to be confirmed. The highest concentration analysed was 0.59 mM, and it presented more sample concentration compared to the previous range, but less precipitation action. This could be referred to the higher DMSO percent presented in the sample, which exceeded 1%, and thus affected the fenofibrate solubility. The

instability results are referred to fenofibrate's low SS stability status, which was reported previously (Skolnik et al., 2018).

In a previous study (Palmelund et al., 2016), the 50 – 100% SS concentrations found for fenofibrate of pathlength 5mm was between 0.13 - 0.25 mM, with induction time range 0.06 – 0.45 hr, and the crystalline solubility was 0.06 mM. This could confirm that the relevant 0.1 mM concentration had a short-lived SS event for 0.17 hr, followed by a precipitation event to 0.07 mM concentration. See Figure 5.14.

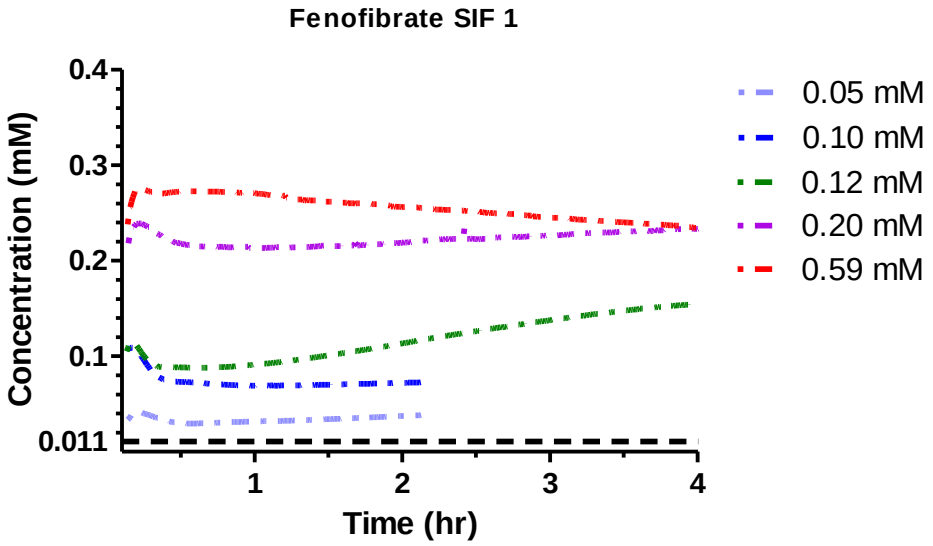

Figure 5.14: Fenofibrate SS assays of SIF medium 1, the black line is representative of the drug's equilibrium solubility.

5.4.7. Probucol

SIF Medium 1 - The Middle Solubility

Path length 10mm only

The MEC spectra analysed between 0.05 – 0.12 mM indicates that even at the lowest concentration, the sample seems to have precipitated, given the absorbance seen across the whole spectrum and the sloping nature of the baseline. From this, it can be inferred that the kinetic solubility of the sample lies below the concentration at which the first spectrum of the MEC was collected, which is 0.05 mM. In the SS assay, at concentration 0.25 mM, a very significant amount of turbidity from the very start of

the assay was shown, which implies the initial spike was above the kinetic solubility, see Figure 5.15.

As the probucol equilibrium solubility found in Chapter 2 was 0.011 mM, this suggests the narrow SS range to be between 0.011 – 0.05 mM, but this range is slightly higher than the previously reported SS concentration, 0.009 mM (Edueng, 2019).

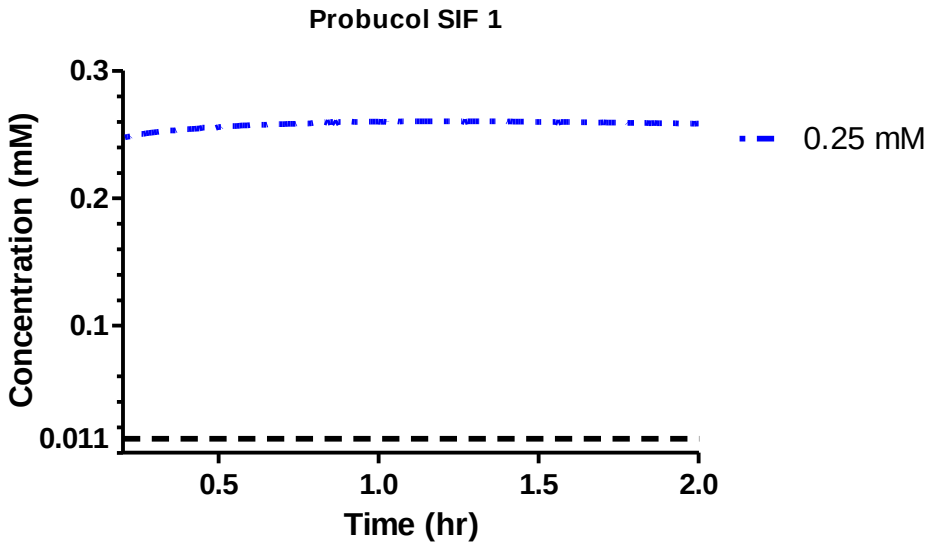

Figure 5.15: Probucol SS assay at concentration 0.25 mM in SIF medium 1, the black line is representative of the drug's equilibrium solubility.

5.4.8. Phenytoin

SIF Medium 1 – The Lowest Solubility

Path length 10mm only

The SS concentration of 1.96 mM seemed to be precipitating from the assay start, judging by the fast onset of concentration drop. Due to how quickly the precipitation occurs, there are not enough points in the upper portion of the plateau to reliably model for the induction time. However, the lower plateau ensures that the precipitation concentration of phenytoin of medium 1 was around 0.3 mM, see Figure 5.16. In a previous study (Skolnik et al., 2018), phenytoin SS concentration was 0.25 mM, which is lower than the precipitation concentration found in this study, but the high concentration analysed, could resulted in this variability.

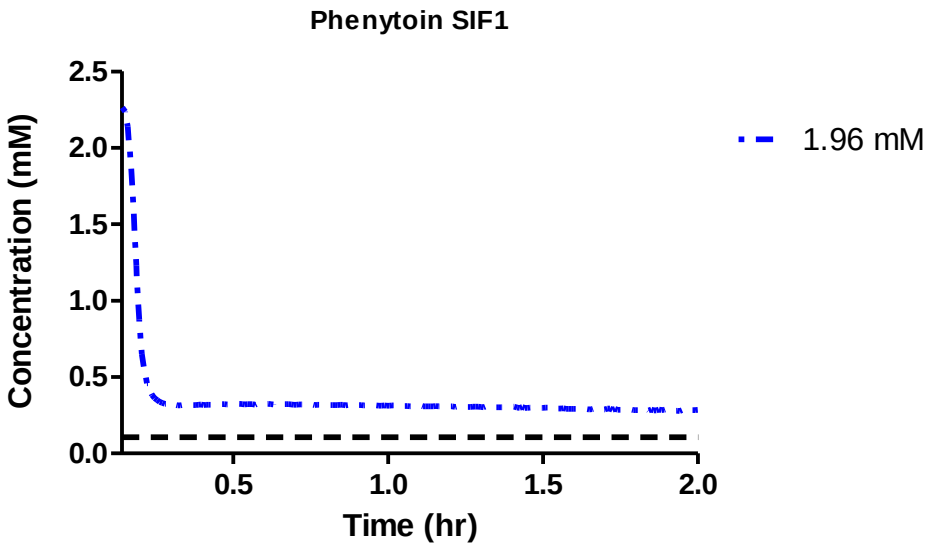

Figure 5.16: Phenytoin SS assay at concentration 1.96 mM in SIF medium 1, the black line is representative of the drug's equilibrium solubility.

5.4.9. Piroxicam

SIF Medium 1 – The Middle Solubility

Path lengths 10- and 5- mm

The concentrations of 0.12 and 0.34 mM of path length 10mm, and 0.16 mM of path length 5mm, all resulted in a stable concentration, for a duration of 2 and 1 hr respectively. This implies that the samples are completely dissolved and in-solution, see Figure 5.17. Referring to Chapter 2 results of piroxicam in SIF medium 1, the solubility result in this medium was approximately 2.5 mM, which indicates that at these concentrations, piroxicam was below its equilibrium solubility. No literature SS studies were found for piroxicam to compare.

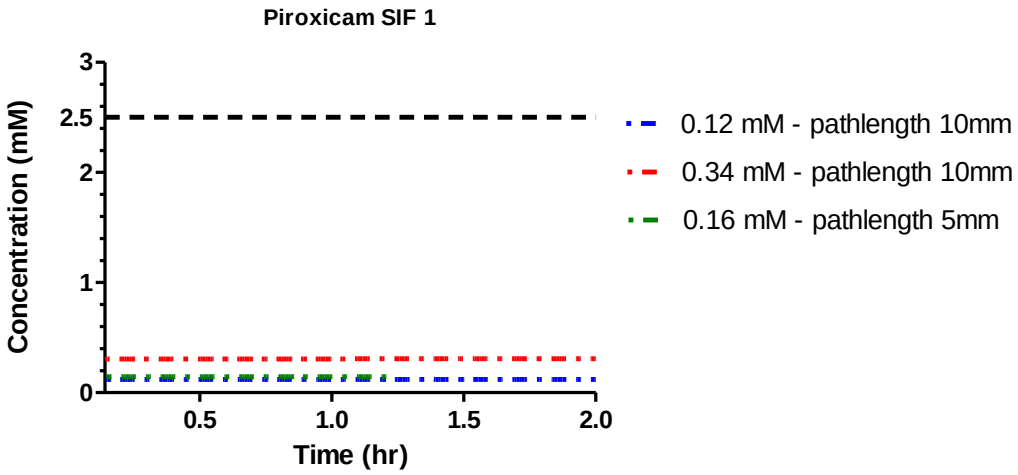

Figure 5.17: Piroxicam SS assays in SIF medium 1 using path lengths of either 5- or 10 mm, the black line is representative of the drug's equilibrium solubility.

5.4.10. Zafirlukast

SIF Medium 1 – The Lowest Solubility

Path lengths 10- and 5- mm

Two SS assays of path length 10mm at concentration 0.14 and 0.60 mM, and two assays of 0.16 and 0.24 mM by path length 5mm, all precipitated from the beginning of the assay, which indicates that those concentrations were above the zafirlukast kinetic solubility, see Figure 5.18. This was confirmed by the MEC assay of concentrations between 0.08 – 0.16 mM, path length 5mm, which showed in-solution profile of the first aliquots 0.08 mM, but the second 0.12 mM triggered precipitation, which could conclude that the SS concentration lies in this range.

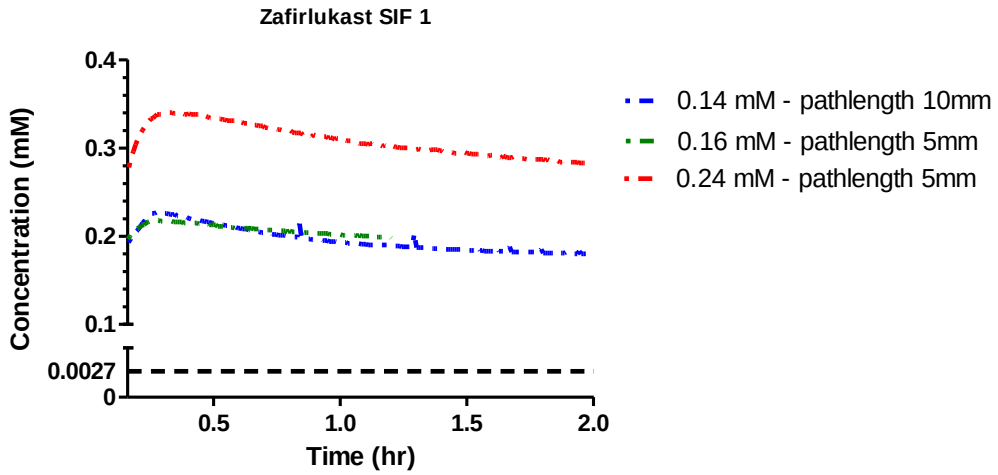

Figure 5.18: Zafirlukast SS assays in SIF medium 1 using path lengths of either 5- or 10 mm, the black line is representative of the drug's equilibrium solubility, Y-scale is segmented.

In a previous study (Madsen et al., 2016), the SS concentration of zafirlukast by using 2mm path length was reported to be 0.14 mM. In this study the path length used was 5- or 10-mm, which also accounted for a relevant possible SS concentration of 0.08 – 0.12 mM, compared to literature.

Table 5.3: A summary of all the results found by the InForm® instrument for 10 drugs.

Drug	SIF media	Path length (mm)	In-solution concentration (mM)	Maximum time (hr)	SS concentration range (mM)	Induction time (hr)	SS plateauing concentration (mM)	Literature SS concentration (mM)
Felodipine	1	10	0.10 - 0.16	1	-	-	-	0.52[1,2]
			-	-	0.3	2	0.22	
		5	0.04 - 0.16	2	0.20 – 0.28	3.1 – 2.2	0.2	
	8	5	0.16 - 0.40	3	0.66 – 0.79	0.9 – 0.6	0.66 – 0.79	
	2	5	0.7 – 0.9	6	2	3	1.9	
Tadalafil	8	5	0.04	3	0.08	2.2	0.08 – 0.14	0.12 - 0.26[1,2]
	2	5	-	-	0.26 – 0.40	2.5 – 0.17	0.26	
Aprepitant	1	10	-	-	-	-	-	0.34[1]
		5	-	-	0.1 – 0.4	< 0.17	0.1 – 0.4	
	2	5	0.2	6	1.19 – 1.77	< 0.17	2.5 – 3.2	
Griseofulvin	1	10	-	-	0.5	0.25	0.45	0.27[3]
		5	0.19	1	-	-	-	
Fenofibrate	1	5	0.02 - 0.05	2	0.1	0.17	0.11	0.25[1]

Drug	SIF media	Path length (mm)	In-solution concentration (mM)	Maximum time (hr)	SS concentration range (mM)	Induction time (hr)	SS plateauing concentration (mM)	Literature SS concentration (mM)
Phenytoin	1	10	-	-	-	-	-	0.25[3]
Zafirlukast	1	10	-	-	-	-	-	0.14[4]
		5	-	-	-	-	-	

NA: not available.

References: 1- (Palmelund et al., 2016) 2- (Plum et al., 2020b) 3- (Skolnik et al., 2018) 4- (Madsen et al., 2016).

Table 5.4: A summary of all the results found by the InForm® instrument for 10 drugs, where precipitation is present.

Drug	SIF medium	Path length (mm)	Sample concentration (mM)	Maximum time (hr)	Measured precipitation rate (µM/min)	Crystalline concentration (mM)	Equilibrium solubility (mM)
Felodipine	1	10	0.3	2	27	-	0.020
		5	0.24	4	5	0.06	
			0.28	4	5.3	0.06	
			0.32	4	2.3	0.045	
			0.36	4	5.2	0.05	
			0.4	4	6.7	0.05	
	8	5	0.66	3	4.8	0.27	0.14
			0.7	6	6.6	-	
			0.75	6	10	-	
			0.79	6	10	-	
	2	5	2.0	6		-	0.40
Tadalafil	8	5	0.12	5.5	6.0	0.026	0.015
			0.14	3	10	-	
	2	5	0.26	6	6.5	0.06	0.032
			0.4	6	16	-	

Drug	SIF medium	Path length (mm)	Sample concentration (mM)	Maximum analysed time (hr)	Measured precipitation rate (µM/min)	Crystalline concentration (mM)	Equilibrium solubility (mM)
Aprepitant	1	10	0.79	2	400	0.2	0.0085
		5	0.1	2	1.2	0.07	
			0.3	2	45	0.1	
			0.4	2	57	0.09	
			0.7	4	174	0.16	
	2	5	1.19	7.5	555	0.25	0.12
			1.77	7.5	1164	0.23	
Griseofulvin	1	10	0.5	2	27	0.078	0.03
Fenofibrate	1	10	0.1	2	5.6	0.07	0.011
Phenytoin	1	10	1.96	2	1100	0.3	0.11
Zafirlukast	1	10	0.14	2	1.3	-	0.0027
		5	0.16	2	0.47	-	
			0.24	1	0.7	-	

5.5. Conclusion

In this chapter, supersaturation was studied by applying solvent shift method on 10 drugs, using fasted bioequivalent SIF media recipes. The results were evaluated by different path lengths and media content, and where available, compared with literature SS results. All the assays were carried out automatically using the inForm® instrument, and the results proved the different propensities of drugs to supersaturate, as found previously (Madsen et al., 2016). For 8 out of the 10 drugs, the SS assays were analysed using concentrations above each drug equilibrium solubility found in Chapter 2, the exception was for carvedilol and piroxicam.

The SS concentration determined for all the drugs was found either precisely by the SS assays, or relatively by the MEC assay range as in probucol and zafirlukast, refer to Table 5.3 and 5.4 for details. The literature SS findings were in the same range of the determined SS concentrations of 4 out of 8 drugs of this study, whereas, 2 drugs were having a lower SS range, and 2 drugs had a higher SS range.

Only 3 out of the 10 drugs were studied using two or/and three SIF media recipes, and all formed SS states which could enable appropriate comparison between different media recipes. The SIF media recipes were different in the components concentrations and had 3 concentration levels, the lowest (medium 1), middle (medium 8), and highest (medium 2). The drugs showed that the media with higher SIF components concentrations, resulted in a higher metastability, and thus higher concentration was needed to form a metastable SS state, which precipitated to a more stable concentration (crystalline solubility). The latter result is comparable to the previous literature studies which found the media components to affect the SS state, along with the precipitation event (Kostewicz et al., 2002), and the higher media components concentration also resulted in a higher SS solubility (Madsen et al., 2016).

One literature study was found to report the time needed to induce SS and the crystalline solubility, which included 4 drugs from this study (Palmelund et al., 2016). Aprepitant and fenofibrate had in-range induction times, whereas the induction times of felodipine and tadalafil were higher in this study.

In this study, 7 out of the 10 drugs reached a stable crystalline solubility, which was higher than the reported equilibrium solubility in Chapter 2 for each of media recipe. This latter result is correlated to the same literature study which found all the 4 drugs to have a precipitation concentration higher than the studied equilibrium solubility they reported (Palmelund et al., 2016), but all had an in-range crystalline solubility values to this study results.

The measured precipitation rate was proportionally correlated with the higher concentrations used. Whereas analyzing relatively equal concentrations with different path lengths (5- or 10- mm) resulted in half precipitation rate value of the 5mm path length in aprepitant and zafirlukast, but 10 times lower precipitation rate was found with felodipine. This is referred to the sensitivity change of the detector to measure the precipitation rate of the precipitated solid particles, where light was scattered. Also, this is referred to the SS stability state which was reported previously as borderline and high, for felodipine and aprepitant respectively (Skolnik et al., 2018). On the other hand, the different media used had no effect on the measured precipitation rate even with doubling the concertation if comparing the lowest to middle, or middle to highest SIF media recipes, which is correlated to the higher SIF media components present that increased the drug stability even with doubled concertation, as seen with felodipine and tadalafil. Whereas, doubling the concentration using the lowest to the highest media recipes resulted in tripling the measured precipitation rate of aprepitant. The reason behind this inconsistency could be related to the higher SIF media concentrations difference. In conclusion, supersaturation and precipitation will vary across the fasted SIF space and therefore across FaHIF space, so once again single measurement will not provide a complete picture and lowest value could be treated as a worst-case scenario.

Chapter 6: The Oral Drugs Bioequivalent Solubility Predictive Effect Using *in-silico* Physiology Based Pharmacokinetic Modelling (PBPK)

6.1. Introduction

The absorption of orally administered drugs from the intestinal tract is a key stage needed for the drug to reach the systemic circulation (Sjögren et al., 2013). The critical factors affecting drug absorption are dissolution controlled by the intestinal solubility, and permeability through the gastrointestinal wall (Amidon et al., 1995). However, multiple studies have emphasized the complexity of the gastrointestinal tract (GIT) absorption process by drawing attention to the other parameters affecting it, such as the drug's physicochemical properties, biopharmaceutical parameters, along with the different physiological properties of the human body (Löbenberg and Amidon, 2000). In order to analyse and understand this complexity, physiology based pharmacokinetic (PBPK) models were introduced to include all those parameters, by the use of different built-in mathematical equations (*in silico* approach). This permitted a link between the *in vivo* physiological data, and the *in vitro* data, for example the GI fluid volumes, transit time, pH, the drug's physicochemical properties, solubility and permeability (Sugano, 2009). Wider aspects were also taken into account to predict the drugs extent of absorption by incorporating the drug's pharmacokinetic (PK) properties, to predict a plasma concentration-time profile (simulated model) for a better comparison with literature clinical data (human observed model) (Sjögren et al., 2013). The application of such models was important, especially for the early stages of drug development to reduce cost, time and effort (Sjögren et al., 2013).

This application of PBPK introduced a need to incorporate accurate input parameters, to precisely predict the PK parameters and the fraction absorbed (Fa%) (Sjögren et al., 2013). This requirement was the main objective of this study, by incorporating a fasted bioequivalent solubility data of six drugs, studied in Chapter 2. This equilibrium solubility data were proven to cover more than 96% of the human intestinal fluid (HIF) composition variability (Pyper et al., 2020), based on the

comparison between the simulated intestinal fluid (SIF) and HIF in Chapter 2 and the determined correlation, this chapter will mainly investigate the impact of the bioequivalent solubility on PBPK simulations.

Several PBPK software packages are available for this purpose, but in this chapter the GastroPlus® software was used for predictions and simulations, depending on the input parameters of literature simulated models (Markovic et al., 2020, Sjögren et al., 2013, Sjögren et al., 2016). Note that this research is not directed at creating, developing, or refining PBPK models, therefore available literature models were used without alteration.

6.2. Aims and Objectives

- Reduce the time, effort, and cost needed for *in vitro* and *in vivo* studies by predicting the PK parameters and the absorption extent of six poorly soluble drugs, using *in silico* modeling approach with fasted bioequivalent lowest and highest solubility data.
- Provide a more accurate prediction accuracy using the current bioequivalent model versus literature *in silico* models.

6.3. Methods

6.3.1. Gastrointestinal Tract Properties

GastroPlus® software depends on an advanced compartmental transit absorption (ACAT) model (Agoram et al., 2001) of 9 compartments: stomach, duodenum, 2 compartments for jejunum, 3 compartments for ileum, caecum, and ascending colon, to predict the oral absorption of the human GIT. See Table 6.1 for the default fasted GI human physiology properties in the software. The version used for GastroPlus® was 9.8.2, and the default setting for the intestinal transit time, as a total, was 3.23 hr.

Table 6.1: The fasted gastrointestinal tract properties incorporated in the GastroPlus® Software.[4]

	pH	Transit time (hr)	Volume (mL)	Length (cm)	Radius (cm)	Bile salt (mM)
Stomach	1.3	0.25, 0.1	48.92	29.19	9.87	0
Duodenum	6	0.26	44.57	14.58	1.56	2.8
Jejunum 1	6.2	0.94	166.6	60.26	1.48	2.3
Jejunum 2	6.4	0.74	131	60.26	1.32	2.03
Ileum 1	6.6	0.58	102	60.26	1.16	1.4
Ileum 2	6.9	0.42	75.35	60.26	1	1.16
Ileum 3	7.4	0.29	53.57	60.26	0.84	0.14
Caecum	6.4	4.36	50.49	13.5	3.45	0
Ascending colon	6.8	13.07	53.55	28.35	2.45	0

6.3.2. The Compounds Properties

Six poorly absorbed drugs were chosen for the modeling (bioavailability in brackets); aprepitant (59%) (Majumdar et al., 2006, Tolou-Ghamari et al., 2013), carbamazepine (75 - 85%) (Tolou-Ghamari et al., 2013), felodipine (15%) (Edgar et al., 1992), fenofibrate (< 35%) (Ling et al., 2013), furosemide (50%) (Waller et al., 1982), and griseofulvin (25 - 70%) (Arida et al., 2007). Those drugs have well-defined PK data, and not completely absorbed in the GIT, and were chosen depending on the availability of literature *in silico* models (Markovic et al., 2020, Sjögren et al., 2013, Sjögren et al., 2016).

The compounds properties are detailed in Table 6.3 and 6.4. The default mean precipitation time used was 900 s, and no enzyme or transporter effects were incorporated in the simulations, for simplification purposes. Solubility data depended on Chapter 2 data of the lowest, highest and FaSSIF-v1 solubility results found for the

[4] Numbers are utilised directly from the software.

6 drugs at a certain pH value, whereas, the permeability values were measured previously (Lennernäs, 2007, Sjögren et al., 2013) and used in modelling.

6.3.3. The pharmacokinetic parameters

The PK parameters of the drugs, except furosemide (Markovic et al., 2020), were obtained from (Sjögren et al., 2013, Sjögren et al., 2016): the first pass extraction % (FPE), total body clearance (CL), volume of distribution (Vd), and the drug's plasma fraction unbound (Fub). The drugs' formulation, dose, water administered, particle size, and individuals body weights were extracted from the same studies the clinical data used, as detailed in Table 6.5 (Sjögren et al., 2013, Sjögren et al., 2016). All the simulations were analysed with 70 kg human body weight.

The ADMET® predictor (version 10) available within the GastroPlus® software, was used in all the drugs' simulation by incorporating each drug's chemical structure, and its results were only used if data was not available in literature, for example for the blood to plasma (B:P) ratio. No data adjustment or model fitting was used in this study.

6.3.4. Analysis

Two models were developed using the lowest and the highest solubility data results from Chapter 2 of each drug, and then both were statistically compared using a paired t-test (p-value < 0.05 were considered significantly different), by graph pad Prism software, version 5.

The simulation results were classified based on the accuracy of the extent of deviation from the clinical data (Sjögren et al., 2016). The accuracy of the results was considered to be highly accurate if the extent of deviation were less than 25%, medium if it was between 25% to 50%, low if it was between 50% - 2 fold, and inaccurate if more than 2-fold (Sjögren et al., 2013). The low and medium accuracies were further classified as acceptably accurate (Sjögren et al., 2016), see Table 6.2.

Table 6.2: Classification criteria of the results accuracy (Sjögren et al., 2013, Sjögren et al., 2016).

Extent of deviation	**Accuracy**
< 25%	High
25% to 50%	Medium (acceptable)
50% - 2-fold	Low (acceptable)
> 2-fold	Inaccurate

Table 6.3: Compound properties for the six drugs simulated using GastroPlus® software.

Drug	**Molecular formula**	**Molecular weight (g/mol)**	**Log D (pH 7.4)**	**pKa**	**Diffusion coefficient (cm^2/s *10^{-5})**	**Drug particle density (g/mL)**	**Permeability (cm/s *10^{-4})**	**Melting point (° C)**
Aprepitant	$C_{23}H_{21}F_7N_4O_3$	534	6.9	9.7	0.63	1.51	7.1	255
Carbamazepine	$C_{15}H_{12}N_2O$	236	1.6	-	0.78	1.27	4.3	190
Felodipine	$C_{18}H_{19}Cl_2NO_4$	384	4.3	-	0.67	1.28	7.8	145
Fenofibrate	$C_{20}H_{21}ClO_4$	361	6.9	-	0.66	1.18	7.7	80
Furosemide	$C_{12}H_{11}ClN_2O_5S$	330	-1.1[1]	4.2[1]	0.73[1]	1.2	0.4[1]	206
Griseofulvin	$C_{17}H_{17}ClO_6$	353	2.9	-	0.7	1.38	7.3	220

References: 1- (Markovic et al., 2020), melting points from PubChem, other data (Sjögren et al., 2013).

Table 6.4: Solubility (mg/mL) input of this study (Chapter 2 results) versus literature data (Markovic et al., 2020, Sjögren et al., 2013).

Drug	**Bioequivalent**				**Literature**	
	Lowest solubility	**pH**	**Highest solubility**	**pH**	**Solubility**	**pH**
Aprepitant	0.0046	6.64	0.064	7.12	0.00037	6.5
Carbamazepine	0.15	6.54	0.25	7.12	0.127	6.5
Felodipine	0.0078	6.64	0.15	7.12	0.001	6.5
Fenofibrate	0.0038	6.64	0.029	7.14	0.00025	6.5
Furosemide	0.397	5.72	15.88	8.04	8.34	7.5
Griseofulvin	0.01	6.64	0.024	7.12	0.015	6.5

Table 6.5: The pharmacokinetics parameters used in the simulation of six drugs.

Drug	**Dose (mg)**	**Particle radius (µm)**	**Water administered (mL)**	**FPE (%)**	**B:P ratio**	**Fub (%)**	**CL (L/h)**	**Vd (L/Kg)**	**Simulation time (hr)**
Aprepitant (Capsule)	125	0.06	240	4	0.73	0.0021	3.7	0.28	96
Carbamazepine (Tablet)	400	75	250	1.3	0.86	1	1.1	0.87	120
Felodipine (Tablet)	10	2.5	240	87	0.70	0.45	58	0.22	8
Fenofibrate (Suspension)	160	1.1	250	NA	0.70	0.0021	0.75	0.097	120
Furosemide (Tablet)	40	25[1]	250	NA	1	1[1]	9.0[1]	0.043[1]	10
Griseofulvin (Suspension)	472	0.6	240	8	0.76	1	7.3	1.3	72

NA: not available, FPE: first pass extraction, B:P: blood to plasma, Fub: fraction unbound, CL: clearance, Vd: volume of distribution. References: 1- (Markovic et al., 2020), other data (Sjögren et al., 2013).

6.3.5. Clinical Data (Human Observed)

The literature was searched for oral clinical data for fasted healthy individuals, of drugs which have a GastroPlus model. For all the drugs, except furosemide (Markovic et al., 2020), the clinical data (mean of individuals concentrations) used were the same data used in literature *in silico* models (Sjögren et al., 2013). The clinical data found for furosemide (Markovic et al., 2020), were extracted using the Web Plot Digitizer tool. Where available, the standard deviation was plotted for the clinical data points, shown in the results section.

6.3.5.1. Furosemide

As a total, seven clinical studies were found for furosemide, the variable properties extracted from each study is detailed in Table 6.6. The previously modeled study (Markovic et al., 2020) used two studies out of the six studies, and only (Beermann and Midskov, 1986) was used in this study comparison.

6.3.5.2. Aprepitant

Two studies were found for aprepitant, one of them was used in the previous *in silico* model (Sjögren et al., 2016) at a dose of 125 mg (Majumdar et al., 2006), see Table 6.7.

Table 6.6: Literature pharmacokinetic studies furosemide oral formulations in healthy fasted individuals.

	Study duration (hr)	**Dose (mg)**	**Formulation**	**Water administered (mL)**	**Number of volunteers**	**Age (yrs)**	**Body weight (kg)**	**F %**
(Beermann, 1982)	24	40	Tablet	150	4 females 8 males	20 – 40	64	NA
(Beermann and Midskov, 1986)	10	40	Tablet	250	10	20-40	NA	NA
(Branch et al., 1977)	5	80	Tablet	NA	6 males	20 – 25	NA	50
(Hammarlund et al., 1984)	8	40	Tablet	200	5 females 3 males	22 – 32	55 - 82	51.3
(Michael et al., 1974)	4	80	Tablet and solution	NA	8	18 - 45	NA	NA
(Tilstone and Fine, 1978)	2.5	44	Solution	NA	14 males	21 - 35	NA	NA
(Waller et al., 1982)	12	40	Tablet	200	18 males	20 - 31	71	71

F: bioavailability, NA: not available.

Table 6.7: The two studies found for aprepitant capsules of healthy fasted individuals.

	Study duration (hr)	**Dose (mg)**	**Formulation**	**Water administered (mL)**	**Number of volunteers**	**Age (yrs)**	**Body weight (kg)**	**CL (L/hr)**	**Vd (L/kg)**	**F %**
(Majumdar et al., 2006)	96	125	Capsule	240	13 females 12 males	28	Normal	3.57	0.89	59
		80						4.3	1	67
(Yang et al., 2020)	96	125	Capsule	240	12 (Females = Males)	25	64	NA	NA	NA

F: bioavailability, CL: clearance, Vd: volume of distribution, NA: not available.

6.3.5.3. Griseofulvin

Six studies were found for griseofulvin with different formulations, particle size, and doses, see Table 6.8. In the literature simulation model (Sjögren et al., 2013), the clinical data were used based on a previous study (Lin et al., 1973).

6.3.5.4. Felodipine

For felodipine, six studies were also found and are detailed in Table 6.9. The reference of the clinical data used in the literature model (Sjögren et al., 2013) couldn't be found, so the data were used based on the supplementary files.

Table 6.8: The six studies found for griseofulvin oral formulations of healthy fasted individuals.

	Study duration (hr)	**Dose (mg)**	**Particle radius (μm)**	**Formulation**	**Water administered (mL)**	**Number of volunteers**	**Age (yrs)**	**Body weight (kg)**
(Straughn et al., 1980)	73	500	4	Suspension, tablet	240	12 Males	21-30	61-91
(Lin et al., 1973)	72	472	4	Suspension	NA	5 Males	NA	70
(Marvel et al., 1964)	48	1000	NA	Tablet	NA	12 (Females=Males)	NA	NA
(Crounse, 1961)	48	1000	NA	Oral	NA	37	NA	NA
(Rowland et al., 1968)	72	500	4	Tablet	200	5 Males	35-48	73
(Lin et al., 1982)	48	500	4	Tablet	NA	16 Males	NA	NA

NA: not available.

Table 6.9: The six studies found for felodipine oral formulations of healthy fasted individuals.

	Study duration (hr)	**Dose (mg)**	**Formulation**	**Water administered (mL)**	**Number of volunteers**	**Age (yrs)**	**Body weight (mean) (kg)**	**F %**
(Jalava et al., 1997)	32	5	ER tablet	150	4 Females, 5 Males	22-26	51-73 (62)	15
(Edgar et al., 1985)	30	27.5	Solution	125	8 Males	22-31	69-82 (74)	16.2
(Madsen et al., 1996)	12	10	ER tablet	200	12 Males	18-50	78.8	NA
(Edgar et al., 1992)	8	5	IR tablet	200	9 Males	40-53	77.2	15
(Landahl et al., 1988)	27	10	IR tablet	NA	12 Males	20-34	69-85 (75)	15
(Edgar et al., 1987)	10	5, 15, 40	Solution	250	10 Males	22-33	66-82 (73)	NA

F: bioavailability, ER: extended release, IR: immediate release, NA: not available.

6.4. Results and Discussion

The bioequivalent simulation results were first inspected for the differences found between the modeled lowest and highest solubility results, and then assessed against the literature simulated models (both used the same input parameters, except the solubility), and finally the literature clinical data. This comparison was based on the results available area under the curve (AUC), peak plasma concentration (Cmax), the maximum time (Tmax) needed to reach Cmax, and the Fa%, for a single drug and for an overall result.

6.4.1. The Bioequivalent Models' Simulation

As seen from Figure 6.1, the plasma concentration simulation profiles were found by using either the lowest or the highest bioequivalent solubility input of six drugs, and were statistically tested, using a paired t-test (p-value < 0.05). Four out of six drugs were significantly different, carbamazepine and griseofulvin (p-value < 0.0001), fenofibrate (p-value 0.0014), and furosemide (p-value 0.0022). This result confirms the importance of using a bioequivalent solubility input. Also, from Figure 6.1 it can be noticed that the corresponding human observed data were closer to the lower solubility simulation model.

The mean AUC, Cmax, and Tmax results found by this study are compared with the results found by the human observed data, see Table 6.10. The mean $AUC_{0-\infty}$ and Cmax findings of the bioequivalent simulated models, were higher than the $AUC_{0-\infty}$ and Cmax findings of the human observed data for all the drugs. Whereas the Tmax of the bioequivalent model was higher in 3 drugs, lower in 2 drugs, and equal in 1 drug, compared to human models. The differences were further assessed by the ratio between the simulated (predicted) model to the human observed (pred/obs) of both the bioequivalent and the literature models, as detailed in next section.

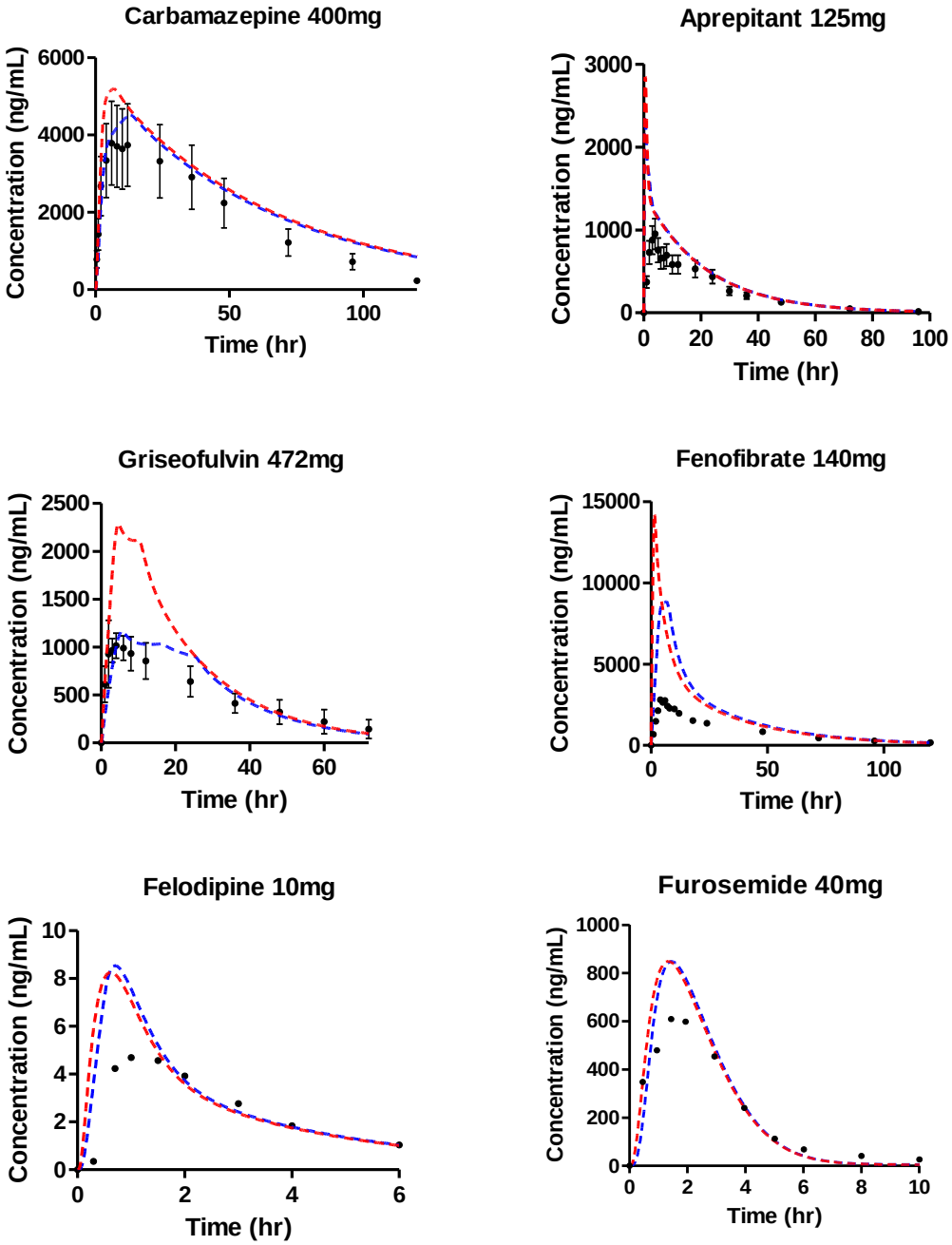

Figure 6.1: The GastroPlus simulation results of six drugs. Where available, error bars represent the standard deviation (refer to tables 6.5 and 6.6 for population number) of the human observed data (black dots), whereas blue and red lines represent the simulation results found using either the lowest or the highest solubility of each drug, respectively.

Table 6.10: The $AUC_{0-\infty}$, Cmax, and Tmax results found for the human observed data, and the bioequivalent models (Markovic et al., 2020, Sjögren et al., 2016).

Drug	**Cmax (µg/mL)**		**Tmax (hr)**		**$AUC_{0-\infty}$ (µg.hr/mL)**	
	Human observed	Bioequivalent model	Human observed	Bioequivalent model	Human observed	Bioequivalent model
Aprepitant	1.0	2.6	4.0	0.52	22.6	32.3
Carbamazepine	3.8	4.9	6.0	9.9	230	343
Felodipine	0.0047	0.0084	1.0	0.66	0.0189	0.0225
Fenofibrate	2.8	11	4.0	4.1	108	213
Furosemide	0.61	0.85	1.4	1.4	2.32	2.39
Griseofulvin	1.0	1.8	4.0	5.2	39.7	49.9

6.4.2. Comparison Between Simulated Models Results for Individual Drug

In this section and the following, the results of the bioequivalent simulation lowest and highest models, were used as a mean, since all the human observed data was below or close to the lowest solubility model. In 5 out of 6 drugs, the mean $AUC_{0-\infty}$ ratio (pred/obs) findings of the bioequivalent model were higher than the literature models (Markovic et al., 2020, Sjögren et al., 2016), exception is furosemide, see Figure 6.2 and Table 6.11. The results, classified based on the deviation extent accuracy detailed in the methods section, found that the $AUC_{0-\infty}$ ratio for all the individual six drugs of the bioequivalent models were predicted in low accuracy. This result is comparable to the literature modeling results, which were found to predict the $AUC_{0-\infty}$ of 5 out of 6 drugs in low accuracy as well, only fenofibrate was predicted in medium accuracy (Markovic et al., 2020, Sjögren et al., 2016).

The Fa% from the bioequivalent solubility simulation was higher than all the literature simulation results, see Figure 6.2, and the deviation extent, between the two simulated models of 3 out of 6 drugs, was between 1.1 – 1.6 (aprepitant, felodipine, and furosemide). Whereas the other three drugs had a deviation extent of more than 2-fold (2.2 – 4.2), see Figure 6.2 and Table 6.11.

The enhancement of both the $AUC_{0-\infty}$ and the Fa% is referred to the bioequivalent solubility used, which covers more than 96% of the HIF composition variability.

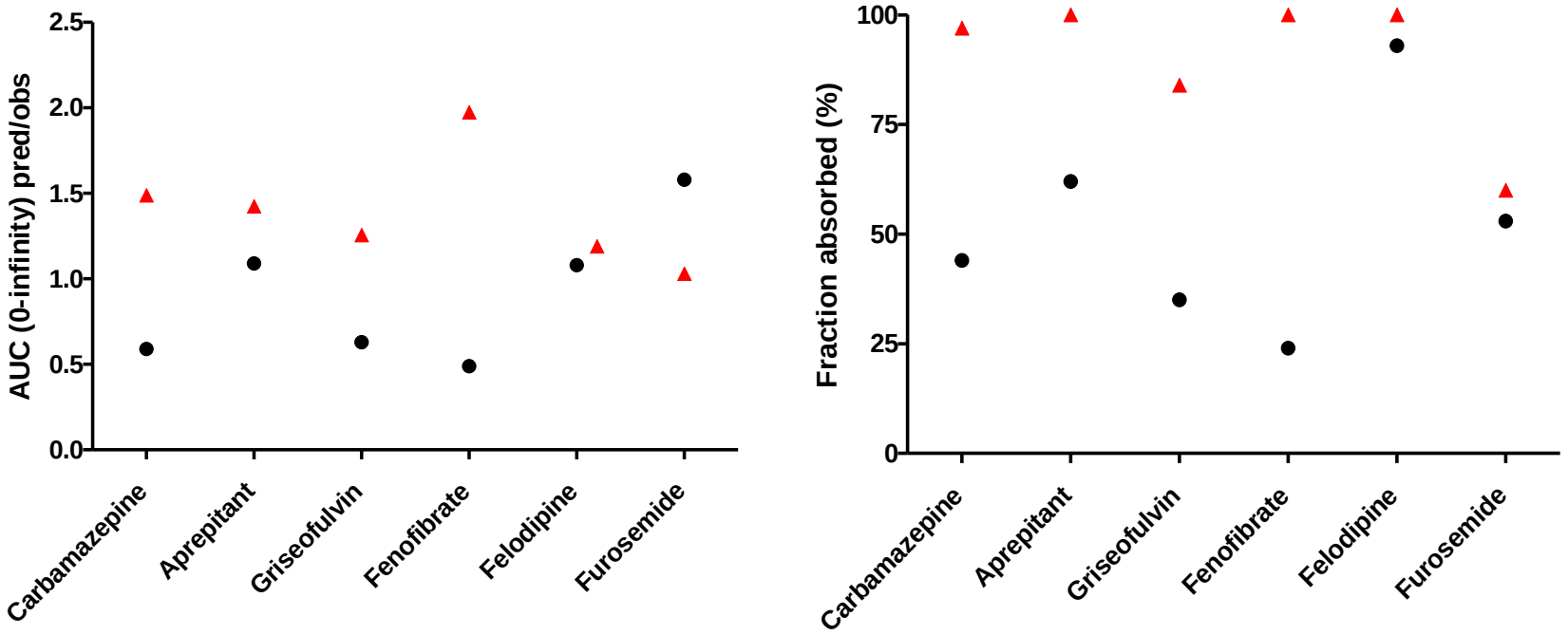

Figure 6.2: The $AUC_{0-\infty}$ ratio (predicted to human observed) to the left, and the predicted fraction absorbed (Fa%) to the right. The mean (lowest and highest) bioequivalent (red triangles) versus literature (black circles) models (Markovic et al., 2020, Sjögren et al., 2016) for 6 drugs.

Table 6.11: The fraction absorbed (Fa%) and $AUC_{0-\infty}$ ratio (pred/obs) simulation results using a bioequivalent mean solubility data from six drugs, compared to literature models (Markovic et al., 2020, Sjögren et al., 2016).

Drug	**Fa (%) Bioequivalent**	**Fa (%) Literature**	**$AUC_{0-\infty}$ ratio Bioequivalent**	**$AUC_{0-\infty}$ ratio Literature**
Aprepitant	100	62	1.43	1.09
Carbamazepine	97	44	1.49	0.590
Felodipine	100	93	1.19	1.08
Fenofibrate	100	24	1.98	0.490
Furosemide	60	53	1.03	1.58
Griseofulvin	84	35	1.26	0.630

6.4.3. Overall Drugs Results Comparison of Simulated Models

The overall simulation accuracy of the bioequivalent models of 6 drugs (mean two levels of solubility), were compared with the overall results found with (Sjögren et al., 2016) of 12 drugs (5 of them are also studied in the bioequivalent model). See Table 6.12 and Figure 6.3. The accuracy of the bioequivalent $AUC_{0-\infty}$ predictions, was acceptable (within 2-fold prediction error) in all the six drugs. This accuracy result was higher compared to the literature model (Sjögren et al., 2016), where only 70% of the $AUC_{0-\infty}$ predictions were within 2-fold prediction error. Whereas the Cmax of the bioequivalent models was predicted in acceptable accuracy (58%), which is lower than the literature predictions accuracy, where 75% of the Cmax predictions were within 2-folds prediction error. Lastly, the bioequivalent model Tmax was predicted in high, acceptable (low and medium), and inaccurate levels of accuracy, in 17%, 75%, and 8% respectively. This result shows a higher accuracy compared to the literature model Tmax predictions, which accounted for 85% within the 2-folds error predictions, compared to 92% of this study.

The reason behind the differences found between this study's predictions and the literature findings is related to the different drugs analysed in each study, along with the usage of a bioequivalent solubility input.

In the previous literature (Sjögren et al., 2013), which studied 12 different acidic, basic, and neutral drugs, they found that there was no specific trend for the predicted

simulation results between the three drugs categories. In this bioequivalent simulation research, one acidic: furosemide, one basic: aprepitant, and four neutral drugs: carbamazepine felodipine, fenofibrate, and griseofulvin, were simulated, and also no general trend was found between the drugs categories.

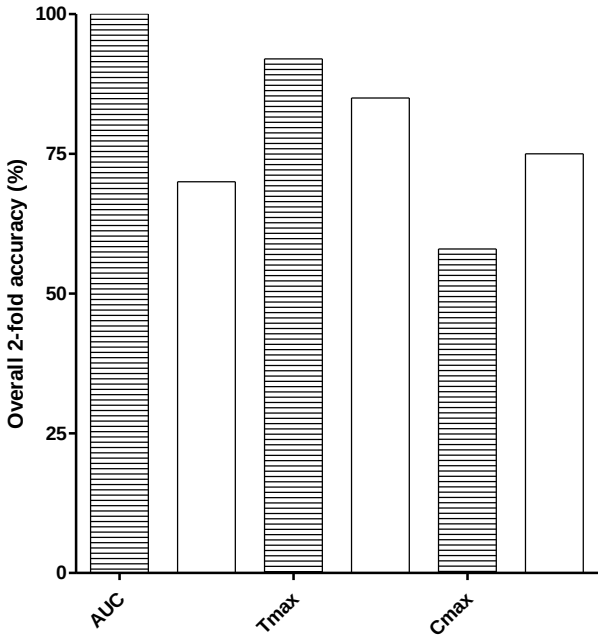

Figure 6.3: Overall accuracy (%) of the simulation results by two-fold deviation. Lined: bioequivalent model of six drugs, plain: literature model of twelve drugs (Sjögren et al., 2016), using GastroPlus® software.

Table 6.12: The overall accuracy results percentage found in the bioequivalent and literature (Sjögren et al., 2016) simulation models.

Accuracy level	**$AUC_{0-\infty}$**		**Cmax**		**Tmax**	
	Bioequivalent	**Literature**	**Bioequivalent**	**Literature**	**Bioequivalent**	**Literature**
High (0 - 25%)	0	40	0	32.5	17	32.5
Medium and Low (acceptable) (25% – 2 folds)	100	30	58	42.5	75	52.5
Inaccurate (> 2 folds)	0	30	42	25	8	15

6.5. Conclusion

This chapter simulated six previously studied drugs, using GastroPlus® modelling software, to predict their PK parameter using a lowest and a highest bioequivalent solubility input, along with the same modelled literature parameters used previously (Markovic et al., 2020, Sjögren et al., 2013).

In four out of six drugs (carbamazepine, fenofibrate, furosemide, and griseofulvin), the two simulated solubility levels, the minimum versus the maximum, were significantly different (p-value < 0.05), and the human observed data were closer to the lower solubility simulation model.

The mean (lowest and highest) simulated $AUC_{0-\infty}$ of the bioequivalent solubility models of 6 studied drugs were higher than the clinical data $AUC_{0-\infty}$, but this was the case only in 3 out of 6 drugs (aprepitant, felodipine, and furosemide) simulated in literature models (Markovic et al., 2020, Sjögren et al., 2016). On the other hand, all the bioequivalent solubility models, of 6 drugs, resulted in a low accuracy (0.25% - 2-fold extent of deviation) of the resulting $AUC_{0-\infty}$ (pred/obs) ratio predictions, compared to 5 out of 6 drugs to be predicted with low accuracy in literature models (Markovic et al., 2020, Sjögren et al., 2016), exception is fenofibrate. Also, the Fa% results were found to be higher in the bioequivalent models compared to the literature models (Markovic et al., 2020, Sjögren et al., 2016), by deviation extents between 1.1 – 1.6 for 3 out of 6 drugs (aprepitant, felodipine, and furosemide), and between 2.2 – 4.2 for the other 3 drugs.

Finally, the overall $AUC_{0-\infty}$ and Tmax accuracy results found by the bioequivalent simulated results was higher than the literature models' accuracy (Sjögren et al., 2016). As within 2-fold deviation, the accuracy of the $AUC_{0-\infty}$ and the Tmax were 100% and 92% for the bioequivalent models, compared to 70% and 85% for the literature models (Sjögren et al., 2016). But the Cmax accuracy was higher in the literature model, 75%, compared to the bioequivalent model, 58%.

The higher accuracy of $AUC_{0-\infty}$ and Tmax detected compared to the clinical data, along with the higher resulting Fa% conclude that the solubility used in these bioequivalent models are more relevant to the human *in vivo* solubility.

In conclusion, the usage of the bioequivalent lowest and highest solubility data, helped in an improved *in silico* prediction of the PK parameters of six poorly soluble drugs, compared to the literature models which used different solubility input. The literature solubility used was lower than the bioequivalent range in four drugs, and only furosemide and griseofulvin literature solubility values were within that range. These results also confirms that the human intestinal solubility should be used as a range, not a single point, to increase the resulting accuracy of the solubility applications. The simulation of a higher number of drugs using the bioequivalent solubility could provide a better understanding of this outcome.

Chapter 7: Conclusion and Future Work

7.1.Conclusion

This study demonstrates that it is possible to assess the fasted intestinal equilibrium solubility envelope using a small number of bioequivalent media recipes obtained from a multi-dimensional analysis of fasted human intestinal fluid (FaHIF). The resulting nine fasted simulated intestinal fluid (Fa9SIF) solubility results enclosed the solubility values of literature FaHIF and fasted simulated intestinal fluids (SIF), and were statistically equivalent to previous design of experiment (DoE) solubility studies, but with a lower range, which correspond to an improved equivalence to *in vivo* solubility. The derivation of the Fa9SIF media coupled with the lower measured solubility range indicate that the solubility results are more likely to reflect the fasted intestinal solubility envelope than previous DoE studies, and highlight that intestinal solubility is a range and not a single value. The Fa9SIF solubility results were analysed to determine the most statistically significant factors within the media influencing solubility, using a custom DoE, which resulted in a lower statistical resolution than a formal DoE, and was not appropriate if determination of media factor significance for solubilization is required.

The measured mid-point solubility value was statistically equivalent to the value determined with the original fasted simulated intestinal fluid recipe (FaSSIV-v1), further indicating similarity and that existing literature results could be utilized as a direct comparison. The analysed drugs all displayed different solubility ranges and behaviour; a result also consistent with previous studies. The lowest solubility represents a worst-case scenario which may be useful in risk-based quality by design biopharmaceutical calculations than the mid-point value. This novel approach was also used to investigate the drugs' developability classification system (DCS), using the dose to solubility ratio, to highlight the individual drug behaviors. The method also permits a dose/solubility ratio frequency distribution determination for the solubility envelope which permits further risk-based refinement, especially where the drug crosses a classification boundary. This novel approach therefore provides greater *in*

vitro detail with respect to possible biopharmaceutical performance *in vivo* and an improved ability to apply risk-based analysis to biopharmaceutical performance.

The Fa9SIF solubility results were also examined for any consistent solubility behavior and found in the majority of cases a structured solubility behaviour that is consistent with physicochemical properties and previous solubility studies. For the acidic drugs (pKa < 6.4) solubility was controlled by media pH, the profile is identical and consistent, and the lowest and highest pH media identify the lowest and highest solubility in over 80% of cases. For weakly acidic (pKa > 8), basic and neutral drugs solubility is controlled by a combination of media pH and total amphiphile concentration (TAC), a consistent solubility behaviour is evident but with variation related to individual drug interactions within the media. The lowest and highest pH × TAC media identify the lowest and highest solubility in over 78% of cases. A subset of the latter category consisting of neutral and drugs non-ionized in the media pH range have been identified with a very narrow solubility range, indicating that the impact of the simulated intestinal media on their solubility is minimal. Two drugs probucol and atazanavir exhibit unusual behaviour. The study also indicated that the use of two appropriate bioequivalent fasted intestinal media, from the nine, will identify *in vitro* the maximum and minimum solubility boundaries for drugs and due to the media derivation, this is probably applicable *in vivo*.

The novel Fa9SIF solubility results provided a drug solubility range bioequivalent to sampled FaHIF solubilities. Statistical comparisons of the Fa9SIF media system against FaHIF did not detect a difference, and individual drug analysis produced an 85% correlation. Correlation is drug physiochemistry dependent, and in agreement with previous statistical solubility behaviour studies. An innovative *in vitro* vs FaHIF correlation window was determined, which enclosed 94% of solubility values from an additional data set, further validating equivalence.

The application of the supersaturation (SS) study on ten drugs, was found to be applicable using the Fa9SIF media, minimum, mid, and/or maximum, with different concentrations and path lengths. The analysed drugs showed that the media with higher SIF components concentrations, resulted in a higher meta stability, and thus higher SS concentration, which precipitated to a more stable concentration. This result was comparable with previous SS literature studies. In 7 out of 10 drugs, the resulting

crystalline solubility was found to be higher than the resulting equilibrium solubility of the Fa9SIF system. A result which was also found in one literature study, but their crystalline solubility results were in the same range to this SS study results. The induction time found in the same literature study, for available four drugs only, enclose the range for two drugs, whereas the other two drugs showed higher induction times. The drugs' precipitation rate was proportionally correlated with the higher concentrations used, and proportionally but variably affected by path length change. On the other hand, the different media used had no effect on the precipitation rate even with doubling the concertation if comparing the lowest to middle, or middle to highest SIF media recipes, which is correlated to the higher SIF media components present which increased the drug stability. Whereas, doubling the concentration using the lowest to the highest media recipes resulted in tripling the precipitation rate, which could be related to the higher SIF media concentrations difference.

Finally, physiology based pharmacokinetic (PBPK) models were used to assess *in silico* modelling in predicting a concentration versus time profiles for six poorly soluble drugs, using the lowest and highest bioequivalent equilibrium solubilities as an input parameter. In four out of six drugs, the two simulated solubility levels were significantly different (p-value < 0.05), and the human observed data compared to the simulated model were closer to the lower solubility simulation model. All the mean (lowest and highest solubility models) $AUC_{0-\infty}$ results were higher than the human observed models' $AUC_{0-\infty}$ results, but only half of those drugs were found to be higher than human observed models in literature models. Also, the fraction absorbed (Fa%) results were found to be higher in the bioequivalent models compared to the literature models by deviation extents between 1.1 – 1.6 for 3 out of 6 drugs, and between 2.2 – 4.2 for the other 3 drugs. The higher accuracy of $AUC_{0-\infty}$ and Tmax detected compared to the clinical data, along with the higher resulting Fa% conclude that the solubility used in these bioequivalent models are more relevant to the human *in vivo* solubility. The simulation of a higher number of drugs using the bioequivalent solubility could provide a better understanding of this outcome.

In conclusion, the Fa9SIF media system could be applied during discovery and development activities to provide a solubility range and rationalize drug and formulation decisions. However, since it's a new methodology, for *in vitro in vivo*

solubility correlation, this radically transforms predictive ability which will benefit drug discovery, development, and formulation studies. The approach is worthy of further investigation and may represent a methodology applicable to other dynamic multicomponent fluids where no single component is responsible for performance.

7.2.Future Work

This study has determined the oral drugs equilibrium solubility in the fasted state only, but this should be linked the fed state solubilities and its effects. The approach is worthy of further development and research to expand the number of drugs analysed, refine the compositional calculations for the bioequivalent points and link *in vitro* solubility to *in vivo* pharmacokinetics. The usage of two media coupled with a central point measurement, limits, and information on the potential Biopharmaceutics Classification System (BCS) or DCS classification and position with respect to the boundaries can be provided. If three media are too onerous, then the lowest solubility media can be applied as a worst-case scenario. This fasted intestinal solubility measurement will provide more information than a single FaSSIF value and can be applied to assess the suitability during drug discovery, and development strategies for oral administration. The structured solubility behavior ensures that the physicochemical properties of each drug should be accounted for in future solubility measurements.

If placement on the BCS/DCS is required along with possible population behaviour, then the Fa9SIF media set can be applied and linked to various biopharmaceutical performance predictors. The Fa9SIF media system is therefore worthy of further investigation with linkage of system results to *in vivo* performance, a key next stage and may represent a methodology applicable to other multicomponent biological fluids where no single component is responsible for performance.

Also, the usage of other modelling software would improve the prediction accuracy of the results.

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Appendix

1. High Performance Liquid Chromatography (HPLC)

1.1.Introduction to HPLC

HPLC is the most used technique to quantitively analyse drug concentrations aided by a detector system, such as the UV system (Watson, 2015). The HPLC device consists of many parts that work as a single unit to achieve the final quantitative analysis (Watson, 2015) see Figure 1.8.

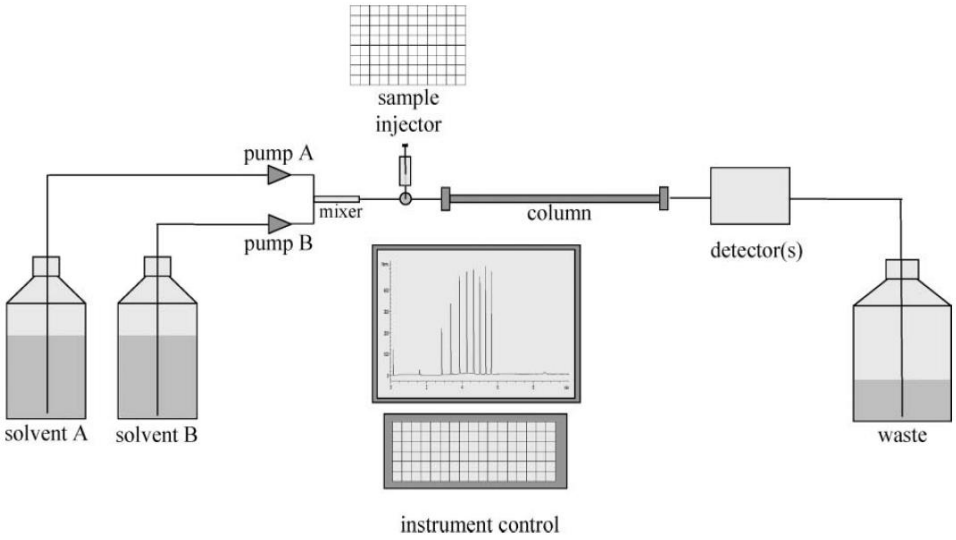

Figure 1.1: A representative diagram for the HPLC device compartments (Welch et al., 2009).

The HPLC device consists of (Watson, 2015):

a. Solvent cabinet, to hold the mobile phase (MP).

b. Pump, responsible for generating the pressure needed to transfer the solvents to the other parts of the device.

c. Loop injector (or auto sampler in modern systems), to withdraw the desired sample volume for analysis (the injection volumes can range from 1 - 200 µL).

d. Column (stationary phase (SP)), the most valuable part because of its capability to separate and retain the analysed samples. One of the most used types is the revered

phase octadecylsilane coated (ODS) silica gel, with different diameter, length, and particle size.

e. UV detector.

f. Computational device to capture the chromatographic data for further quantitative analysis.

For a good analysis, an appropriate MP solvent and a SP column need to be chosen, which should be in opposite or variable states of polarities (Watson, 2015). The most used SP is the non-polar reversed phase column, with a more polar MP (Watson, 2015). The compounds' separation depends on the degree of lipophilicity of the analysed compounds, so choosing a non-polar SP column means the more polar compounds will elute first, whereas the least polar will retain on the column and elute last (Watson, 2015). The elution pattern is visualised by a data capturing system (computer) represented by peaks with different retention times, and area under the curve (AUC) (Watson, 2015). Those eluted peaks should be sharp in shape with a good resolution properties (well-separated peaks) (Watson, 2015). However, good resolution is relatively hard to achieve with structurally related molecules. So, a gradient change in the percent of the organic MP during the analysis run is needed, which is named as the gradient method (Watson, 2015). Another way to change the peaks elution order is to change the pH of the MP, but this is not applicable for non-ionisable drugs, and must only be in pH range of 2 - 8.5, otherwise the SP column may lose its internal bonds and degrade (Watson, 2015). However, the best ionization results of pH changing is achieved within one pH unit of the drug's pKa. For example, setting one pH unit above an acidic drug's pKa, will ionize the drug, leading to a faster elution and a lower retention time (Watson, 2015). Also, the different composition of the MP can affect the retention time of the analytes, generally there are three solvents used, methanol (MeOH), acetonitrile (ACN), and tetrahydrofuran (THF) (Watson, 2015). According to Dolan's rule, a 10% decrease of MeOH in the MP, will increase the capacity (retention) factor three times (Watson, 2015). On the other hand, decreasing ACN by 10%, will increase the capacity factor by 2;
Relatively 40% of MeOH = 33% of ACN = 23% of THF (Watson, 2015).

As a result of the close affinity to the MP of some analysed compounds, a mixture of the MP solvents are used together (Watson, 2015).

1.2.Elution Types

a. Isocratic Elution

The isocratic elution technique uses one MP containing a fixed proportion of organic and aqueous solvents together during the whole run time. It's the easiest separation method, but it will result in a long retention time if many compounds are to be separated, and if the organic proportion of the MP was increased, it will result in bad peaks resolution (Watson, 2015).

b. Gradient Elution

For a shorter elution time and better peaks separation, this type of elution is used (Watson, 2015). The gradient elution is accomplished by changing the MP proportions through the run, using two MP reservoirs, MP A is normally the aqueous solvent, and MP B is the organic solvent.

2. The Inform instrument

The inForm®[5] is a multitasking analytical instrument which can work on different compounds and sample types to measure various drugs' measurements, such as the molar extinction coefficient (MEC), the dissolution process, the controlled SS assays, the pKa either by pH or UV change, and the solubility determination. The inForm® has two main modules: the dispenser bank, and the assay deck. First, the dispenser bank holds the reagent bottles which are connected by pipes and a 6-way valve capable of loading six different reagents, at once, into the assay deck through glass syringes, see Table 5.1. It is connected to a nitrogen gas source to get rid of any CO_2 gas which could spoil the reagents, and to reduce the reagents loss by evaporation. Also, the dispenser bank is attached to a UV/visible spectrometer and a light connected to a fiber optic dip probe.

[5]Unless mentioned, the following information in this section were taken from the instrument's user manual.

Table 2.1: Main reagent bottles used in the inForm® instrument assays.

Reagent	Concentration and material
Ionic strength water	0.15 M NaCl
Acid titrant	0.5 M HCl
Base titrant	0.5 M NaOH
Vial arm's needle cleaner	100% IPA
Clean-up assays	80% Methanol with 20% 0.15 M IPA water
Pre-saturated water	Saturated with nitrogen gas

IPA: isopropanol.

Second, is the assay deck, which consists of two arms, operating separately, a titrator (probe) arm and a gripper (vial) arm, a 20-position vials rack (each vial accommodates 60 ml volume), a rack capable to hold small high pressure liquid chromatography (HPLC) excipients vials (each accommodates 2- or 4- ml), and a dedicated five-position static sample rack, see Figure 5.1 below. This static rack contains daily-changed solutions, the first vial from the right, contains deionized water for storing the probe arm, followed by a buffer vial of pH 7 for cleaning and calibrating the pH electrode, a surfactant wash vial and a solvent rinse vial for biphasic dissolution assays, and a solvent wash vial containing 50% Methanol for clean-up assays. To the left side of this static rack, the drain and the flowing water wash are positioned, which both are connected to separated waste and water drums.

The probe arm has a collection of probes mainly: a pH electrode (preserved in a 3 M KCl solution), two different UV dip probe (with a changeable path length), a dual stirrer, a temperature sensor, and a bundle of capillaries to add the various reagents from the dispenser bank. It's stored in the storage vial when there are no running assays, or when the vial arm is working.

The vial arm is used for gripping the different sample vials from the 20-position vial tray into the sample position, which is temperature controlled by a Peltier device. This arm also has a needle which takes the samples held in the HPLC rack into the sample stage position, and this needle is washed by the isopropanol reagent bottle before and after each sample addition.

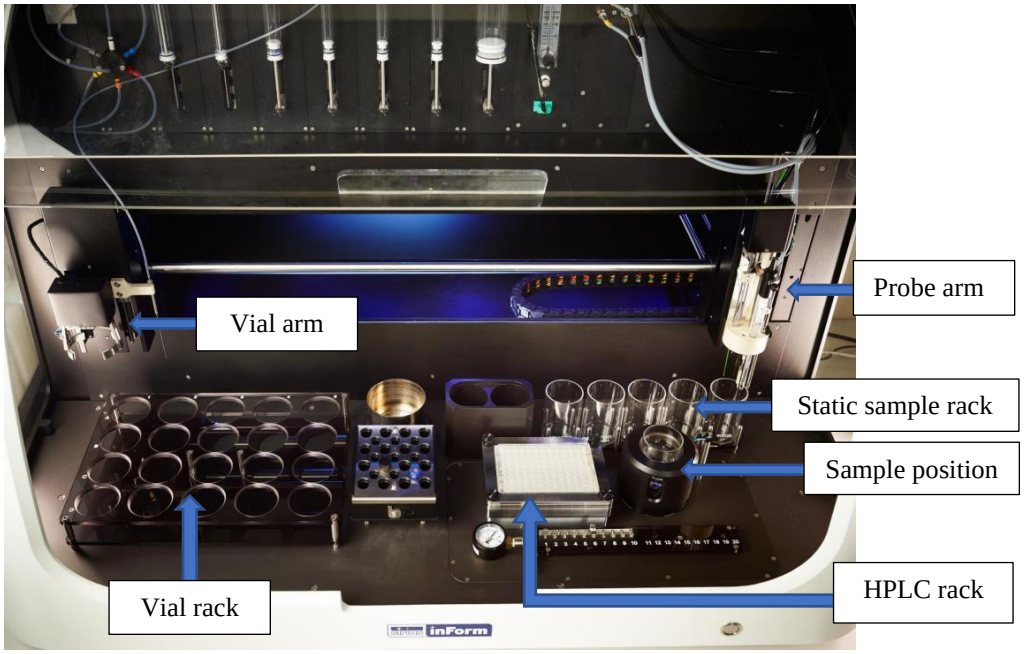

Figure 2.1: The InForm® instrument assay deck's main parts.

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Small scale in vitro method to determine a bioequivalent equilibrium solubility range for fasted human intestinal fluid

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Zafirlukast
Tadalafil
Fenofibrate
Griseofulvin
Felodipine
Probucol

ABSTRACT

Drug solubility is a key parameter controlling oral absorption, but intestinal solubility is difficult to assess in vitro. Human intestinal fluid (HIF) aspirates can be applied but they are variable, difficult to obtain and expensive. Simulated intestinal fluids (SIF) are a useful surrogate but multiple recipes are available and the optimum is unknown. A recent study characterised fasted HIF aspirates using a multi-dimensional approach and determined nine bioequivalent SIF media recipes that represented over ninety percent of HIF compositional variability. In this study these recipes have been applied to determine the equilibrium solubility of twelve drugs (naproxen, indomethacin, phenytoin, piroxicam, aprepitant, carvedilol, zafirlukast, tadalafil, fenofibrate, griseofulvin, felodipine, probucol) previously investigated using a statistical design of experiment (DoE) approach. The bioequivalent solubility measurements are statistically equivalent to the previous DoE, enclose literature solubility values in both fasted HIF and SIF, and the solubility range is less than the previous DoE. These results indicate that the system is measuring the same solubility space as literature systems with the lower overall range suggesting improved equivalence to in vivo solubility, when compared to DoEs. Three drugs (phenytoin, tadalafil and griseofulvin) display a comparatively narrow solubility range, a behaviour that is consistent with previous studies and related to the drugs' molecular structure and properties. This solubility behaviour would not be evident with single point solubility measurements. The solubility results can be analysed using a custom DoE to determine the most statistically significant factor within the media influencing solubility. This approach has a lower statistical resolution than a formal DoE and is not appropriate if determination of media factor significance for solubilisation is required. This study demonstrates that it is possible to assess the fasted intestinal equilibrium solubility envelope using a small number of bioequivalent media recipes obtained from a multi-dimensional analysis of fasted HIF. The derivation of the nine bioequivalent SIF media coupled with the lower measured solubility range indicate that the solubility results are more likely to reflect the fasted intestinal solubility envelope than previous DoE studies and highlight that intestinal solubility is a range and not a single value.

1. Introduction

The preferred method for the self-administration of drugs is the oral route where tablets and capsules account for over seventy percent of the marketed products available. Since solid drug is not absorbed from the gastrointestinal tract the process of drug dissolution is a critical stage during oral absorption. This is recognised in the biopharmaceutics classification system (BCS) [1] where drug solubility and permeability through the intestinal membrane are the two key parameters controlling absorption. Drugs can be categorised as exhibiting a high solubility (dose soluble in the pH range 1.2–7.5 and a fluid volume of 250 mL) or low solubility. This approach was refined in the Developability Classification System (DCS) [2,3] through application of the dose/solubility ratio and splitting the low solubility category into two. Category IIa, for drugs where a dissolution rate limitation was likely and IIb where a solubility limited absorption would be the dominant feature. Knowledge of a drug's solubility and position within the BCS/DCS, especially for poorly soluble drugs [4], is therefore an important parameter during drug development and for the application of quality by design concepts to the formulation and development of oral products [2].

An obvious route to the determination of a drug's solubility in human intestinal fluid (HIF), is to use sampled intestinal fluid [5]. Fasted HIF

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sampling requires normal volunteers to fast overnight, which is followed by the insertion of an oral catheter and then determination of its anatomical position to ensure that it is in the small intestine. Intestinal fluid is then collected by application of a vacuum for a period of time, typically 1–2 h [6]. The volume of fluid collected depends on the protocol and volunteer and post collection has to be frozen to preserve properties. Several groups have applied this procedure [7,8] to determine a range of drugs' solubility in fasted [9] HIF samples. These studies have demonstrated the issues around the routine collection of HIF, the variability of the measured solubility due to variations in the HIF composition [10–12] and indicate that this approach will not provide a reproducible solubility determination. In addition the HIF sampling process limits the sample volumes available.

To circumvent HIF availability issues simulated intestinal fluids (SIF) have been developed [13] based on the components of HIF and with the fluid designed to match HIF solubility values [7,8]. Multiple recipes are available in the literature [14] displaying variations in the content of bile salt, phospholipid, pH, buffer salt and presence or absence of additional components. This provides variability in the solubility value determined [15] by the different SIF media and presents an additional question of which media recipe is appropriate. As part of the EU IMI Oral Biopharmaceutical Tools research program [16] this group conducted a design of experiment (DoE) study into equilibrium solubility in simulated fasted media [17]. This examined seven media components (bile salt, lecithin, buffer, salt, pH, enzyme and fatty acid) using a fractional factorial design that required 66 experiments. A similar study was also conducted using six components (bile salt, lecithin, pH, buffer capacity, osmolarity and bile salt phospholipid ratio) and a different DoE protocol that required 24 experiments [18]. These studies [17–22] quantified the significance of individual media factors for drug solubilisation, and permitted a simple classification of drug behaviour based on acidic, basic or neutral properties. For acidic drugs pH was the major driver of solubility by around a factor of 10 when compared to other media components and for basic and neutral drugs an almost equivalent combination of pH, bile salt, phospholipid and fatty acid controlled solubility. The studies also identified that there were two-way interactions between the media components that influence solubility and a further study also hinted at three-way component interactions [20,23] affecting solubility. Indicating that these simulated systems and therefore also the natural systems exhibit complex drug solubilisation behaviour, that is difficult to fully re-capitulate using small numbers of components. Although wonderful at revealing and quantifying drug solubilisation, the DoE approaches are experimentally cumbersome [17,18,23] and not likely to be applied on a routine basis. To reduce experimental load, low experimental number DoE studies have been performed utilising a mixed dual level (fasted and fed) [19] or a full range (fasted + fed) designs [21] that only required 20 or 32 experiments respectively. A further dual level modification has been published that only requires 9 experiments for the fasted state [22]. Whilst these protocol adaptations reduce experimental load they remain based on a DoE, which aims to statistically determine solubility variation using conditions that are hypothesized to reflect the component variation within the experimental system or simulated fluid. Statistically this links a high concentration value of one component with a low concentration value of another (see Fig. 4 in [17]) a combination in the SIF system (for example bile salt with phospholipid) that may not arise in vivo in HIF and therefore be bioequivalent. DoE approaches therefore do not have a direct relationship to HIF.

In order to address the issues with SIF and DoE approaches a recent publication has examined HIF composition using a multidimensional mathematical analysis that treated the fluid as a 5 dimensional system [24] consisting of the following components, pH, bile salt, phospholipid, fatty acid and cholesterol. This utilised a data set of fasted and fed HIF samples obtained from volunteers [25] and identified 8 bioequivalent media compositions that statistically characterised over 90% of the variation within the sample set in the fasted and fed states. In addition a centre point was identified in each state using a Euclidean approach in 5-dimensional space, rather than the mean (or similar) value for each component since the component distributions were not normal. To achieve the multidimensional analysis the measured concentrations of components were summed and treated as a single variable, for example six bile salt species were analysed but only a single concentration calculated (Table 1). This simplification applies to bile salts, phospholipids and fatty acids were in HIF multiple species will be present. This is a situation also applicable to SIF and for bile salts it is known that the concentration has a greater influence on solubilisation than species [26]. However, it does represent an ever present challenge between simulation by simplification and the native fliud.

In this paper we have applied the calculated fasted state compositions from the multidimensional analysis to determine the equilibrium solubility of the twelve drugs (naproxen, indomethacin, phenytoin, piroxicam, aprepitant, carvedilol, zafirlukast, tadalafil, fenofibrate, griseofulvin, felodipine, probucol) investigated in the original DoE study [17]. The equilibrium solubility data has also been compared, to the original DoE [17] and where appropriate, to the reduced experiment fasted DoE distributions [19,22] and literature HIF and SIF values. The aim of this study is to provide a comparison of these two approaches into investigating the solubility of drugs in simulated fasted intestinal fluid systems.

2. Materials and methods

2.1. Materials

Sodium taurocholate, cholesterol, sodium chloride (NaCl), sodium oleate, ammonium formate, potassium hydroxide (KOH), hydrochloric acid (HCl), naproxen, phenytoin, piroxicam, fenofibrate, probucol, griseofulvin, carvedilol, tadalafil, and indomethacin were purchased from Merck Chemicals Ltd. Aprepitant and felodipine were provided through OrBiTo by Dr. R. Holm, Head of Preformulation, Lundbeck, Denmark. Zafirlukast was purchased from Stratech Scientific Ltd. Phosphatidylcholine from soybean (lecithin) was purchased from Lipoid company. Chloroform from Rathburn Chemical Company. FaSSIF-v1 media was purchased from Biorelevant.com Ltd. Sodium phosphate monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$) and formic acid from Fisher Scientific. All acetonitrile (ACN) and methanol (MeOH) solvents were HPLC gradient (VWR). All water was ultrapure Milli-Q.

2.2. Methods

2.2.1. Solubility media preparation

Biorelevant media stock solutions

For each media recipe (Table 1) a concentrated lipid stock was prepared. The required (×15) weight of bile salt (sodium taurocholate), phospholipid (soybean lecithin) and fatty acid (sodium oleate) for each media recipe was dissolved in chloroform (3 mL) – stock A. The required

Table 1
Bioequivalent media compositions.

Media	Bile Salt (mM)	Phospholipid (mM)	FFA (mM)	Cholesterol (mM)	pH
1	1.06	0.16	1.04	0.01	6.64
2	11.45	2.48	2.88	0.38	7.12
3	3.4	0.33	2.88	0.09	8.04
4	3.56	1.18	1.04	0.06	5.72
5	3.62	1.25	3.43	0.03	7.14
6	3.35	0.31	0.87	0.17	6.62
7	5.33	0.4	2.96	0.07	6.42
8	2.27	0.96	1.01	0.08	7.34
centre point (9)	3.46	0.52	1.64	0.032	6.54

Values from [24].

weight of cholesterol (×1500) for each media recipe was dissolved in chloroform (10 mL) – stock B. An aliquot of stock B (0.1 mL) was added to each stock A, mixed and the stock A chloroform solution evaporated under a stream of dry nitrogen gas. The dry lipid film was resuspended in water, quantitatively transferred to a volumetric flask (5 mL) and made to volume with water. Stock aqueous solutions of buffer (sodium phosphate monobasic monohydrate; 28.4 mM) and salt (sodium chloride; 105.9 mM) were prepared in water.

Fasted simulated small intestine fluid (FaSSIFv1) media

Pre-prepared media from Biorelevant company was used as described by the manufacturer.

2.2.2. Equilibrium solubility measurement

The method was based on multiple previous papers [17,21,22]. Into a centrifuge tube (15 mL Corning® tubes) was added aliquots (267 μL) of the lipid, buffer and salt stock solutions, an excess of the solid drug under test and water (3.199 mL) to make a final aqueous system volume of 4 mL. Tube pH was adjusted to the required value (Table 1, target value ± 0.05) using KOH or HCl as required. FaSSIF-v1 media (4 mL) was added to the tube along with an excess of the solid drug under test and pH adjusted if required. The tubes were capped and placed at room temperature into an orbital shaker (Labinco BV model L28) for 1 h, and the final pH was re-adjusted if required. Tubes were then placed in the shaker at 37 °C for 24 h. Post incubation an aliquot (1 mL) of each tube was transferred to a 1.5 mL Eppendorf tube, and centrifuged for 15 min, 10000 rpm and the supernatant analysed by HPLC for drug content. For each drug this process was repeated three times and the average value is used.

2.2.3. HPLC analysis

Analysis was performed on a Shimadzu Prominence-i LC-2030C HPLC system using a gradient method for all the drugs except probucol. Column Xbridge® C18 5 μm (2.1 × 50 mm) at 30 °C, mobile phase A 10 mM ammonium formate pH 3 (adjusted with formic acid) in water, and mobile phase B 10 mM ammonium formate in acetonitrile:water (9:1), flow rate 1 mL/min (except carvedilol 0.7 mL/min), gradient start 70:30 (A:B), 3 min 0:100, 4 min 0:100, 4.5 min 70:30 total run time 8 min. The retention time, analysis wavelength and injection volume for each drug are provided in Table 2. For probucol an isocratic method was used [17] mobile phase ACN, MeOH, and water 45:45:10 and the column was Speck and Burke, ODS-H optimal 5 μm (30 × 150 mm). For each drug a concentration curve was prepared using five or six standards that bracketed all the measurement concentrations, for all drugs correlation coefficient > 0.99.

2.2.4. Data analysis

Data comparison using non-parametric Kruskal-Wallis test with Dunn's multiple comparison correction was conducted in Prism 9 for MacOSX, only comparisons indicated in the figures was analysed. Media bioequivalent factor concentrations/values (Table 1) was used as an input for a factorial custom design of experiment using Minitab®19 and the significant factors influencing solubility calculated.

Table 2
HPLC conditions.

Drug	Retention time (min)	Wave-length (nm)	Injection volume (μL)
Naproxen	1.6	254	10
Indomethacin	2.1	254	10
Phenytoin	1.1	254	20
Piroxicam	1.07	254	10
Aprepitant	2.27	254	50
Carvedilol	1.6	254	10
Zafirlukast	2.6	254	25
Tadalafil	1.4	291	50
Fenofibrate	3	291	10
Felodipine	2.4	254	10
Griseofulvin	1.5	291	10
Probucol	4.87	220	100

3. Results and discussion

3.1. Equilibrium solubility

The equilibrium solubility results from this bioequivalent nine point fasted study are presented in Fig. 1 for the acidic drugs and in Figs. 2 and 3 for the basic and neutral drugs. For each drug the comparable data set from the initial 66 point DoE (DoE 66) fasted study [17] is included along with, where available, results from the smaller sample number fasted DoE studies, DoE 10 [19] and DoE 9 [22]. Literature values for equilibrium solubility in fasted HIF or fasted SIF media [10] (NB One FaSSIF value is from this study) are provided for visual comparison but are not included in the statistical analysis.

A statistical comparison of the bioequivalent equilibrium solubility distribution with the DoE results (Figs. 1, 2 and 3) indicates that in the thirty cases where a comparison is possible, twenty five (just over 80%) are statistically equivalent. Indicating that in the majority of cases the bioequivalent approach is measuring the same solubility space as the previous DoE approaches. A comparison against available HIF solubility values indicates that of the nine possible drug-based comparisons the literature fasted HIF equilibrium solubility data points lie within the bioequivalent envelope in seven cases, almost eighty percent. A similar analysis of the fasted SIF equilibrium solubility values indicates that in nine out of twelve (seventy five percent) possible drug based comparisons, the data points lie within the bioequivalent solubility envelope. The comparisons against literature HIF and SIF values contain an unavoidable error since multiple protocols have been applied in the determination of these values. However, the HIF and SIF comparison provides a similar level of agreement (approximately eighty percent) with the DoE comparison and collectively indicates that in the majority of cases the bioequivalent approach is measuring the same equilibrium solubility space as literature DoE, HIF and SIF approaches.

A striking feature of the original DoE 66 was equilibrium solubility variability, with some drugs exhibiting a greater than three log range between the lowest and highest values measured. In Fig. 4a the calculated solubility multiple (highest solubility ÷ lowest solubility) is presented for each drug in the DoE 66 and bioequivalent test systems. There is a statistically significant reduction in the solubility multiple in the bioequivalent system where for nine out of the twelve drugs the value is smaller. There is no available comparison with literature data but there are several possible reasons for this result. The bioequivalent system only contains nine measurement points and therefore the possibility for variability is lower, but will depend upon the variability of the media compositions examined. The range of media factors and factor values assessed between the systems is not equivalent and this will influence the solubility measurements, for example the DoE 66 pH range was between 5 and 7, whilst the bioequivalent range is greater at between 5.7 and 8. In contrast the fatty acid range is lower in the bioequivalent (0.9–3.4 mM) when compared to the DoE 66 (0.5–10 mM). In addition cholesterol is present in the bioequivalent system but not in the DoE 66. The combined solubility influence of these various differences is difficult to predict. However, the pH difference between DoE systems (all pH range 5–7) with the bioequivalent system (pH range 5.7–8) is probably the reason for the statistical difference determined for piroxicam in DoE9 (Fig. 1). No difference is detected for piroxicam in the bioequivalent with the DoE 66 due to the difference in the data point numbers. Finally, the bioequivalent system does not contain statistically driven measurement points that combine a high value of one factor with a low value of another (see Introduction). It is known from the previous high number DoE systems that media factors interact [17,20] to influence solubility. This is likely to produce increased solubility variability but would require a more detailed analysis to separate this effect out from points that do not contain this issue. Overall the bioequivalent

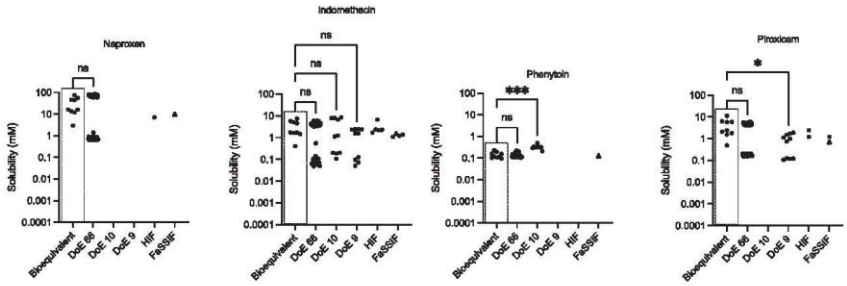

Fig. 1. Measured Equilibrium Solubility of Acidic Drugs. Bioequivalent – this study; DoE 66 [17]; DoE 10 [19]; DoE 9 [22]; HIF (Fasted Human Intestinal Fluid) data from [10]; FaSSIF (Fasted Simulated Intestinal Fluid) data from [10], plus one point (△) from this study. ns no significant difference; * p 0.0172; *** p 0.0003.

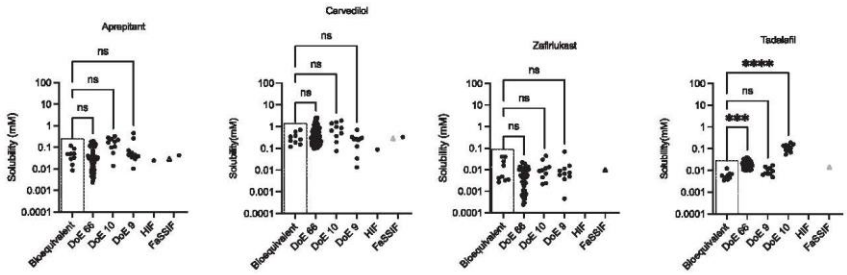

Fig. 2. Measured Equilibrium Solubility of Basic Drugs. Bioequivalent – this study; DoE 66 [17]; DoE 10 [19]; DoE 9 [22]; HIF (Fasted Human Intestinal Fluid) data from [10]; FaSSIF (Fasted Simulated Intestinal Fluid) data from [10], plus one point (△) from this study. ns no significant difference; *** p 0.0002; **** p 0.0001.

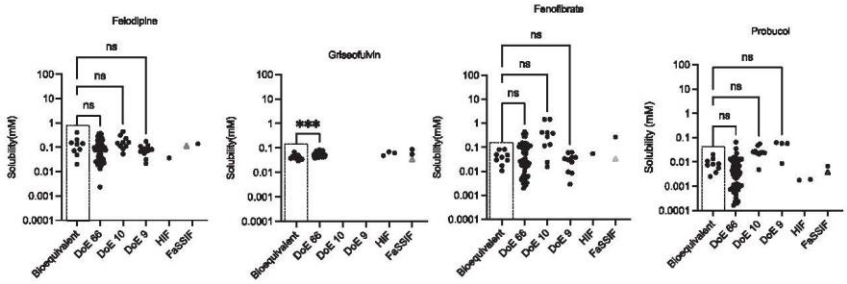

Fig. 3. Measured Equilibrium Solubility of Neutral Drugs. Bioequivalent – this study; DoE 66 [17]; DoE 10 [19]; DoE 9 [22]; HIF (Fasted Human Intestinal Fluid) data from [10]; FaSSIF (Fasted Simulated Intestinal Fluid) data from [10], plus one point (△) from this study. ns no significant difference; *** p 0.0006.

system is providing a reduced solubility range that due to the method applied to derive the measurement points' composition [24] represents a more realistic fasted intestinal solubility window than DoE based investigations into the media, its factors and factor ranges.

There are three drugs (phenytoin, tadalafil and griseofulvin) where the difference in the solubility multiple between the two systems is minimal (Fig. 4b). These drugs also have the lowest solubility multiple values and represent three of the four drugs (and four of the five cases) where there is a statistical difference between the solubility data sets. This multi-point assessment process reveals a behaviour that has not been previously reported in the literature, possibly since studies only examine a single point or SIF recipe [27] but with multiple drugs. The behaviour is different to the rest of the drug test set and surprisingly is one example from each of the three drug categories (acidic, basic and neutral) examined. The solubility distributions indicate that these drugs have a very low solubility variability within a simulated intestinal media system and presumably therefore HIF, and the solubility window moves as the media factors and factor values are varied. The latter statement is

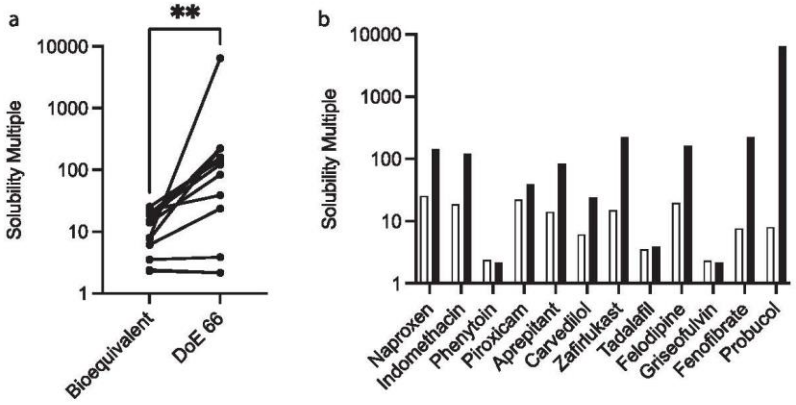

Fig. 4. a. Collected Solubility Multiple Values. Bioequivalent – this study; DoE 66 [17]; ** p 0.0024. Solubility multiple (highest measured solubility : lowest measured solubility). b. Individual Solubility Multiple Values. Bioequivalent – this study – open bar; DoE 66 [17] – closed bar. Solubility multiple (highest measured solubility : lowest measured solubility).

self-evident but the consistent low solubility range is not and overall this result is an example of drug dependent solubility behaviour in these systems, which is present [17,20], but is very difficult to visualise [20,23]. It is interesting that these three drugs have relatively a low molecular weight and log P value and molecularly have similar compact structures with predominantly flat aromatic rings. This simple chemical property analysis could also be applied to naproxen (pKa 4.15), indomethacin (pKa 4.5) and piroxicam (pKa 6.3), but the solubility multiple for these drugs is much larger (Fig. 4b). However, for the acidic drugs it is known that pH is the major solubility driver [17] and these drugs have pKa values within the DoE 66 or bioequivalent pH range. This is evident in the DoE 66 results (Fig. 1) where points cluster in either high or low groups (pH values tested 5, 6 and 7), however the solubility multiple within a pH cluster is low. It is interesting that this low solubility multiple is present in both ionised and non-ionised states for naproxen, indomethacin and piroxicam. The limited solubility variability in the ionised state is understandable, since this represents aqueous solubility of the ionised molecule, but the tight solubility of the non-ionised which should partition into the amphiphilic micellar structure is comparable to the behaviour of phenytoin (pKa 8.33), tadalafil (0.85) and griseofulvin (neutral), with the latter two not ionised. This is most likely to be related to molecular structure and properties and indicates that molecular structure sits within the three categories in controlling solubility behaviour in fasted intestinal media systems. There are not sufficient examples in this study to assess this effect, however this is an indication of a link between molecular structure or shape and solubility in the intestinal media systems over and above more general properties such as pKa and log P [28]. Further more focussed studies will be required to fully elucidate this behaviour.

3.2. Media factor analysis

Although the bioequivalent media composition is based on a multi-dimensional analysis of fasted intestinal media [24] it is possible to fit the factor values into a tailored DoE structure [21]. This allows a standardised effect value to be calculated for the impact of each media factor on drug solubility, but does not permit the calculation of two-way or higher effects. The results are presented in Table 3 along with effect values from the three previous equilibrium fasted solubility DoE studies [17,19,22]. For the bioequivalent system significant media factors were

Table 3
Significant media factors affecting compound solubility in the systems.

	Bioequivalent	DoE 66	DoE 10	DoE 9
Naproxen	pH	pH	NT	NT
Indomethacin	pH	pH, bile salt, buffer, oleate	pH	pH
Phenytoin	NSF	pH, bile salt, lecithin, oleate, buffer, salt, pancreatin	pH, oleate, cholesterol, BS: PL ratio	NT
Piroxicam	pH	pH	NT	NT
Aprepitant	lecithin, oleate	oleate, pH, lecithin	Oleate, lecithin, monoglyceride	NSF
Carvedilol	lecithin	bile salt, oleate	NSF	bile salt, pH
Zafirlukast	NSF	pH, oleate, lecithin, bile salt	pH, cholesterol, monoglyceride	pH, oleate, bile salt, lecithin
Tadalafil	lecithin	bile salt, pH, buffer, lecithin, oleate, salt	NS	pH
Fenofibrate	lecithin	oleate, bile salt, pH, lecithin, buffer, salt	pH, oleate, lecithin	oleate
Felodipine	lecithin	pH, oleate, lecithin, bile salt	pH, oleate, lecithin, monoglyceride	oleate
Griseofulvin	NSF	pH, bile salt, lecithin, oleate, buffer, salt	NT	NT
Probucol	NSF	pH, oleate	Oleate, BS:PL ratio	pH

NT: drug not tested in this system.
NSF: no significant factors detected.

detected for eight out of the twelve drugs (sixty seven percent). This rate is lower than either of the two reduced number DoEs (note comparison only based on the drugs analysed in this study and present in either DoE) which are at seventy seven percent (DoE 10 [19]) and eighty seven percent (DoE 9 [22]), whilst the large number DoE (DoE 66 [17]) is at

one hundred percent. The lower number of factors identified when comparing DoE 66 to DoE 10 or DoE 9 can be attributed to the lower number of experimental points in these systems reducing the statistical power of the experimental design [22]. The further reduction in the bioequivalent system can be attributed to the fact that the experimental points measured are also not statistically designed for the DoE process.

The majority of factors identified (eight out of nine) in the bioequivalent analysis are identified by DoE 66 with the one exception for carvedilol where lecithin is the sole significant biorelevant factor but is not identified in DoE 66. A comparison with the small scale DoEs indicates that the correlation is reduced to below 50% and in some cases detection of factor significance is variable and there are several reasons for the differences. The already reduced statistical power of the smaller number of experiments and the variations in the factors present within each media system (see comment on cholesterol above) combined with variations in the levels of the factors (see comments on pH and oleate above).

Since the bioequivalent system was designed not to be a DoE the reduced number of factors identified and limited number of correlations with DoE results is to be expected. The identification of a significant factor that corresponds to the DoE 66 factors could therefore be considered a bonus and if identification of media factors influencing solubility is required a DoE approach is preferable.

4. Conclusions

This study demonstrates that it is possible to assess the fasted intestinal equilibrium solubility distribution using a small number of bioequivalent media recipes obtained from a multi-dimensional analysis of sampled fasted human intestinal fluid. The solubility distribution obtained is statistically equivalent to those determined using DoE studies, which indicates that this approach is examining the same solubility space. In addition, the data from this paper in combination with the results from multiple design of experiment papers [17–19,22] and other single point solubility measurements [10] indicates that the use of simulated media system, utilising the same media factors and concentrations are likely to provide similar solubility distributions.

By creating a custom design of experiment using the bioequivalent media recipe factor values it is possible to calculate the factors significantly influencing drug solubility. However, the number of factors identified is reduced when compared to statistically designed small scale studies [19,22], which are again lower than the large scale studies [17]. Therefore small scale studies using bioequivalent media compositions are not useful for the identification of the media factors or factor combinations that significantly influence a drug's solubility and to assess this property large scale DoE studies are required.

For three drugs (phenytoin, tadalafil and griseofulvin) this study identifies a very narrow solubility distribution that is consistent with behaviour in previous studies [17,19,22]. This indicates that molecular structure impacts solubilisation in these systems on top of basic physicochemical parameters such as pKa and log P. However, there is insufficient data within this study to fully analyse this result. The detection of this behaviour was only possible through the application of multiple point solubility studies rather than the single point studies more commonly applied [28]. This might indicate that in order to understand and predict intestinal solubilisation behaviour of drugs, multiple point solubility assessments should be applied.

The solubility variability measured by this study is statistically significantly lower than the variability from the initial large scale design of experiment [17] study. The two studies are not directly comparable and multiple factors could be responsible for this difference. However, based on the source for the media recipe compositions in this study, the lower solubility range measured is more likely to reflect the fasted intestinal solubility envelope than a design of experiment approach. The results also indicate that intestinal solubility is a range, not a single point, and this should be accounted for when assessing solubility impact in the BCS or DCS. Further studies would be useful in an attempt to link this in vitro measurement with in vivo performance and also other important biopharmaceutical properties such as dissolution and supersaturation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Fasted intestinal solubility limits and distributions applied to the biopharmaceutics and developability classification systems

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ABSTRACT

After oral administration, a drug's solubility in intestinal fluid is an important parameter influencing bioavailability and if the value is known it can be applied to estimate multiple biopharmaceutical parameters including the solubility limited absorbable dose. Current in vitro measurements may utilise fasted human intestinal fluid (HIF) or simulated intestinal fluid (SIF) to provide an intestinal solubility value. This single point value is limited since its position in relation to the fasted intestinal solubility envelope is unknown. In this study we have applied a nine point fasted equilibrium solubility determination in SIF, based on a multi-dimensional analysis of fasted human intestinal fluid composition, to seven drugs that were previously utilised to investigate the developability classification system (ibuprofen, mefenamic acid, furosemide, dipyridamole, griseofulvin, paracetamol and acyclovir). The resulting fasted equilibrium solubility envelope encompasses literature solubility values in both HIF and SIF indicating that it measures the same solubility space as current approaches with solubility behaviour consistent with previous SIF design of experiment studies. In addition, it identifies that three drugs (griseofulvin, paracetamol and acyclovir) have a very narrow solubility range, a feature that single point solubility approaches would miss. The measured mid-point solubility value is statistically equivalent to the value determined with the original fasted simulated intestinal fluid recipe, further indicating similarity and that existing literature results could be utilised as a direct comparison. Since the multi-dimensional approach covered greater than ninety percent of the variability in fasted intestinal fluid composition, the measured maximum and minimum equilibrium solubility values should represent the extremes of fasted intestinal solubility and provide a range. The seven drugs all display different solubility ranges and behaviours, a result also consistent with previous studies. The dose/solubility ratio for each measurement point can be plotted using the developability classification system to highlight individual drug behaviours. The lowest solubility represents a worst-case scenario which may be useful in risk-based quality by design biopharmaceutical calculations than the mid-point value. The method also permits a dose/solubility ratio frequency distribution determination for the solubility envelope which permits further risk-based refinement, especially where the drug crosses a classification boundary. This novel approach therefore provides greater in vitro detail with respect to possible biopharmaceutical performance in vivo and an improved ability to apply risk-based analysis to biopharmaceutical performance. Further studies will be required to expand the number of drugs measured and link the in vitro measurements to in vivo results.

1. Introduction

1.1. Oral drug administration

The oral route is the most common method of drug administration. It permits self-administration, which provides patient acceptability, assists compliance and allows the pharmaceutical industry to meet this demand through the provision of adaptable and stable solid oral dosage forms. The apparent simplicity of this approach, however, hides a complexity arising from the combination of gastro-intestinal tract anatomy and physiology along with the physicochemical properties of the administered drug and dosage form. It has been recognised [1] that amongst the

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factors controlling drug absorption is the drug's solubility in intestinal fluid since solid drug particles are not absorbed. This was formalised in the Biopharmaceutics Classification System [2], that linked solubility and permeability with in vitro and in vivo performance, with applicability to regulatory situations covering oral products especially around solubility and dissolution.

1.2. Intestinal solubility calculations

Solubility as a factor in oral absorption permitted, by using a range of assumptions, the development of drug absorption models that could be applied to calculate or estimate the absorption of drugs. One of the first values proposed was the Absorption Potential (AP, Eq. (1)) [1],

$$AP = \log\left(P \times F_{non} \times \frac{S_0 V_L}{X_o}\right) \quad (1)$$

where P is effective gut wall permeability to the drug, F_{non} is the fraction non-ionised at pH 6.5, S_0 is the intrinsic solubility (aqueous solubility of the non-ionised species at 37 °C), V_L is the small intestinal water volume (mL), and X_o is the dose administered. A further variation is the Maximum Absorbable Dose (MAD), which could be calculated using Eqs. (2) [3] and (3) [4];

$$MAD = S \times K_a \times SIWV \times SITT \quad (2)$$

$$MAD = P_{eff,human} \times S \times A \times T_{si} \quad (3)$$

where, S is the solubility at pH 6.5, K_a is the transintestinal absorption rate constant (min ^{1}), SIWV is the small intestinal water volume (mL), SITT and T_{si} are the small intestinal transit time (min), $P_{eff\ human}$ is human jejunal drug permeability (cm s ^{1}), and A is the absorption surface area (7.54×10^{4} cm^{2}). These equations utilise aqueous solubility at pH 6.5, however, it was recognised that aqueous solubility is not identical to intestinal solubility [5] due to the presence of solubilising agents such as bile salts and phospholipids. This approach was further modified with the dimensionless Dose Number (D_o, Eq. (4)) [6];

$$D_o = \frac{D/V_0}{S} \quad (4)$$

where D is the dose administered, V_o is the volume of water taken and S is the physiological solubility. This introduces the dose/solubility ratio concept, which is further expanded in the Developability Classification System (DCS) [7] and also required the use of a "physiological" solubility value rather than a simple aqueous value. This led to the Solubility Limited Absorbable Dose (SLAD, Eq. (5)) [7,8];

$$SLAD = S_{si} \times V \times M_p \quad (5)$$

where S_{si} is the estimated small intestinal solubility (mg mL ^{1}), V is the volume of fluid (500 mL) and M_p is the permeability dependent multiplier. For a high permeability drug M_p is equal to the absorption number (A_n, Eq. (6)); for low permeability drugs is set equal to 1. The A_n is defined as the ratio between the mean small intestinal transit time (T_{si} 3.32 h) to absorption time (R/P_{eff}), where R is the intestinal radius (1.25 cm [6]) and P_{eff} the effective permeability of the intestine to the drug.

$$A_n = \frac{P_{eff} \times T_{si}}{R} \quad (6)$$

The solubility value required to calculate SLAD is the intestinal equilibrium solubility [7], which can be measured in intestinal fluid or simulated intestinal fluids. A recent refinement of the DCS [8] proposes standardisation of the solubility criteria with the use of fasted human intestinal fluid (HIF) as a "gold standard" approach, since this is the most biorelevant. However, the authors recognise that "fasted HIF samples are difficult to handle and quite expensive" and that a surrogate of fasted simulated intestinal fluid (SIF) "is an attractive alternative". The recommendation proposed using solubility values in either fasted HIF or fasted SIF and a correlation between the two systems, based on literature results, is presented.

1.3. Intestinal solubility measurement

Equilibrium solubility measured in aspirates of fasted HIF is known to be variable [9,10], as is the composition of HIF aspirates [11]. The application of fasted HIF as a solubility determination medium is also restricted, as discussed in the refined DCS [8], by the limited volumes extracted during sampling and the intrusive nature of the sampling process [12]. Multiple fasted SIF recipes have been developed to overcome HIF limitations [13,14] and a correlation between drug solubility in HIF and SIF [8,15] can be determined. Despite this relationship, it is not evident which SIF recipe is optimal [13], new recipes are still in development [16] and the measured solubility for drugs varies between recipes [16] and measurements [9]. Recent design of experiment (DoE) guided studies of the impact of SIF media components on drug solubilisation [14,17–19] highlight the variation and complexity that is inherent within these SIF media systems. In order to unify the various approaches, a recent study performed a multi-dimensional analysis of an extensive fasted HIF chemical compositional data set [11] to calculate eight points or HIF compositions [20] that provided a greater than ninety percent coverage of the compositional space in five dimensions. Along with a central distribution point, the nine compositions have been applied as a set of fasted SIF media recipes to explore equilibrium intestinal solubility [21]. The resulting solubility distributions are statistically equivalent to the previous DoE guided studies of the fasted state [14,17,22], and encompass published solubility values in either fasted HIF samples or SIF recipes. This recent result indicates that intestinal solubility is a range and not a single value. Application of a single solubility value measured either in fasted HIF or SIF in the calculations detailed above will therefore represent a mid-point and will not provide information on the potential range or distribution of the solubility due to the inherent variability of intestinal conditions that influence solubility.

1.4. Intestinal solubility and developability classification system

In this study, we have applied the fasted intestinal fluid media compositions identified using the multi-dimensional analysis [20] to the drugs (excluding digoxin) assessed in the original Developability Classification System [7] for the fasted state. The equilibrium solubility of ibuprofen, mefenamic acid, furosemide, dipyridamole, griseofulvin, paracetamol, and acyclovir, has been determined in the nine media recipes [20,21], along with a value in simulated fasted simulated intestinal fluid (FaSSIFv1) version 1 [14]. The nine media recipes provide a range of solubility values that, due to the derivation from sampled HIF, covers the fasted HIF range and can therefore be considered bioequivalent. The solubility values therefore can be applied to the DCS grid and associated calculations that predict absorption to provide the limits for likely in vivo solubility behaviour. Finally, a solubility frequency distribution within those limits can be determined to assess solubility behaviour across the population range, based on the twenty volunteers sampled in the original study [11]. It should be noted that the frequency distribution represents the aggregated measured HIF compositions from all volunteers and therefore intra- and inter-subject variability cannot be analysed using this approach.

2. Materials and methods

2.1. Materials

Sodium taurocholate, cholesterol, sodium chloride (NaCl), sodium oleate, ammonium formate, formic acid, potassium hydroxide (KOH), hydrochloric acid (HCl), griseofulvin, furosemide, dipyridamole, and

Table 1
Physicochemical properties and molecular structures of drugs.

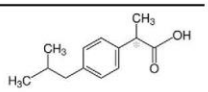
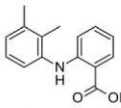
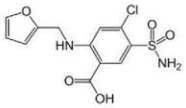
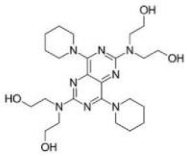
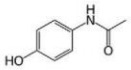
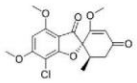
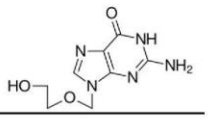
Compound	a/b/n	pKa	LogP	Structure
Ibuprofen	a	5.3	3.97	
Mefenamic Acid	a	4.2	5.12	
Furosemide	a	3.9	2.03	
Dipyridamole	b	6.2	3.77	
Paracetamol	n	–	0.46	
Griseofulvin	n	–	2.18	
Acyclovir	n	2.52/9.35	−1.56	

Table 2
Compositional values of the 8 points, centre point and FaSSIFv1.

Samples	Bile Salt (mM)	Phospholipid (mM)	FFA (mM)	Cholesterol (mM)	pH
1	1.06	0.16	1.04	0.01	6.64
2	11.45	2.48	2.88	0.38	7.12
3	3.4	0.33	2.88	0.09	8.04
4	3.56	1.18	1.04	0.06	5.72
5	3.62	1.25	3.43	0.03	7.14
6	3.35	0.31	0.87	0.17	6.62
7	5.33	0.4	2.96	0.07	6.42
8	2.27	0.96	1.01	0.08	7.34
Centre point (9)	3.46	0.52	1.64	0.032	6.54
FaSSIFv1	3	0.75	1.64	–	6.5

Values from [20], FaSSIFv1 from [14].

acyclovir were purchased from Merck Chemicals Ltd. Ibuprofen was obtained from BSAF chemical company, Paracetamol was from Mallinckrodt Pharmaceuticals and mefenamic acid from Sigma Aldrich. Phosphatidylcholine from soybean (PC S) was purchased from Lipoid company. See Table 1 for physicochemical properties and molecular structures. Chloroform was from Rathburn Chemical Company, FaSSIF media was purchased from Biorelevant.com, and sodium phosphate monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$) was purchased from Fisher Scientific. All acetonitrile (ACN) and methanol (MeOH) solvents were HPLC gradient (VWR). All water is ultrapure Milli-Q water.

Table 3
HPLC conditions.

Drug	Injection volume (μL)	Wave-length (nm)	Retention time (min)
Ibuprofen	100	254	2
Mefenamic acid	10	291	2.3
Furosemide	10	291	2.5
Dipyridamole	10	291	2.5
Griseofulvin	10	291	1.5
Paracetamol	10	254	1.07
Acyclovir	10	254	1.52
Zafirlukast	25	254	2.6
Felodipine	10	254	2.4

2.2. Methods

2.2.1. Solubility media preparation

2.2.1.1. Bioequivalent media stock solutions. For each media recipe (Table 2), a concentrated lipid stock was prepared as follows. The required (×15) weight of bile salt (sodium taurocholate), phospholipid (soyabean lecithin) and free fatty acid (sodium oleate) for each media recipe was dissolved in chloroform (3 mL) – Stock A. The required weight of cholesterol (×1500) for each media recipe was dissolved in chloroform (10 mL) – Stock B. An aliquot of Stock B (0.1 mL) was added to each Stock A, mixed and the Stock A chloroform solution evaporated under a stream of dry nitrogen gas. The dry lipid film was resuspended in water, quantitatively transferred to a volumetric flask (5 mL) and made to volume with water. Stock aqueous solutions of buffer (sodium phosphate monobasic monohydrate; 28.4 mM) and salt (sodium chloride; 105.9 mM) were prepared in water.

2.2.1.2. Fasted state simulated intestinal fluid (FaSSIF). Pre-prepared media, purchased from Biorelevant.com, were used as described by the manufacturer.

2.2.2. Equilibrium solubility measurement

The method is based on previous papers [14] aliquots (267 μL) of the lipid, buffer and salt stock solutions, an excess of the solid drug and water (3.199 mL) were added Into a centrifuge tube (15 mL Corning® tubes) to make a final aqueous system volume of 4 mL. The pH was adjusted to the required value (Table 1) using 1 M KOH or HCl as required. FaSSIF medium (4 mL) was added to the tube along with an excess of the solid drug and pH was adjusted if required. Tubes were capped and placed into a shaker (Labinco L 28 Orbital shaker) for 1 h at room temperature and the final pH was re-adjusted as required. Tubes were then placed in the shaker at 37 °C for 24 h. Post incubation, an aliquot (1 mL) of each tube was transferred to a 1.5 mL Eppendorf tube and then centrifuged for 15 min, 10,000 rpm. The supernatant was analysed by HPLC for drug content. For each drug, this process was repeated three times and the average value is used.

2.2.3. HPLC analysis

Analysis was performed on a Shimadzu Prominence-i LC-2030C HPLC system using a gradient method for all the drugs. Mobile phases A 10 mM ammonium formate pH 3 (adjusted with formic acid) in water, and mobile phase B 10 mM ammonium formate pH 3 (adjusted with formic acid) in acetonitrile:water (9:1), flow rate 1 mL/min (except acyclovir 0.5 mL/min), time start 70:30 (A:B), 3 min 0:100, 4 min 0:100,

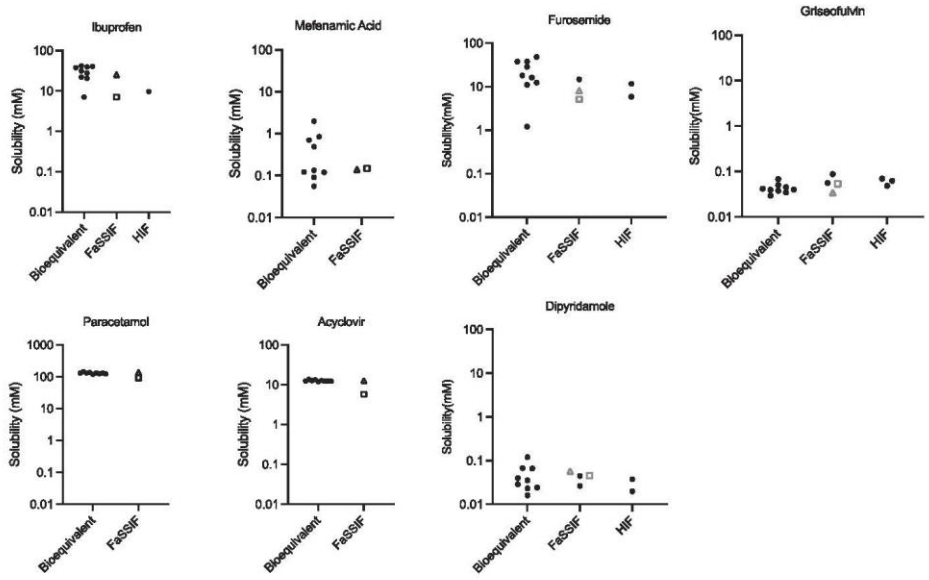


Fig. 1. Measured Equilibrium Solubility Distributions. Bioequivalent (mean, n 3) this study; FaSSIFv1 (Fasted Simulated Intestinal Fluidv1) △this study (mean n 3); □ from [7]) ● from [4]; HIF (Fasted Human Intestinal Fluid) data from [4]. NB Paracetamol y-axis different scale.

4.5 min 70:30 total run time 8 min. The following columns were used (all at 30 °C): Xbridge® C18 5 μm (2.1 × 50 mm) for ibuprofen, mefenamic acid and griseofulvin, Speck and Burke, ODS-H optimal 5 μm (30 × 150 mm) for acyclovir, furosemide and dipyridamole, and Kromasil 60-5-SIL (3 mm, 15 cm) for paracetamol, The retention time, detection wavelengths and injection volume for each drug are provided in Table 3. For each drug, a concentration curve was prepared using five or six standards that bracketed all the measurement concentrations. For all drugs, the correlation coefficient of the calibration curve was >0.99.

2.2.4. Data analysis

Data analysis and comparison was conducted using Graphpad Prism 9 for MacOSX.

3. Results and discussion

3.1. Equilibrium solubility measurements

The equilibrium solubility results using the nine point bioequivalent media recipes and FaSSIFv1 for the drugs analysed are presented in Fig. 1. The drugs in Fig. 1 (with the exception of griseofulvin) have not previously been analysed in DoE guided fasted media solubility experiments [14,17,19,22] and therefore no comparison with these data sets is possible. Griseofulvin has been previously analysed in the aforementioned DoE guided solubility experiments and comparisons to these data sets are analysed in a previous publication [21]. Where available, literature solubility measurement values for either fasted HIF or SIF are included in Fig. 1. The drugs display solubility behaviour that is consistent with the drug's physicochemical properties (Table 1) and the solubility drivers identified in the DoE studies [14,17,19,22], see Section

Table 4
Equilibrium solubility data and analysis.

Drug	Dose (mg)	Estimated Human Peff (cms $^{-1}$ × 10^{-4})	FaSSIFv1 Solubility (mg ml $^{-1}$)	Centre Point Solubility (mg ml $^{-1}$)	Minimum Solubility (mg ml $^{-1}$)	Maximum Solubility (mg ml $^{-1}$)	Solubility Multiplier[1]	Skew[2]
Ibuprofen	400*	12*	5.26	4.27	1.46	6.44	4.41	0.772
Mefenamic Acid	250*	14*	0.0341	0.0289	0.0134	0.481	35.9	29.2
Furosemide	80*	0.6*	4.84	4.12	0.398	15.9	40.0	3.16
Dipyridamole	100*	1.5*	0.0133	0.0145	0.00813	0.0608	7.48	7.23
Paracetamol	500*	1.3*	20.6	19.9	18.0	22.0	1.22	1.10
Griseofulvin	500*	8.7*	0.0122	0.0133	0.0104	0.0240	2.32	3.63
Acyclovir	800*	0.25*	2.86	2.87	2.67	3.07	1.15	0.929

* Data from Butler [7] #9838; **Data from Martindale Extra Pharmacopoeia.
[1] Solubility Multiplier (Maximum Solubility)/(Minimum Solubility).
[2] Skew ((Maximum Solubility Centre Point Solubility))/((Centre Point Solubility Minimum Solubility)).

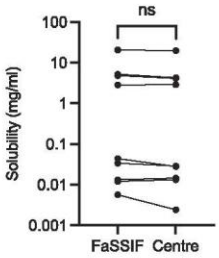


Fig. 2. FaSSIFv1 vs Centre Point Solubility Comparison. FaSSIFv1 (Fasted State Simulated Intestinal Fluid) and Centre this study. ns no significant difference ($P > 0.05$), each point mean n 3.

3.3. In addition, for all drugs the majority (for exceptions see next paragraph) of published fasted HIF or SIF solubility values sit within the solubility range measured using the nine bioequivalent media recipes, indicating that the measured bioequivalent solubility envelope is consistent with the available fasted HIF or fasted SIF data.

Three drugs (griseofulvin, paracetamol and acyclovir) present a different behaviour with a very narrow solubility range (solubility multiplier <4, Fig. 1 and Table 4) in the bioequivalent media recipes, indicating that their solubility is not greatly influenced by media composition variation. This narrow solubility range behaviour was present for griseofulvin, as well as for tadalafil and phenytoin, in the initial statistically guided study [14] and has been replicated in a recent study that re-examined the behaviour of the original twelve drugs in the bioequivalent media system [21]. This result adds a further two drugs to this behaviour list (paracetamol and acyclovir), indicating that the multi-point solubility analysis is revealing an intestinal solubility property or behaviour that a single point measurement would miss. For these drugs, three points out of eleven literature solubility values for either fasted HIF or SIF sit outside the bioequivalent solubility range, indicating a level of variability between values. However, there are variations in the measurement protocols applied and based on the very narrow solubility range for the drugs this difference is probably related to the measurement protocols.

This narrow solubility range behaviour is not restricted to a single BCS/DCS class (paracetamol – class I; griseofulvin – class II; acyclovir – class III) and is probably related to drug molecular structure and properties since these three compounds are relatively simple planar molecules with a low logP value (Table 1). This consistent solubility behaviour, irrespective of media composition might be interesting to examine in relation to possible biopharmaceutical performance implications and biowaivers. Further experimental studies will be required to confirm and fully elucidate this interesting observation and maybe link to drug structure and properties.

3.2. Solubility range

Collected solubility data are presented in Table 4, along with data from the original Developability Classification System paper [7]. A statistical comparison of the calculated mean FaSSIFv1 and centre point solubility values (Fig. 2) indicates that there is no statistically significant difference between the two data sets using a Wilcoxon matched pairs signed rank test. Individual drug based non-parametric statistical comparisons of FaSSIFv1 vs centre point measurements ($n = 3$ per drug for both systems) does not detect a statistically significant difference between the values ($P < 0.05$) for any drug, results not shown. This indicates that existing FaSSIFv1 results for these drugs could be utilised as a direct comparison to centre point solubility values measured using the bioequivalent system. However, due to the small number of drugs tested, the inherent spread between the values and utilisation of a non-parametric ranking based comparison, it would be prudent to check this relationship either as further results become available or through multiple measurements of individual drugs.

Using the measured bioequivalent maximum and minimum solubility values, a solubility multiplier can be calculated. The values range from 1.15 for acyclovir to 40 for furosemide and reflect the visual point distributions already presented in Fig. 1. The multiplier's magnitude is smaller than in the original fasted DoE study [14] where for some drugs a three log variation was detected and this reflects the smaller variation

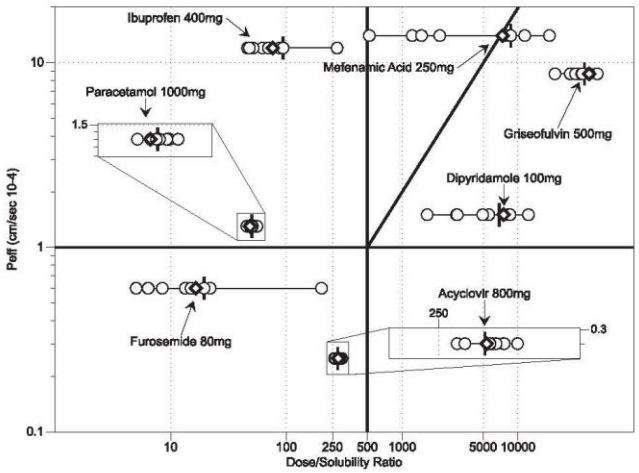


Fig. 3. Bioequivalent Systems on Developability Classification System Grid. ◇FaSSIFv1 (Fasted State Simulated Intestinal Fluid); ○ Bioequivalent data points, I Bioequivalent centre point. Inset expanded scale for acyclovir and paracetamol. Individual drugs and doses as labelled. Each point mean n 3.

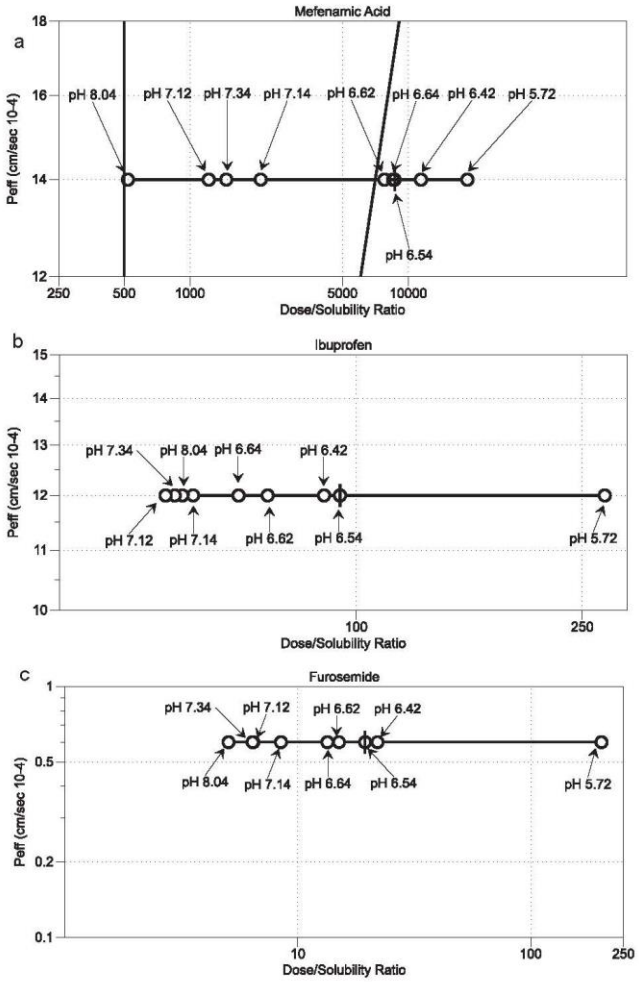


Fig. 4. Acidic Drugs pH solubility behaviour. (a) Mefenamic Acid. (b) Ibuprofen. (c) Furosemide. ○ Bioequivalent data points, I Bioequivalent centre point. Measurement pH values as labelled. Each point mean n 3.

noted in the bioequivalent media system [21] study. This was attributed to the elimination of statistically driven and non-biorelevant combinations of high and low media component concentrations. Using the centre point it is possible to calculate a skew value to determine distribution symmetry, with values ranging from 0.772 to 29.2. Generally, the drugs with the lowest solubility multiplier also have the lowest skew value, however, furosemide deviates from this trend having the largest solubility multiplier with a low skew value. This variation indicates the individualistic drug behaviour in these complicated media systems [23,24] and further results and discussion with respect to this issue are in the next section. This is the first experimental study that permits the calculation of these values and a greater number of examples is required to assess the utility of this information. At this stage it could be surmised that for drugs with a low solubility multiplier and skew value in vivo bioavailability variability will not be influenced by intestinal solubility variability and other factors permeability and/or metabolism will be more important. For high solubility multiplier and skew drugs intestinal solubility variability along with permeability and/or metabolism will contribute to in vivo bioavailability variability.

Based on these results, and Section 3.1 above, the bioequivalent media system is detecting a relevant solubility range and this range is dependent upon the drug's physicochemical properties, molecular

Table 5
Calculated biopharmaceutical data.

Drug	SLAD (mg)		Particle Radius (μm)	
	Centre Point Solubility	Minimum Solubility	Centre Point Solubility	Minimum Solubility
Ibuprofen	24,519	8380	253	148
Mefenamic Acid	193	90	20	14
Furosemide	1181	114	248	77
Dipyridamole	10	6	15	11
Paracetamol	12,357	11,183	545	519
Griseofulvin	55	43	14	12
Acyclovir	3434	3186	207	200

Solubility Limited Absorbable Dose – SLAD $S_{INT} \times V \times A_n$ where S_{INT} is the intestinal solubility (mg/ml) measurement as indicated in column header (see Table 3), V is the volume of intestinal fluid (500 mL) and A_n is the absorption number ($A_n \quad \frac{P_{eff} \times T_{si}}{R}$) where P_{eff} is the effective permeability of the intestine to the drug (see Table 3), T_{si} is the small intestinal transit time (3.32 h) and R is the intestinal radius (1.25 cm).

Particle radius $\sqrt{3D \times S_{INT} \times T_{si}/D_n \times \rho}$ where D is the diffusion coefficient (typically at 5×10^{-6} cms^{-1}), S_{INT} and T_{si} are as above, D_n is the dissolution number (set to 1) and ρ is the drug density (typically 1.2 g cm^{-3}).

structure and media composition.

3.3. Developability classification system range

Using the drug's human intestinal permeability values along with the normal oral dosages (Table 4) [7], it is possible to plot the results on the Developability Classification System grid by calculating a dose/solubility ratio for each measurement point. This is presented in Fig. 3 using FaSSIFv1 and the nine bioequivalent media system measurements. The plot highlights the solubility range along with the multiplier and distribution issues discussed above. The behaviour of the acidic drugs, mefenamic acid, ibuprofen and furosemide with respect to measurement pH is illustrated in Fig. 4a–c. A predominant effect is that solubility increases (therefore dose/solubility volume decreases) with increasing pH with minor variation due to the amphiphilic factors present in the media. This is consistent with the solubility drivers identified for acidic drugs in the original fasted DoE study [14] and other related studies [17,19,22]. This indicates that although the media component concentrations and ratios have been changed to provide equivalence to the measured HIF samples [20], the system's solubility behaviour remains consistent with previous DoE studies.

The bioequivalent point compositions describe greater than ninety percent of the compositional variation present in the analysed HIF samples [20] from twenty, healthy young adult (18–31 years) volunteers [11]. Therefore, it is reasonable to assume that the measured range for each drug in Fig. 3 represents greater than ninety percent of a drug's solubility behaviour in the measured fasted intestinal space and the calculated maximum and minimum values indicate a drug's intestinal fasted solubility range. The lowest solubility or largest dose/solubility volume ratio could be taken to represent a worst case scenario with greater than ninety percent of the distribution above this extreme limit, exhibiting a higher solubility. Therefore compound screening or formulation selection based on the lowest solubility point rather than a centre point or average fasted SIF point might be useful as a worst case for a cautious risk based quality by design approach [8]. In addition, this eliminates the inherent risk associated with solubility range distributions if a centre point or average fasted SIF value is utilised, without any knowledge of the solubility range.

For the drugs presented in Fig. 3 only mefenamic acid crosses a DCS boundary from IIb solubility limited to IIa dissolution limited and of note is that the centre point and FaSSIFv1 value is located at or close to the boundary. The additional range based information arising from the multi-point measurement indicates that a worst case formulation approach for mefenamic acid should be based on solubility limited performance rather than dissolution approaches. This demonstrates the utility of using a range over a single value measured either in fasted HIF or SIF. Investigation of more drugs will reveal further candidates where different aspects of these scenarios are likely to arise.

By applying the biopharmaceutical calculations detailed in the introduction a solubility limited absorbable dose (SLAD) and a target particle size to avoid dissolution rate limiting issues [7,8] can be calculated. The calculation has been applied to the measured centre point and lowest solubility value as a worst case situation (Table 5), using Peff values for each drug from the literature [7] and standard values for other properties. A comparison between the outputs arising from the centre point and lowest solubility measurements exhibit the same relationship described above and in Sections 3.1 and 3.2. For the narrow solubility distribution drugs (paracetamol, acyclovir and griseofulvin) there is minimal difference between the values, whilst for the other drugs the difference reflects the discussion above. This hints that a narrow intestinal solubility range might be a useful drug development target, since the drug would then be intrinsically resistant to intestinal solubility variability. The authors recognise this might be an unrealistic target based on current medicinal chemistry structures. For four drugs (paracetamol, ibuprofen, furosemide and acyclovir), the calculated lowest SLAD is above the administered dose (Tables 4 and 5) and therefore minimal solubility based absorption issues are possible, reflective of their positions on the BCS/DCS grid. For three drugs (mefenamic acid, dipyridamole, and griseofulvin), the calculated lowest SLAD is below the dose (Tables 4 and 5) and therefore the lowest solubility based calculation could be applied as a quality by design parameter for particle size to reduce the risk of absorption issues [8].

3.4. Fasted solubility distributions

The bioequivalent point compositions (Table 2) were calculated to describe the compositional variation present in the 152 fasted HIF samples within the analysed data set [20]. Through the application of 5-dimensional Euclidean space it is possible to calculate the proximity of each data set point to an individual bioequivalent point composition to produce a frequency distribution based on the number of data set points closest to each bioequivalent point. Since the study has measured the equilibrium solubility of each bioequivalent point, this can then be converted to a dose/solubility volume frequency distribution, see Fig. 5a and b. It should be noted that this frequency distribution arises from the sampled fasted HIF point compositions [11,20] and cannot be related to measured in vivo pharmacokinetic variability [25] at this stage. NB Drugs split between figures on basis of presentation clarity.

In Fig. 5a the distributions for paracetamol, acyclovir, griseofulvin and dipyridamole are presented. Based on the presentation in Fig. 1 and associated discussion in Section 3.1, paracetamol, acyclovir and, griseofulvin all have very narrow frequency distributions with almost vertical cumulative lines, related to the very narrow solubility range for these drugs. The points are not evenly distributed on the cumulative plot and only for paracetamol does the centre point occur in the middle of the distribution. Dipyridamole has a broader distribution range but the points are not evenly distributed on the cumulative plot and centre point is towards the lower end of the cumulative plot. In Fig. 5b the distributions for mefenamic acid, ibuprofen and furosemide are presented. Since these are all acidic drugs [26] the distributions will be predominantly controlled by pH (see Section 3.3 and Fig. 4a–c), but also display the same characteristics previously described with points not evenly distributed and centre point towards the lower end of the cumulative plot. Mefenamic acid and furosemide also exhibit an increased degree of structure in the cumulative plot with steps indicative of peaks in the distribution.

Statistical analysis of the distributions either for normal or log normal behaviour did not produce significant results. Previous statistical analysis of fasted SIF DoE solubility distributions [17,19] highlighted

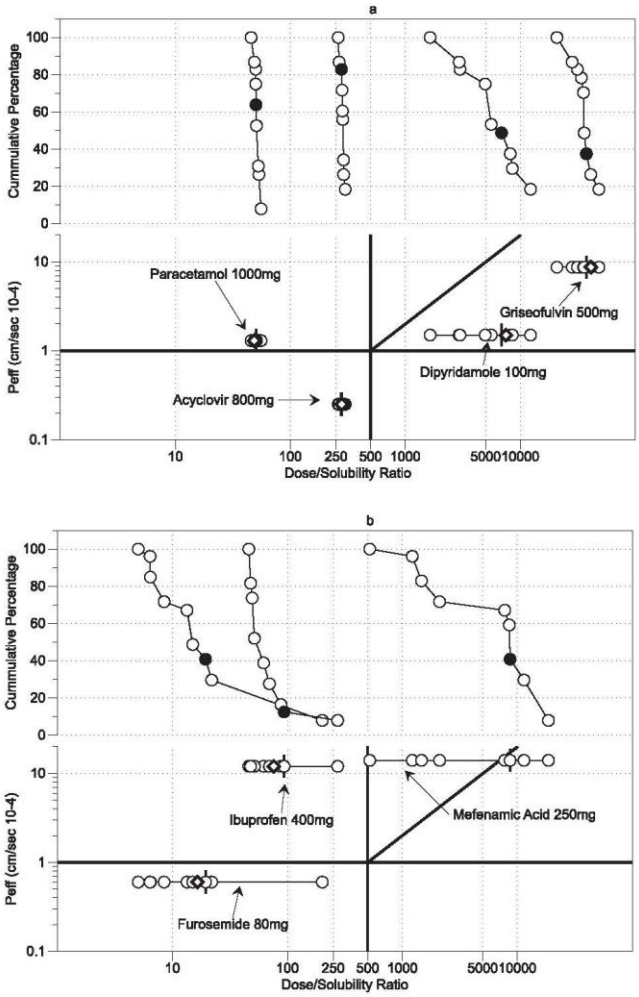


Fig. 5. Cumulative dose/solubility ratio distributions. Lower graph: Developability Classification System Grid, ◇ FaSSIFv1 (Fasted State Simulated Intestinal Fluid); ○ Bioequivalent data points, I Bioequivalent centre point. Each point mean n 3. Upper graph: Cumulative percentage incidence of HIF data points, ○ Bioequivalent data points, ● Bioequivalent centre point.

that the distributions were not normal, also the fasted HIF data points used to calculate the bioequivalent points [20] were not normally distributed. This result might reflect the well known variability of these fluids [27,28] and the measurement of solubility in them [10,15,24]. Within this bioequivalent system, and presumably HIF as well, the traverse from low to high solubility points is not a simple vector based on a single concentration of a media component, where a solubilisation relationship might be expected [29,30], but a five dimensional [23] (and in HIF more) transit through a complex compositional space. Therefore, the lack of an organised statistical distribution when traversing the solubility range based on individual discrete points is to be expected. This might represent an evolutionary aspect to HIF providing variability that maximises nutrient solubilisation, but also impacts administered drugs. This highlights why a single HIF aspirate will not be representative of the entire HIF space and single measurements limited by a lack of knowledge of the sample's position in the space, which will be further complicated when drug properties are superimposed. This makes prediction difficult and points that knowledge of the solubility distribution via measurement is required with the information potentially useful for performing, as discussed, a risk analysis for the likely impact of

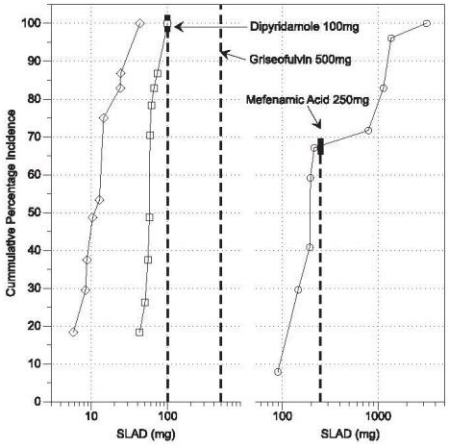


Fig. 6. Cumulative Percentage Incidence of Solubility Limited Absorbable Dose. ○ Mefenamic Acid; ◇ Griseofulvin; □ Dipyridamole. Vertical line drug dose, value as indicated.

solubility variability on absorption behaviour.

3.5. Solubility limited absorbable dose distribution

In Section 3.3 a calculated SLAD based on the centre point and an extreme worst case scenario based on the lowest solubility indicated that for mefenamic acid, dipyridamole and griseofulvin solubility and dissolution rate limiting issues are likely to occur upon oral administration. For mefenamic acid (weak acid) and dipyridamole (weak base) [26], modifications could be applied to account for pH changes during transit through the gastric compartment and down the intestinal tract [31,32]. Investigation of intestinal tract pH indicates that this source of variation in the upper tract diminishes as material transits down the tract. Since griseofulvin is not ionisable, a pH based adaptation is not applicable. However, for mefenamic acid even the centre point (see Table 5) calculation highlights a solubility issue with respect to the dose. By calculating the SLAD values for all bioequivalent points and linking to the cumulative percentage incidence (see Section 3.4), it is feasible to determine where solubility limitations no longer apply. This is presented in Fig. 6 for mefenamic acid, dipyridamole and griseofulvin. Dipyridamole and griseofulvin plots do not reach the required oral dose value of 100 mg or 500 mg respectively and will not be discussed further. For mefenamic acid the plot indicates that solubility limitations will only be resolved in approximately thirty percent of fasted HIF compositions (vertical line Fig. 6) and this information could be applied for a risk assessment based approach to development and formulation. This represents a further advantage of solubility range knowledge and frequency distribution within the range to asses solubility associated biopharmaceutical issues, especially where the drug crosses a classification boundary. As above investigation of more drugs will reveal further candidates where this scenario is likely to arise.

4. Conclusions

The results in this paper indicate that the nine bioequivalent media recipes are simple to apply and provide solubility measurements in agreement with literature fasted HIF and SIF values and drug solubility behaviour in agreement with previous DoE studies. Three drugs exhibit a very narrow solubility range that has been revealed by the multi-point analysis and which has not been previously picked up using a single point measurement. This might represent an interesting behaviour category for further biopharmaceutical consideration. Application of the dose/solubility calculation to the bioequivalent points allows a DCS range to be plotted, which represents greater than ninety percent of drug's intestinal solubility based on the derivation of bioequivalent points. The calculated range provides greater information than single point measurements and the lowest solubility value represents a worst case scenario that could be applied to quality by design approaches during drug screening, development and formulation. The bioequivalent points can be linked to the original HIF data set to provide a frequency distribution for the measured solubility value. The solubility distributions do not follow a normal or log normal pattern, which it can now be concluded is in part due to the measurement points being distributed in multidimensional space. Therefore, the traverse from low to high solubility points is not a simple vector based on a single concentration or property. In addition, the distribution can be used to refine quality by design risk assessments since it provides a population value for solubility behaviour. Overall the results indicate that the small scale fasted bioequivalent study provides greater information than single point measurements in either fasted HIF or SIF, by determining a fasted intestinal solubility range, with a population frequency distribution (based on the original population [11] and analysis [20]) that can be applied to biopharmaceutical calculations and quality by design approaches. The approach is therefore worthy of further development and research to expand the number of drugs analysed, refine the compositional calculations for the bioequivalent points and link in vitro solubility to in vivo pharmacokinetics.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Structured solubility behaviour in bioequivalent fasted simulated intestinal fluids

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ABSTRACT

Drug solubility in intestinal fluid is a key parameter controlling absorption after the administration of a solid oral dosage form. To measure solubility in vitro simulated intestinal fluids have been developed, but there are multiple recipes and the optimum is unknown. This situation creates difficulties during drug discovery and development research. A recent study characterised sampled fasted intestinal fluids using a multidimensional approach to derive nine bioequivalent fasted intestinal media that covered over 90% of the compositional variability. These media have been applied in this study to examine the equilibrium solubility of twenty one exemplar drugs (naproxen, indomethacin, phenytoin, zafirlukast, piroxicam, ibuprofen, mefenamic acid, furosemide, aprepitant, carvedilol, tadalafil, dipyridamole, posaconazole, atazanavir, fenofibrate, felodipine, griseofulvin, probucol, paracetamol, acyclovir and carbamazepine) to determine if consistent solubility behaviour was present. The bioequivalent media provide in the majority of cases structured solubility behaviour that is consistent with physicochemical properties and previous solubility studies. For the acidic drugs (pKa < 6.3) solubility is controlled by media pH, the profile is identical and consistent and the lowest and highest pH media identify the lowest and highest solubility in over 70% of cases. For weakly acidic (pKa > 8), basic and neutral drugs solubility is controlled by a combination of media pH and total amphiphile concentration (TAC), a consistent solubility behaviour is evident but with variation related to individual drug interactions within the media. The lowest and highest pH × TAC media identify the lowest and highest solubility in over 78% of cases. A subset of the latter category consisting of neutral and drugs non-ionised in the media pH range have been identified with a very narrow solubility range, indicating that the impact of the simulated intestinal media on their solubility is minimal. Two drugs probucol and atazanavir exhibit unusual behaviour. The study indicates that the use of two appropriate bioequivalent fasted intestinal media from the nine will identify in vitro the maximum and minimum solubility boundaries for drugs and due to the media derivation this is probably applicable in vivo. These media could be applied during discovery and development activities to provide a solubility range, which would assist placement of the drug within the BCS/DCS and rationalise drug and formulation decisions.

1. Introduction

1.1. Oral drug administration

The oral route is the most common method of drug administration, permitting self-medication which assists patient compliance and acceptability and allows the pharmaceutical industry to meet this requirement through the provision of adaptable and stable solid oral dosage forms. For a patient oral administration is a simple procedure, however for a pharmaceutical scientist this simplicity masks a complexity that spans through drug discovery, development, physicochemistry, manufacturing, gastro-intestinal physiology (normal and diseased) and patient behaviour, for example food consumption. A constant for solid oral dosage forms is that post administration the solid drug must dissolve and form a solution within the gastro-intestinal tract in order to be absorbed, enter the portal and then systemic circulation and elicit the desired pharmacological response. A fundamental property of oral administration is therefore drug solubility within the gastro-intestinal tract since this controls dissolution [38] and calculated values such as the maximum absorbable dose [13,17,19] and the solubility limited absorbable dose [10,37]. The critical importance of drug solubility for the oral route was formalised in the Biopharmaceutics Classification System [4], that linked solubility and permeability with in vitro and in vivo performance, and refined in the Developability Classification System [10,37]. Solubility is also of increasing importance due to the trend towards development of poorly soluble drugs with respect to dose [28,38]. The ability to measure in vivo intestinal solubility in vitro is therefore a critical stage in a drug development program [16].

Abbreviations: TAC, Total Amphiphile Concentration; FaSSIF, Fasted simulated intestinal fluid; DoE, Design of Experiment.

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Equilibrium solubility can be measured experimentally however physicochemical principles dictate that the value will be controlled by the drug's solid state properties and the "solvent" into which the drug dissolves [22]. It has been recognised that within the gastrointestinal tract different solvent systems are present based on the normal tract physiology and anatomy [8], and patient populations [35,39], and therefore solubility measured in simple aqueous buffers is not applicable [19]. In order to overcome this limitation two approaches have been applied measuring solubility in sampled intestinal fluids [6,27] or simulated intestinal fluids [9,18] that can be utilised in vitro to asses in vivo solubility in various anatomical locations of the intestinal tract and simulating either the fasted or fed states.

1.2. Fasted simulated intestinal fluids

Multiple fasted simulated intestinal fluid (FaSSIF) recipes are now available in the literature [9] ranging from simple systems consisting of a buffer, bile salt and lecithin to more complex systems which include free fatty acid, monoglyceride and enzyme components [25]. Media development has been based around compositional data from sampled fasted human intestinal fluid (FaHIF) along with measured drug solubilities in FaHIF compared to the solubility in the proposed FaSSIF media. This has provided a correlation between drug solubility in FaHIF and FaSSIF [11,37] but despite research it is still not clear which FaSSIF media is optimal [9], new versions are still in development [21] and the measured solubility for drugs varies between media [21]. In addition a single media is unable to simulate the inherent variation that is present in FaHIF [14,34] and therefore biopharmaceutical predictions are limited since only a single solubility value is determined and the value's position in a population is unknown nor is the potential range of solubility behaviours.

Recent design of experiment (DoE) guided studies have examined the impact of FaSSIF media components on drug solubilisation [3,25,29,33], highlighting the potential solubility variability inherent in these systems but also identifying the key media components influencing solubility and the complex interactions between them [40]. Whilst these statistically guided studies are excellent for determining the intricate range of interactions present they are complex (the original DoE required 66 experiments per drug [25], which limits application in a drug development setting and they are also potentially not biorelevant due to the statistically constructed media recipes. In order to remove the biorelevant limitation a recent study [34] has performed a multidimensional analysis of FaHIF samples [36] to calculate eight FaHIF compositions that cover greater than 90% of the variability present in the sampled fasted intestinal fluid and can be applied to create bioequivalent FaSSIF media. This has been coupled with a central distribution point, based on the FaHIF samples to create a set of nine bioequivalent FaSSIF media recipes [2] that can be used to explore fasted intestinal solubility. Whilst this approach substantially reduces the experimental load and improves the biorelevance of the test media by removing the statistical aspects associated with DoE generated media, the lower number of samples [30] along with the media structure derivation [2] limits the ability to asses individual media components' contribution to solubility.

1.3. Bioequivalent fasted simulated intestinal media solubility

A recent paper [2] has compared the equilibrium solubility distribution of naproxen, indomethacin, phenytoin, piroxicam, zafirlukast, aprepitant, carvedilol, tadalafil, fenofibrate, griseofulvin, felodipine, and probucol measured using the nine bioequivalent fasted media against results from the original fasted DoE [25] and other DoE references which included the fasted state [3,30,33]. There were no statistically significant differences between the data sets indicating that all the systems were examining an identical solubility space. In addition, the measured solubility range for the nine bioequivalent media was, due to the elimination of non-biorelevant statistical media compositions, smaller than the DoE range.

A further paper [1] has utilised the equilibrium solubility measured in the nine bioequivalent fasted media for ibuprofen, mefenamic acid, furosemide, dipyridamole, griseofulvin, paracetamol and acyclovir to expand the original Developability Classification System grid [10]. The results indicate that the single point based on measurement in a FaSSIF recipe [9] sits within a range of points extending from the highest to the lowest measured solubility based on the nine bioequivalent fasted media. This indicates that for some drugs (mefenamic acid for example) the range crosses DCS boundaries, which indicates that development and formulation strategies should be adapted to suit the range and not the single measurement [26].

1.4. Solubility behaviour and measurement reduction

In this paper using the nine bioequivalent fasted media [2,34], we have examined the equilibrium solubility of the twelve drugs (naproxen, indomethacin, phenytoin, piroxicam, aprepitant, carvedilol, zafirlukast, tadalafil, fenofibrate, griseofulvin, felodipine, probucol) investigated in the original DoE study [25] with the additional drugs (ibuprofen, mefenamic acid, furosemide, dipyridamole, griseofulvin, paracetamol, and acyclovir) investigated in order to replicate the Developability Classification System study [1,10] along with posaconazole, atazanavir and carbamazepine. The aim is to investigate the solubility behaviour of the nine bioequivalent fasted media recipes to determine if this is consistent between the drugs and drug categories. Consistent solubility behaviour might permit a further reduction or refinement of the number of media required to determine a FaHIF solubility range in vitro using FaSSIF media. Determination of an intestinal solubility range, with minimum addition of required media would be useful during early drug development, when API material is limited but crucial decisions concerning for example API physical form and formulation are made [7,16,17].

2. Materials and methods

2.1. Materials

Sodium taurocholate, cholesterol, sodium chloride (NaCl), sodium oleate, ammonium formate, formic acid, potassium hydroxide (KOH), hydrochloric acid (HCl), furosemide, dipyridamole, naproxen, phenytoin, piroxicam, fenofibrate, probucol, griseofulvin, carvedilol, tadalafil, carbamazepine, indomethacin and acyclovir (see Table 1 for basic physicochemical data) were purchased from Merck Chemicals Ltd. Aprepitant and felodipine were provided through OrBiTo by Dr. R. Holm, Head of Preformulation, Lundbeck, Denmark. Zafirlukast was purchased from Stratech Scientific Ltd. Ibuprofen was obtained from BSAF chemical company, paracetamol was from Mallinckrodt Pharmaceuticals and mefenamic acid from Sigma Aldrich. Atazanavir and posaconazole were purchased from Chemshuttle company. Phosphatidylcholine from soybean (PC S) was purchased from Lipoid company. See Table 1 for physicochemical properties and molecular structures. Chloroform was from Rathburn Chemical Company, and sodium phosphate monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$) was purchased from Fisher Scientific. All acetonitrile (ACN) and methanol (MeOH) solvents were HPLC gradient (VWR). All water is ultrapure Milli-Q water.

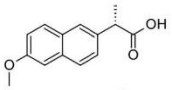
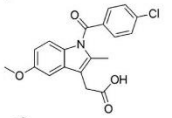
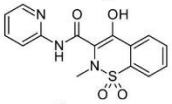
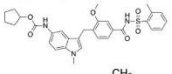
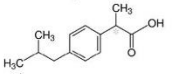
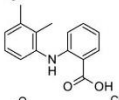
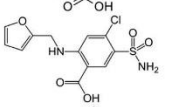
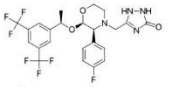
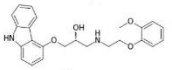
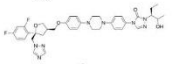
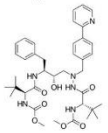
2.2. Methods

2.2.1. Solubility media preparation

Bioequivalent media stock solutions.

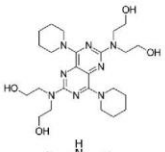
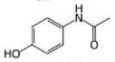
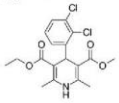
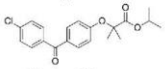
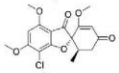
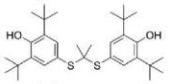
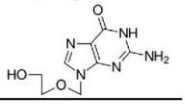
For each media recipe (Table 2), a concentrated lipid stock was prepared as follows. The required (x15) weight of bile salt (sodium taurocholate), phospholipid (soyabean lecithin) and free fatty acid

Table 1
Physicochemical properties and molecular structures of drugs.

Compound	a/b/n	pKa	Log P	Structure
Naproxen	a	4.15	3.18	
Indomethacin	a	4.5	4.27	
Piroxicam	a	6.3	3.06	
Phenytoin	a	8.33	2.47	
Zafirlukast	a	4.94	2.3	
Ibuprofen	a	5.3	3.97	
Mefenamic Acid	a	4.2	5.12	
Furosemide	a	3.9	2.03	
Aprepitant	b	9.7	4.5	
Carvedilol	b	7.8	4.19	
Tadalafil	b	3.5	1.7	
Posaconazole	b	3.6 & 4.6	4.6	
Atazanavir	b	4.7	5.9	

(continued on next page)

Table 1 (*continued*)

Compound	a/b/n	pKa	Log P	Structure
Dipyridamole	b	6.2	3.77	
Paracetamol	n	–	0.46	
Felodipine	n	–	3.86	
Fenofibrate	n	–	5.2	
Griseofulvin	n	–	2.18	
Probucol	n	–	11.3	
Carbamazepine	n	–	2.45	
Acyclovir	n	2.52/ 9.35	−1.56	

(sodium oleate) for each media recipe were dissolved in chloroform (3 mL) – Stock A. The required weight of cholesterol (x1500) for each media recipe was dissolved in chloroform (10 mL) – Stock B. An aliquot of Stock B (0.1 mL) was added to each Stock A, mixed and the Stock A chloroform solution evaporated under a stream of dry nitrogen gas. The dry lipid film was resuspended in water, quantitatively transferred to a volumetric flask (5 mL) and made to volume with water. Stock aqueous solutions of buffer (sodium phosphate monobasic monohydrate; 28.4 mM) and salt (sodium chloride; 105.9 mM) were prepared in water.

2.2.2. Equilibrium solubility measurement

The method is based on previous papers [25], aliquots (267 μL) of the lipid, buffer and salt stock solutions, an excess of the solid drug and water (3.199 mL) were added into a centrifuge tube (15 mL Corning® tubes) to make a final aqueous system volume of 4 mL. The pH was adjusted to the required value (Table 2) using 1 M KOH or HCl as required. The required bioequivalent FaSSIF medium (4 mL) was added to the tube along with an excess of the solid drug and pH was adjusted if required. Tubes were capped and placed into a shaker (Labinco L 28 Orbital shaker) for 1 h at room temperature and the final pH was re-adjusted as required. Tubes were then placed in the shaker at 37 °C for 24 h. Post incubation, an aliquot (1 mL) of each tube was transferred to a 1.5 mL Eppendorf tube and then centrifuged for 15 min, 10,000 rpm. The supernatant was analysed by HPLC for drug content. For each

Table 2
Compositional values of the 8 points, centre point and FaSSIF.

Media	Bile Salt (mM)	Phospholipid (mM)	FFA (mM)	Cholesterol (mM)	pH	Total Amphiphile Concentration (mM) × pH
1	1.06	0.16	1.04	0.01	6.64	15.07
2	11.45	2.48	2.88	0.38	7.12	122.4
3	3.56	1.18	1.04	0.06	5.72	33.40
4	3.4	0.33	2.88	0.09	8.04	53.87
5	3.35	0.31	0.87	0.17	6.62	31.11
6	3.62	1.25	3.43	0.03	7.14	59.48
7	2.27	0.96	1.01	0.08	7.34	31.71
8	5.33	0.4	2.96	0.07	6.42	56.24
centre point (9)	3.46	0.52	1.64	0.032	6.54	36.96
FaSSIFv1	3	0.75	1.64	–	6.5	35.04

Values from [34].

Table 3
HPLC conditions.

Drug	Injection volume (μL)	Wave-length (nm)	Retention time (min)
Naproxen	10	254	1.6
Indomethacin	10	254	2.1
Phenytoin	20	254	1.1
Piroxicam	20	254	1.07
Mefenamic acid	10	291	2.3
Furosemide	10	291	2.5
Ibuprofen	100	254	2
Zafirlukast	25	254	2.6
Aprepitant	50	254	2.27
Carvedilol	10	254	1.6
Tadalafil	50	291	1.4
Dipyridamole	10	291	2.5
Posaconazole	10	254	1.9
Atazanavir	10	254	1.7
Fenofibrate	10	291	3
Felodipine	10	254	2.4
Griseofulvin	10	291	1.5
Probucol	100	220	4.9
Paracetamol	10	254	1.07
Acyclovir	10	254	1.52
Carbamazepine	10	291	1.9

drug, this process was repeated three times and the average value was used.

2.2.3. HPLC analysis

Analysis was performed on a Shimadzu Prominence-i LC-2030C HPLC system using a gradient method for all the drugs except probucol. Gradient Mobile phases A 10 mM ammonium formate pH 3 (adjusted with formic acid) in water, and mobile phase B 10 mM ammonium formate pH 3 (adjusted with formic acid) in acetonitrile:water (9:1) flow rate 1 mL/min (except for acyclovir and carbamazepine 0.5 mL/min, and carvedilol 0.7 mL/min), time start 70:30 (A:B), 3 min 0:100, 4 min 0:100, 4.5 min 70:30, total run time 8 min. For probucol an isocratic method was used [25] mobile phase ACN, MeOH, and water 45:45:10. The following columns were used (all at 30 °C): Speck and Burke, ODS-H optimal 5 μm (30 × 150 mm) for acyclovir, furosemide, probucol, and dipyridamole, Kromasil 60–5-SIL (3 mm, 15 cm) for paracetamol, and

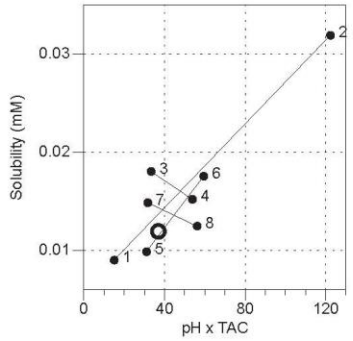


Fig. 1. Representative plot of Solubility vs pH × TAC. Legend: Point label indicates media number (see Table 2). Media 1 and 2 are based on the major axis of the multi-dimensional ellipse that described the FaHIF data cloud [34] and 3 and 4 on the minor axis. Media points 5 and 6 and 7 and 8 are based on further major and minor axes in other dimensions. The statistical analysis means that media are arranged in approximately two columns of three, based on pH × TAC values (3, 5 and 7 and 4, 6 and 8).

the rest of drugs were analysed by Xbridge® C18 5 μm (2.1 × 50 mm), the retention time, detection wavelength and injection volume for each drug are provided in Table 3. For each drug, a concentration curve was prepared using five or six standards that bracketed all the measurement concentrations. For all drugs, the correlation coefficient of the calibration curve was >0.99.

2.2.4. Data analysis

Data analysis and comparison was conducted using Graphpad Prism 9 for MacOSX.

3. Results and discussion

3.1. Solubility analysis

The multi-dimensional analysis of FaHIF composition included five factors pH, bile salt, phospholipid, free fatty acid and cholesterol [34] and in order to plot solubility data on x-y co-ordinates each media recipe has been reduced to a single value by either by calculating the total amphiphile concentration (mM) multiplied by the media pH value (Table 2) or using pH alone. The rationale for the former unusual data manipulation is based on three behaviour properties of these media systems. Previous studies have used the total amphiphile concentration (TAC) to correlate solubility, either individually [32], or in combination [23]. The fasted DoE study [25] indicated that for basic and neutral drugs the media components' (pH, free fatty acid, bile salt and phospholipid) standardised effect values on solubility were similar and that the majority of significant two way interactions involved pH along with an ionisable amphiphile. Finally, a topographical analysis of solubilisation in simulated fluids that employed a four component (bile salt, phospholipid, free fatty acid and monoglyceride) mixture design with varying pH and TAC [20] noted that solubility generally increased as both TAC and pH increased. The pH X TAC manipulation is not completely applicable to acidic drugs where in the fasted DoE the standardised effect value for pH dominated solubility behaviour by a factor of twenty fold greater than any of the amphiphilic media factors. Therefore for acidic drugs a plot using media pH only is presented for the more soluble drugs and comparison (pH and pH × TAC) plots for poorly soluble.

A representative plot of solubility against pH × TAC is presented in Fig. 1, which illustrates the data structure of the media compositions induced by the multidimensional analysis. Media 1 and 2 are based on the major axis of the multi-dimensional ellipse that described the FaHIF data cloud [34] and 3 and 4 on the minor axis. Media points 5 and 6 and 7 and 8 are based on further major and minor axes in other dimensions and the eight points cover 96.4% of the variability in the HIF samples. The statistical analysis means that media are arranged in approximately two columns of three, based on pH × TAC values (3, 5 and 7 and 4, 6 and 8) with the solubility measured in each media dependent upon the drug under investigation.

3.2. Acidic drugs

3.2.1. Solubility behaviour

The solubility plots for the acidic drugs are presented in Fig. 2a and b. Visual analysis indicates that there is a relationship of increasing solubility with media pH and the drugs generally have a very similar behaviour with variation linked in the main to pKa but with minor influences from other amphiphilic media components. For example the solubility ranking of media 2, 6 and 7 is not identical for all the drugs even although the pH values (7.12, 7.14 and 7.34 respectively) are very similar. For the majority of the drugs the pKa value (Table 1) is below the pH of the lowest media (Table 2) and therefore the measurement is of ionised drug solubility in the media. For piroxicam the pKa value is within the media range and for phenytoin the pKa is greater than the highest media pH value. This pH dependent solubility behaviour is

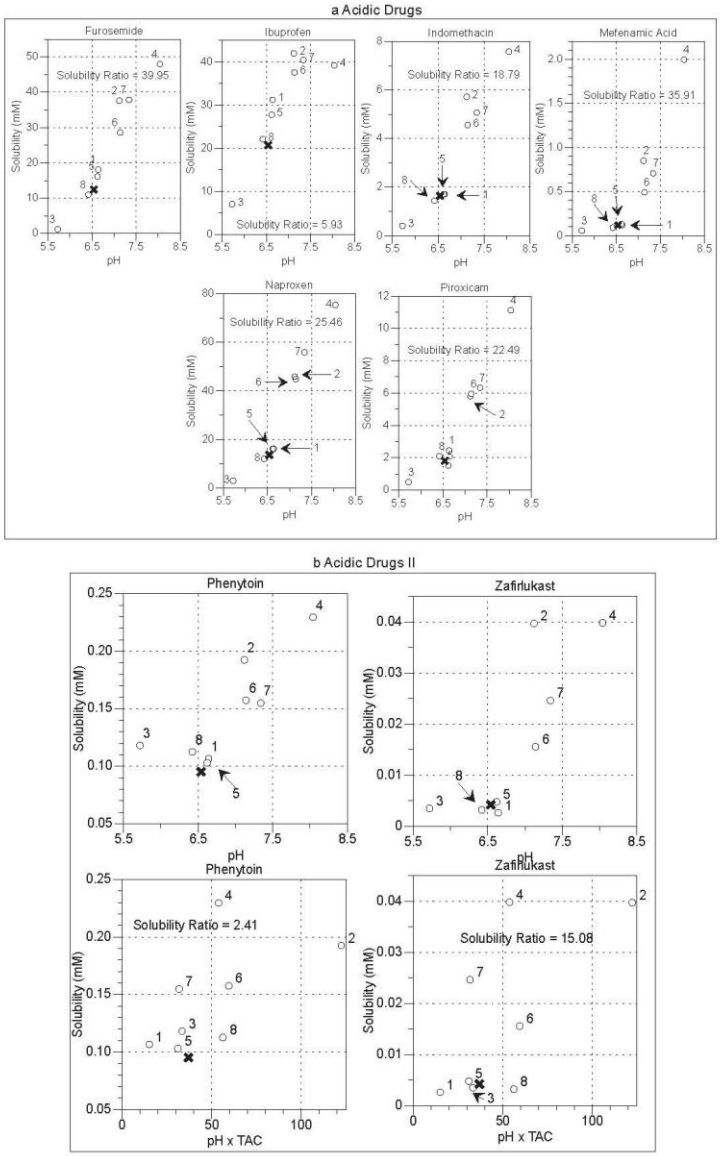


Fig. 2. a and b. Acidic Drugs – Solubility Plot Legend: Point label indicates media number (see Table 2); x centre point - media 9. Calculated solubility ratio (highest solubility/lowest solubility) value in text.

Table 4
Biorelevant fasted simulated intestinal fluids – solubility behaviours.

Category	1 pH controlled TAC minimal variation	2 pH × TAC controlled	3 Minimal pH × TAC control	4 Non-categorised solubility behaviour Drug controlled
Solubility Behaviour	Solubility increases with increasing pH, minimal impact from amphiphilic media components	Gross solubility increases with increasing pH and total amphiphile content, solubility granularity controlled by individual drug interactions with media components	Minimal impact of media components on solubility	No gross solubility relationship between pH and total amphiphile content, drug dependent behaviour, increasing pH and total amphiphile content might reduce solubility
Description	Acidic drugs pKa < 6.3[A]	Basic, neutral drugs, and weak acidic drugs pKa > 8[B]	Neutral drugs[C]	Basic and neutral drugs – categorisation based on solubility behaviour
Drugs	Naproxen, piroxicam, indomethacin, zafirlukast, ibuprofen, mefenamic acid, furosemide	Aprepitant, carvedilol, tadalafil, dipyridamole, posaconazole, fenofibrate, felodipine, griseofulvin, phenytoin	Paracetamol, carbamazepine, acyclovir, griseofulvin[D], phenytoin[D]	Probucol, atazanavir
Comment	Five out of seven examples from non-steroidal anti-inflammatory therapeutic category, expansion into other therapeutic modalities required	Examples varied physicochemical properties, increased drug examples required	Increased drug examples required	Insufficient data for conclusive analysis, increased drug examples required
Lowest Solubility Media[E] Number and Frequency	3 71% 5 out of 7 examples	1 89% 8 out of 9 examples	1 40% 2 out of 5 examples	Not assigned
Highest Solubility Media[E] Number and Frequency	4 86% 6 out of 7 examples	2 78% 7 out of 9 examples	2 80% 4 out of 5 examples	Not assigned
Mean Solubility Ratio (Highest/Lowest) ± Standard Deviation/Ratio Range	23.4 ± 11.8/34 (n = 7)	7.34 ± 5.96/17.37 (n = 9)	1.74 ± 0.596/1.26 (n = 5)	–

TAC Total Amphiphile Concentration; A: Based on highest pKa of acidic drugs measured – piroxicam; B: Based on the single example of phenytoin.
C: Category could include acidic and basic drugs that have pKa values outside of the media pH ranges – see drugs section; D: Added to category based on solubility ratio < 3 – phenytoin and griseofulvin therefore in category 2 and 4; E: Values not equal to Fig. 4 or 9, consult drugs list for values included in each category.

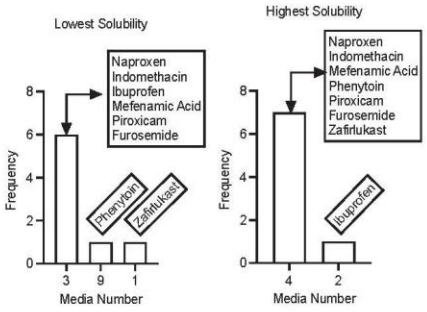


Fig. 3. Acidic Drugs – Lowest and Highest Solubility Media Frequency. Legend: Frequency of lowest and highest solubility media for drugs in Fig. 2a and b. Drugs as listed in boxes.

described as Category 1 in Table 4. The solubility of phenytoin and zafirlukast in Fig. 2b is also presented based on the media pH × TAC value, since for both these drugs media 2 the highest pH × TAC value is providing a solubility greater than expected based simply on pH. This indicates that for these two drugs solubilisation by the amphiphilic components is important, see Catergory 2 in Table 4.

3.2.2. *Solubility behaviour analysis*

This solubility behaviour is consistent with previous literature for acidic drugs in intestinal media [12] and with the various DoE studies [3,25,30,33] in fasted simulated media that identify pH as the major media component driving solubility. The initial DoE study [25] reported that for acidic drugs the average standardised effect value of pH on solubility was twenty times greater than any of the individual amphiphilic media components an observation that is replicated by the behaviour in this study with these different simulated media recipes. The DoE also identified two way interactions between media components [25], however for acidic drugs the largest interaction was between pH and free fatty acid, but with a magnitude around a tenth of pH alone. Therefore, variations in concentrations and interactions between the amphiphilic media components are not interfering with the major solubility driver pH. The initial data analysis performed by applying a DoE structure to the media did detect pH as a significant factor for naproxen indomethacin and piroxicam [2], but with only nine data points the significance of other factors is, due to statistical limitations, not detected [30].

Mefenamic acid, furosemide and ibuprofen have not been assessed in the large sized DoE protocol, therefore it is not possible to analyse their solubility behaviour with respect to known standardised effect values for the media components. However, the consistent distribution of the points in Fig. 2a indicate that these drugs are behaving in a similar manner to the drugs that have been subjected to a DoE investigation. The point distribution may therefore represent a useful tool for determining major features of drug solubility behaviour without conducting a DoE.

3.2.3. *Media frequency analysis*

In Fig. 3 the frequency of each media recipe as either the highest or lowest solubility value for an acidic drug is presented. In 7 out of 8 cases (87%) media number 4 provides the highest solubility value and is not surprisingly the media with the highest pH value (Table 2). The one exception is ibuprofen, where media 2 provides the highest solubility, examination of the data indicates that there is minimal solubility difference (media 2 = 42 mM, media 4 = 39 mM) between the media and for ibuprofen media numbers 7, 4, 6 and 2 are very similar. In 6 out of 8 cases (75%) media number 3 provides the lowest solubility value and again this is not surprising since this media has the lowest pH value. The

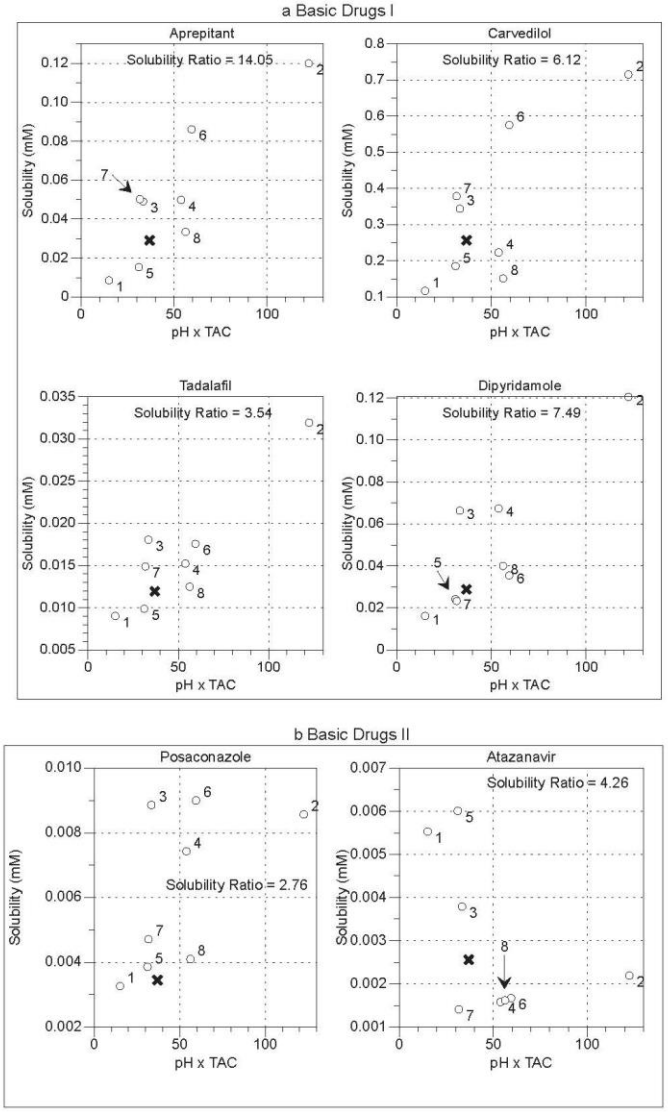


Fig. 4. a and b. Basic Drugs – Solubility Plot Legend: Point label indicates media number (see Table 2); x centre point - media 9. Calculated solubility ratio (highest solubility/lowest solubility) value in text.

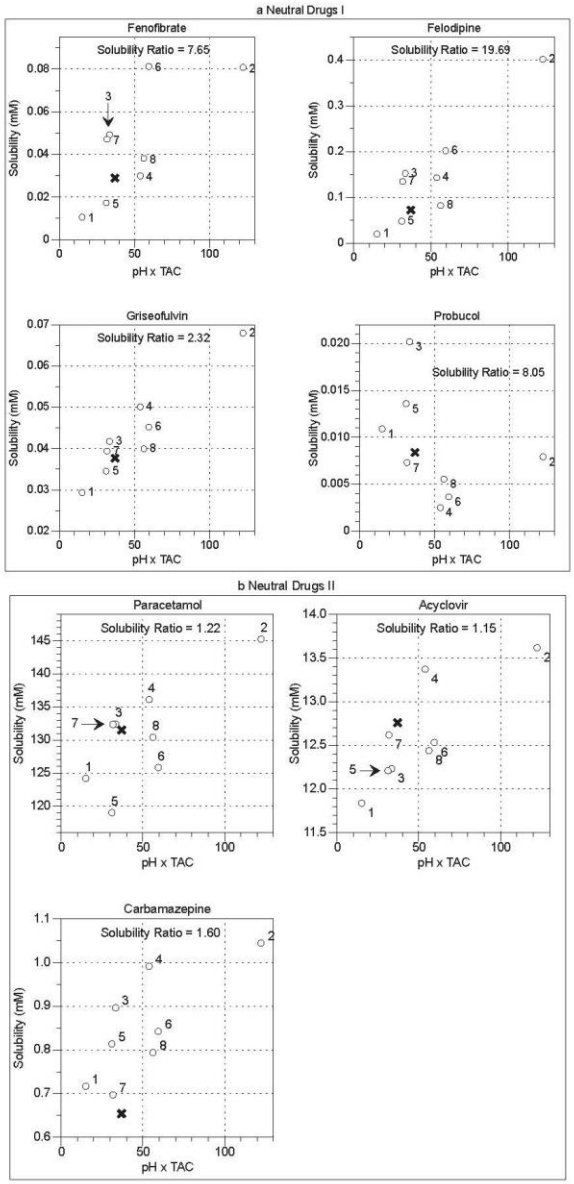


Fig. 5. a and b. Neutral Drugs – Solubility Plot. Legend: Point label indicates media number (see Table 2); x centre point - media 9. Calculated solubility ratio (highest solubility/lowest solubility) value in text.

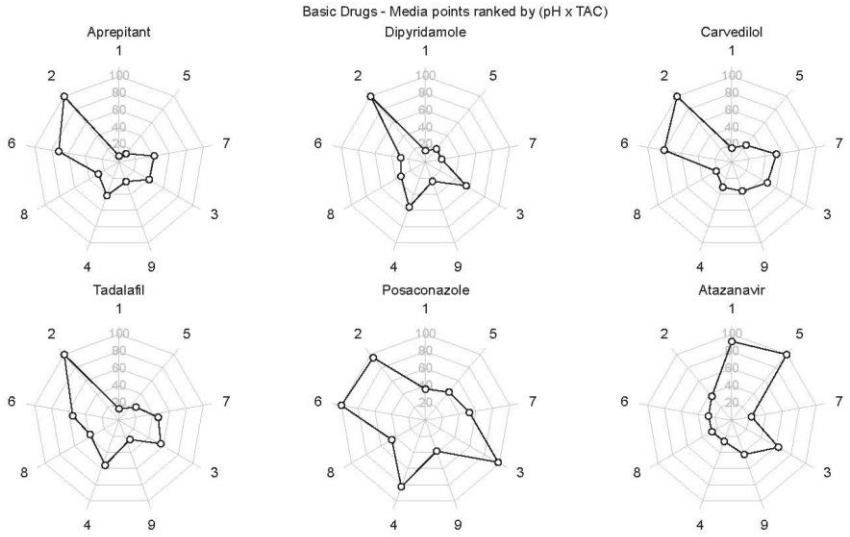


Fig. 6. Basic Drugs – Spider Plot Legend:Highest solubility value normalised to 100; point label indicates media number (see Table 2) arranged in a clockwise order of increasing pH × TAC – lowest pH at 12o'clock.

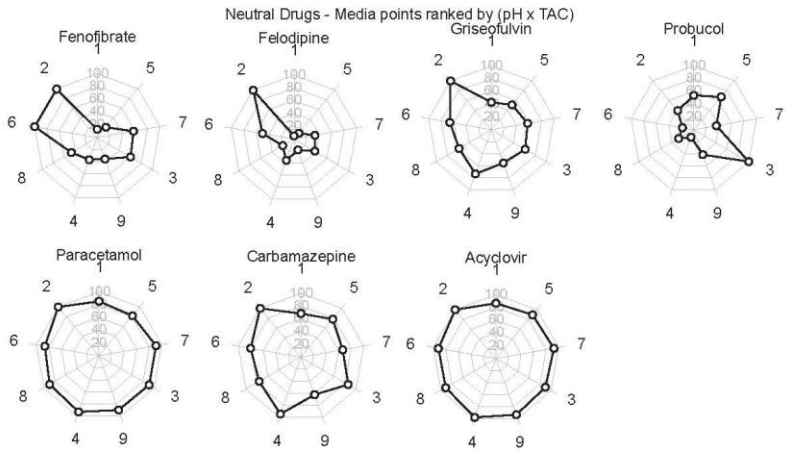


Fig. 7. Neutral Drugs – Spider Plot Legend: Highest solubility value normalised to 100; point label indicates media number (see Table 2) arranged in a clockwise order of increasing pH × TAC – lowest pH at 12o'clock.

exceptions are phenytoin and zafirlukast, where the lowest solubility media is number 9 (centre point) and 1 respectively. For phenytoin due to pKa this represents the interaction of the un-ionised molecule with the media components and overall is an unusual result since media 9 or the centre point usually is located within the point cloud, see other figures. However, phenytoin is a class of drug that has a very narrow solubility distribution (solubility ratio = highest solubility/lowest solubility) in FaSSIF systems [2,25] and the solubility values for media systems 1, 3, 5 and 8 are very similar, see category 3 Table 4. Zafirlukast is acidic but is known to be very poorly soluble in aqueous systems [15] therefore the lowest solubility in media 1 even although it's pH is higher than the pKa (pH 6.64 vs pKa 4.94) can be rationalised due to the low pH × TAC value

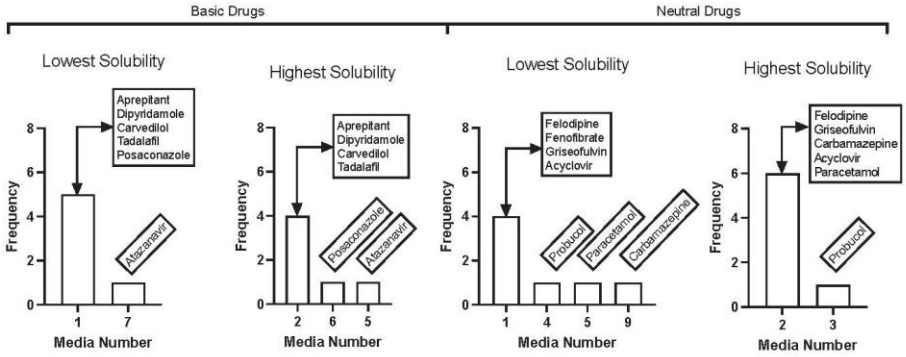


Fig. 8. Basic and Neutral Drugs – Lowest and Highest Solubility Media Frequency. Legend:Frequency of lowest and highest solubility media for drugs in Fig. 4a, b, 5a and 5b. Drugs as listed in boxes.

of the media, see next section. In a similar manner to phenytoin for zafirlukast the solubility in media systems 1, 3, 5 and 8 are very similar, therefore even if the lowest pH value media (number 3) was applied a low solubility value would be determined.

3.3. Basic and neutral drugs

3.3.1. Solubility behaviour

The solubility plots for the basic drugs are presented in Fig. 4a and b with the neutral drugs in Fig. 5a and b. Visual analysis indicates that there is a general structure with the lowest solubility measured in media 1, the highest in media 2 and spatial arrangement of the intermediate media similar although unlike the acids, this is not identical or consistent between drugs. There are also noticeable exceptions in probucol and atazanavir. The data has been transformed into a spider or polar plot in Figs. 6 and 7, where solubility is normalised to the highest value (set to 100) and arranged in a clockwise order around the plot starting at 12o'clock with the lowest pH × TAC media value (media 1, see Table 2) and running to the highest (media 2). This also highlights further noticeable exceptions in addition to atazanavir and probucol, with paracetamol, carbamazepine and acyclovir displaying an almost circular polar plot. A universal solubility behaviour is not evident, but three categories can be identified.

For aprepitant, dipyridamole, carvedilol, tadalafil, posaconazole, fenofibrate, felodipine, and griseofulvin there is a general increase in solubility from media 1 around the plot to media 2, but the increase is not smooth and there are variations in the profile. This variation is evident in Figs. 4 and 5 where for example in Fig. 4a the solubility rank (highest to lowest) of media 4, 6 and 8 is for aprepitant, carvedilol and tadalafil 6, 4 then 8 but for dipyridamole is 4, 8 then 6. A similar variation analysis can be applied to posaconazole, fenofibrate, felodipine, griseofulvin, paracetamol, acyclovir and carbamazepine and the other media numbers 3, 5, and 7. This solubility behaviour pH × TAC dependent is described in Category 2 in Table 4.

The circular polar plots for paracetamol, carbamazepine and acyclovir indicate that there is minimal variation in solubility with changing media (see solubility range values in Fig. 5b compared to other similar Figs. 2a/b, 4a), a feature that has been previously highlighted for griseofulvin, phenytoin and tadalafil [2]. This behaviour was also evident in the original DoE [25] and a comparison drugs (see Figs. 2a, 4a and 5a) also indicates a small solubility range is measured. As reported [1,2] the identification of this solubility property is a feature of measurement using multiple media and there is no direct literature comparison available. However, the reported standard deviation for fenofibrate solubility in multiple pooled FaHIF samples is one hundred and thirty two percent [11] whilst for griseofulvin it is twenty nine percent [5]. An arbitrary ratio cut off at phenytoin (solubility ratio 2.41) has been applied and this solubility behaviour, which is a subset of category 2, with a low solubility ratio is described in Category 3 in Table 4. This subset categorisation might be excessive, since it is based on a limited number of examples.

For probucol media 1 has a higher solubility than media 2 and the polar plot shape is unique amongst the neutral drugs with a flat top (between media 1 and 5) and a point out to media 3. Atazanavir has a very similar shape and although it is basic, has a pKa value (4.7) below the pH of the lowest pH media and therefore the solubility measurements are on the neutral molecule. This behaviour is counter intuitive, probucol has the highest log P (Table 1) and solubility would be expected to increase with TAC, see next section. The solubility is therefore not linked to pH × TAC and is described in Category 4 in Table 4. With only two examples, further research and examples are required.

3.3.2. Solubility behaviour analysis

For basic and neutral drugs, the average standardised effect values from the original DoE studies for pH, free fatty acid, bile salt and phospholipid[1] were equal indicating that these media components all impact solubility. For the basic drugs (tadalafil, carvedilol and aprepitant) the standardised effect fingerprint is variable, pH and free fatty acid were generally not as significant as bile salt and phospholipid with only aprepitant displaying a positive value for the four factors. For fenofibrate, felodipine, and griseofulvin the standardised effect values for pH, free fatty acid, bile salt and phospholipid were positive [25]. There is therefore a generally increasing solubility with increasing pH × TAC. The variation of standardised effect value for media components for each drug is the reason behind the individual drug changes in media number solubility behaviour discussed above, within the background of an overall solubility increase related to increasing pH × TAC [20]. There will therefore be a gross solubility trend of increasing solubility with increasing pH × TAC for the media, hence why point 1 universally has a lower solubility than point 2, atazanavir and probucol excepted. This average behaviour will be modified by each drug's individual

[1] [1] NB Cholesterol was not examined as a media factor in the original DoE.

fingerprint of standardised effect values, hence the inconsistent solubility ranks previously mentioned. This behaviour is consistent with the original DoE [25] and topographical analysis [20]. The original DoE also identified for basic and neutral drugs two way interactions between media components were equivalent contributors to solubility as the components acting alone. For example, for both drug categories pH with free fatty acid was the third and bile salt with free fatty acid the sixth most significant solubility drivers along with free fatty acid, pH, bile salt and phospholipid. These interactions will influence the analysis presented above and highlight that the media (Table 2) due to the method of calculation are not optimised for a DoE, therefore only gross single component effects can be determined [2]. Dipyridamole and posaconazole, have not been measured in any of the fasted DoE protocols, therefore analysis with respect to media component standardised effect values is not possible. However, dipyridamole and posaconazole have a similar shape to the other basic drugs (aprepitant, carvedilol and tadalafil) and therefore indicates that these drugs are behaving in a comparable manner to the DoE drugs.

The behaviour of paracetamol, acyclovir and carbamazepine with essentially circular polar plots represent a category that has been previously recognised for phenytoin and griseofulvin and for these drugs media variation has very limited solubility impact. Paracetamol, acyclovir and carbamazepine have not been measured in the fasted DoE, and with only 5 examples it is difficult to determine which parameters are involved in the property. However, it is interesting that these drugs have the simplest molecular structures amongst all the drugs in the study and are obviously different to probucol and atazanavir described below (Table 1). Possibly indicating that molecular structure somehow has to considered as a property over and above the total molecule physicochemical measurements of melting point, intrinsic solubility, pKa, and log P. A narrow solubility range might also be a useful solubility property to design into oral drug candidates [1] and would provide resistance to variation in human intestinal fluid composition.

For probucol in the DoE [25] bile salt and phospholipid had no significant standardised effect value on solubility and free fatty acid and pH were only just significant. This maybe the reason behind the unusual and paradoxical solubility behaviour (Fig. 5a or 7) with no correlation between solubility and pH × TAC, media 1 (lowest pH × TAC) has a higher solubility than media 2. This behaviour is also present for atazanavir, which as discussed will be behaving as a neutral molecule. With only two examples it is very difficult to rationalise this solubility behaviour, but it does indicate for these drugs a complex solubility behaviour across the fasted intestinal media space. It is also interesting that the solubility ratio for atazanavir is relatively low (4.26), which in a similar manner to category 3 is indicating that it is not interacting with the media components, but has a complex molecular structure. For poorly soluble drugs the use of micellar surfactant solutions as a surrogate for intestinal fluids has been suggested with bile salt one of the initial systems examined [9,31]. For a range of drugs solubility in D-α-tocopheryl polyethylene glycol 1000 succinate increased with increasing amphiphile concentration [11]. A recent paper has published a similar relationship with atazanavir [24] using sodium dodecyl sulphate. However, the result for atazanavir in Fig. 4b indicates that in the multi component bioequivalent media systems the relationship between solubility and TAC is not applicable. This is only a single example but potentially indicates that for some drugs use of single surfactant systems could provide misleading results, a situation that can only be discovered if a multiple FaSSIF media measurement is conducted.

3.3.3. Media frequency analysis

In Fig. 8 the frequency of each media recipe as either the highest or lowest solubility value for the basic and neutral drugs is presented. For the basic drugs media number 2 provides the highest solubility in 4 out of the 6 (66%) drugs analysed with posaconazole registering media number 6 and atazanavir number 5. With posaconazole the highest measured solubility in media 6 was very close to the value of media 2 (see Fig. 4b and 5a) indicating that the difference is minor. For the neutral drugs media number 2 provides the highest solubility in 6 out of 7 (86%) cases, with probucol registering media number 3. For the basic drugs the lowest solubility is measured in media number 1 in 5 out of 6 (83%) drugs analysed with atazanavir registering in media number 7. For the neutral drugs the lowest solubility occurs in 4 out of 7 cases (57%) in media 1 with probucol registering media 4, paracetamol media 5 and carbamazepine media 9. Due to the low solubility variability exhibited by paracetamol and carbamazepine the difference in solubility measurement between media 1 and the lowest solubility media will be low. Probucol and atazanavir are also two of the three drugs that do not register media number 1 for the lowest solubility and as discussed above exhibit solubility behaviour that is very different from the other drugs.

3.4. Solubility behaviour categorisation

The grouping of the drugs analysed in this study, as discussed above, is based on a simple classification around ionisation for comparability with previous studies [25]. Based on the results in this study the categorisation can be modified to reflect the drugs solubility behaviour in the bioequivalent fasted simulated intestinal media but also utilising the ionisation properties, see Table 4. This provides four categories of solubility behaviour, with two defined by physicochemical properties, a third as a subset based on behaviour in the bioequivalent fasted simulated intestinal media, with the final category based on solubility behaviour alone.

The first category is acidic drugs with a pKa value <6.3 (defined by the highest pKa in the sample set) where pH is the main solubility driver, resulting in a consistent pH dependent solubility behaviour and the lowest and highest solubility is measured in media number 3 and 4 respectively in over 70% of cases. This category also exhibits a high solubility ratio and range of solubilities, reflective of the impact of pH on ionisation and solubility. Further studies would be required to expand this set and refine the behaviour pattern, especially since the majority of examples are derived from the non-steroidal analgesic therapeutic category. The second category includes, weakly acidic drugs with a pKa value greater than 8, and basic and neutral drugs where the main solubility driver is a combination of pH and TAC resulting in a general trend of increasing solubility with increasing pH × TAC. The solubility behaviour in the bioequivalent fasted simulated intestinal media will be drug dependent but the lowest and highest solubility is measured in media number 1 and 2 respectively in almost 80% of cases. With only 9 examples it would be sensible to expand the data set as for category 1. Based on physicochemical principles an acidic drug with a pKa between 6.3 and 8 and with a low solubility (comparable to zafirlukast) would be likely to exhibit solubility behaviour that is associated with both categories.

Category 3 drugs have a very low solubility ratio, which is logical based on physicochemical properties and present on polar plots as an almost circular distribution. An arbitrary (based on phenytoin) solubility ratio of<3 has been applied for this category, which means that in this analysis it includes neutral and acidic drugs. Solubility measurement in media number 2 would identify the highest solubility in 80% of cases, but the lowest solubility if media 1 was applied would only be identified in 40% of cases. However, the low solubility ratio would assist in identification of the categorisation and the low range would indicate that the low value solubility error is likely to be small. In Category 4 drug solubility does not increase with pH and TAC and both drugs (probucol and atazanavir) in this category exhibit different solubility behaviour to all other measured drugs. With only two examples in this category it is not possible to fully define properties and the categorisation could be an artefact of the inclusion of these two drugs in the study. However, if media numbers 1 and 2 where applied to measure solubility the signature of lower solubility in media 2 than 1 would identify the behaviour.

3.5. Drug solubility and media component interactions

The solubility behaviour analysis above is predicated on the results from the original fasted design of experiment study [25] which applied a quarter fraction factorial design and required 66 experiments per drug. This measured a standardised effect value for the impact of each media component and two way interactions between components on solubility, although in the latter case some of these were conflicted due to the reduced (quarter fraction) design. The DoE focus was to understand the importance of the media components on drug solubility whereas the current study has applied bioequivalent media derived from a mathematical analysis of FaHIF samples to determine fasted intestinal fluid solubility limits. The two approaches are complementary, with the current (only three media required, see Conclusions) less resource intensive than the DoE. Simple statistical issues around sample numbers indicate that this low media number approach [30] will never provide the depth of information available from a fully structured DoE. During drug development a combination approach would be sensible, solubility screen with limited media numbers with exemplar candidates investigated by DoE, to link the statistical with the bioequivalent to guide development and reduce the possibility for solubility surprises.

4. Conclusions

This is the first study that has examined the solubility behaviour of a range of drugs in a structured set of bioequivalent fasted intestinal fluid media and no consistent solubility behaviour that covers all the drugs tested is evident [6,9]. However, the measured solubility behaviours can be categorised into two types using physicochemical properties and two further categories based on media solubility behaviour.

For acidic drugs (pKa < 6.3) (Category 1, Table 4) equilibrium solubility is directly linked to media pH, an identical solubility behaviour is present for all drugs and the lowest and highest solubility can be determined in the lowest (media 3) and highest (media 4) pH media with a greater than 70% frequency.

For weakly acidic (pKa > 8), basic and neutral drugs (Category 2, Table 4) equilibrium solubility is correlated to increasing media pH $\times$ TAC but the solubility behaviour is not consistent between drugs. The lowest and highest solubility can be determined in the lowest (media 1) and highest (media 2) pH $\times$ TAC media with a greater than 70% frequency. Category 3 is a subset of category 2 including neutral or drugs not ionised within the media pH range and characterised by solubility behaviour that is not sensitive to media composition, leading to a very narrow solubility range. The lowest and highest solubility can be determined in the lowest (media 1) with a 40% certainty and highest (media 2) with an 80% certainty. Any possible error in the low solubility measurement would be easily spotted by the narrow solubility ratio and likely to be minimal due to the narrow range.

The final category with only two drug examples is not well defined but would be detected if media numbers 1 and 2 were applied since the solubility in media 1 would be higher than 2, which is opposite to categories 2 and 3. This category requires further examples to fully define and for all categories increased example numbers and analysis would be prudent.

This indicates that if a drug can be categorised as an acid or weak acid, base or neutral then two simulated intestinal fluid media, either 3 and 4 (representing minimum and maximum pH values for for acidic drugs) or 1 and 2 (representing minimum and maximum pH $\times$ TAC values for weak acid, basic and neutral drugs) can be used to determine in vitro the fasted intestinal solubility range (minimum to maximum). The measurement can then be applied to refine the categorisation and relate solubility behaviour to previous DoE studied examples [25]. Coupled with a central point measure, three media can provide limits and information on the potential BCS or DCS classification and position with respect to the boundaries [1]. If three media are too onerous then the lowest solubility media can be applied as a worst case scenario. This fasted intestinal solubility measurement will provide more information than a single FaSSIF value and can be applied to assess the suitability during drug discovery [16] and development strategies [7] for oral administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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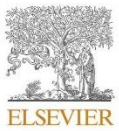

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A novel simulated media system for in vitro evaluation of bioequivalent intestinal drug solubility

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ABSTRACT

Orally administered solid drug must dissolve in the gastrointestinal tract before absorption to provide a systemic response. Intestinal solubility is therefore crucial but difficult to measure since human intestinal fluid (HIF) is challenging to obtain, varies between fasted (Fa) and fed (Fe) states and exhibits inter and intra subject variability. A single simulated intestinal fluid (SIF) cannot reflect HIF variability, therefore current approaches are not optimal. In this study we have compared literature Fa/FeHIF drug solubilities to values measured in a novel in vitro simulated nine media system for either the fasted (Fa9SIF) or fed (Fe9SIF) state. The manuscript contains 129 literature sampled human intestinal fluid equilibrium solubility values and 387 simulated intestinal fluid equilibrium solubility values. Statistical comparison does not detect a difference (Fa/Fe9SIF vs Fa/FeHIF), a novel solubility correlation window enclosed 95% of an additional literature Fa/FeHIF data set and solubility behaviour is consistent with previous physicochemical studies. The Fa/Fe9SIF system therefore represents a novel in vitro methodology for bioequivalent intestinal solubility determination. Combined with intestinal permeability this provides an improved, population based, biopharmaceutical assessment that guides formulation development and indicates the presence of food based solubility effects. This transforms predictive ability during drug discovery and development and may represent a methodology applicable to other multicomponent fluids where no single component is responsible for performance.

1. Introduction

Oral drug administration is preferred by patients but solid drug must dissolve in the gastrointestinal tract (GIT) to enable absorption and produce a response. Intestinal solubility controls [1] absorption and the Developability Classification System [2] (DCS) links intestinal solubility, volume and dose administered with permeability to classify absorption behaviour. Most drug development candidates are poorly soluble (DCS Class II and IV) [3] and during drug discovery and development an accurate in vitro intestinal solubility measurement is essential to assess in vivo biopharmaceutical properties [4] and potential formulation strategies.

The gold standard for measuring intestinal solubility is sampled human intestinal fluid (HIF) [2]. However, HIF is a multicomponent system containing in the fasted (Fa) state endogenous solubilising agents e.g. bile salts and phospholipids, with in the fed (Fe) state additional food digestion products such as fatty acids and glycerides [5]. Average bile salt concentration varies from 3 mM in the fasted state to 15 mM in the fed increasing drug solubility and absorption, leading to a potential "food effect" [6]. This prandial variation is superimposed on intra and inter subject variability [5,7], along with population and disease changes [8]. Obtaining HIF requires nasogastric intubation, only provides small volumes (1–2 mL) and exhibits intra and inter subject variability [5]. Drug solubilities measured in sampled Fa/FeHIF are therefore due to HIF compositional variability highly variable [9] and single values are difficult to correlate to in vivo biopharmaceutical performance.

To mitigate HIF availability, fasted and fed simulated intestinal fluid (Fa/FeSIF) based on average HIF component values was introduced as an in vitro surrogate. Several versions were developed [10] by comparing drug Fa/FeHIF solubilities vs Fa/FeSIF and adjusting SIF media composition. However, there is solubility variability between Fa/

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FeSIF recipes [11] and between Fa/FeHIF samples and therefore no consensus on the optimal Fa/FeSIF media.

Statistically guided studies on SIF composition and solubility [12,13] identified the media components driving solubility either individually or in combination [14]. These studies also revealed that drug molecular structure and physicochemical properties influence solubility variability in combination with media variability [14]. Due to these inherent properties of the drug and the media intestinal solubility is therefore a range. A single solubility value determined in a sampled (Fa/FeHIF) or fixed simulated intestinal media (Fa/FeSIF) composition is therefore incapable of representing the potential in vivo solubility range (which can vary by orders of magnitude) due to HIF variability [8].

To capture HIF compositional variability and therefore solubility variability, a study [7] reported a five-dimensional (pH, bile salt, phospholipid, free fatty acid and cholesterol) analysis of Fa/FeHIF samples [5]. The dimensions or media constitutents included were those that had the major individual impact on drug solubility [12,13]. For both prandial states, eight intestinal media that incorporated 95 % of HIF compositional variability were determined along with a centre point (Fa9SIF and Fe9SIF). Each media is a novel FaSIF [15] or FeSIF [16] directly linked to Fa/FeHIF composition with all 9 in combination covering 95 % of either the fasted or fed compositional variability. There is a fed state limitation since the original study [5] administered the liquid feed Ensure Plus™, which is not equivalent to solid meals.

Previous studies have compared Fa/Fe9SIF solubility [15,16] to Design of Experiment (DoE) studies [12,13,17,18], the DCS [2] with calculation of a new solubility population distribution [19,20] and to determine structured solubility behavior [21,22] that identifies the lowest and highest solubility media. Due to Fa/Fe9SIF's derivation [7] from Fa/FeHIF composition [5], measured drug solubility ranges should be bioequivalent and include measured Fa/FeHIF values. In this paper we have compared Fa/Fe9SIF solubility data for twenty three drugs in the fasted and twenty in the fed state to published Fa/FeHIF solubilities (see **Supplementary Tables 3 and 4**). Establishing an in vitro in/ex vivo intestinal solubility correlation along with the ability to determine a drug's intestinal solubility variability will introduce a transformational change throughout drug discovery, development and formulation [4].

2. Materials and methods

2.1. Materials

Sodium taurocholate, cholesterol, sodium chloride (NaCl), sodium oleate, ammonium formate, formic acid, potassium hydroxide (KOH), hydrochloric acid (HCl), were from Merck Life Science UK Limited, Dorset, UK. Phosphatidylcholine from soybean (PC S) was from Lipoid GmbH, Ludwigshafen, Germany. Chloroform was from Rathburn Chemical Company, Walkerburn, Scotland and sodium phosphate monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$) was from Fisher Scientific, Leicestershire, UK. All acetonitrile (ACN) and methanol (MeOH) solvents were HPLC gradient (VWR). All water is ultrapure Milli-Q water.

Aprepitant and felodipine were through OrBiTo by Dr. R. Holm, Head of Preformulation, Lundbeck, Denmark. Zafirlukast was from Stratech Scientific Ltd, Ely, UK and ibuprofen was obtained from BSAF chemical company. Atazanavir and posaconazole were from Chemshuttle, Burlingame, CA, USA. Carbamazepine, carvedilol, danazol, diazepam, dipyridamole, fenofibrate, furosemide, griseofulvin, indomethacin, itraconazole, naproxen, phenytoin, piroxicam, prednisolone, probucol, tadalafil, valsartan were from Merck Chemicals Ltd, Dorset, UK. See **Supplementary Table 1** for physicochemical data on all drugs.

2.2. Methods

2.2.1. Bioequivalent media stock solutions

For each media recipe (Table 1a Fasted, Table 1b Fed), concentrated stock solutions were prepared [15,16]. The required (x15) weight of bile salt (sodium taurocholate), phospholipid (soyabean lecithin) and free fatty acid (sodium oleate) for each media recipe was dissolved in chloroform (3 mL) – Stock A. The required weight of cholesterol (x1500) for each media recipe was dissolved in chloroform (10 mL) – Stock B. An aliquot of Stock B (0.1 mL) was added to each Stock A, mixed and the chloroform evaporated under a stream of dry nitrogen gas. The dry lipid film was resuspended in water, quantitatively transferred to a volumetric flask (5 mL) and made to volume with water. Stock aqueous solutions of buffer (sodium phosphate monobasic monohydrate; 28.4 mM) and salt (sodium chloride; 105.9 mM) were prepared in water.

2.2.2. Equilibrium solubility measurement

The general equilibrium solubility measurement method has been applied in previous published papers [12,13,15,16,19–22]. In a centrifuge tube (15 mL Corning® tubes), an excess amount of solid drug, exceeding its solubility limit, was weighed, followed by the addition of appropriate concentrated media stock solutions and water. The pH of each tube was adjusted to ± 0.02, using KOH or HCl if necessary, and shaken for an hour at room temperature. The pH was readjusted if needed. The tubes were then placed in an orbital shaker (Labinco L28 Orbital Shaker) and incubated for 24 h at 37 °C and 240 rpm.

Following the 24-hour incubation period, the contents of all tubes were inspected for the presence of solid drug. Then, 1 mL of each solution was transferred to 1.5 mL Eppendorf tubes and centrifuged at 10,000 rpm (RCF approx. 14,000) for 15 min using the Hettich Zentrifugen Mikro 20. Centrifugation is only intended to remove excess solid drug that has not dissolved in the media. Micellar material should remain in the supertnatant as a critical solubilisation component. The supernatant from each tube was analysed for drug content using HPLC. Three separate solubility measurements were taken for each media point to ensure accuracy [15,16].

2.2.3. HPLC analysis

Analysis was performed on a Shimadzu Prominence-i LC-2030C HPLC system using a gradient method for all the drugs. The mobile phase, column (all at 30 °C), retention time, detection wavelengths and injection volume for each drug are provided in **Supplementary Table 2**. For each drug, a concentration curve was prepared using five or six standards that bracketed all the measurement concentrations. For all drugs, the correlation coefficient of the calibration curve was > 0.99 [12,15,16].

2.2.4. Statistical analysis

Statistical analysis was conducted using Graphpad Prism 9 for MacOSX. Correlation analysis were performed using Datagraph 4.7.1 for MacOSX. The variable number of FaHIF measurement values do not permit a simple direct statistical comparison between the data sets. Thirteen drugs have 3 or more available FaHIF values, with seven for FeHIF and these have been compared as a group using a Wilcoxon matched pairs signed rank test, $P < 0.05$ (Two-tailed). Each drug has also been individually compared using a Mann-Whitney test, $P < 0.05$ (Two-tailed). Previous papers have highlighted that the simulated data sets [17] and Fa/FeHIF chemical compositions [7] do not follow a normal distribution and therefore non-parametric statistical comparison is appropriate. The nine media minimum and maximum solubility values (x_{min},y_{max}; x_{max},y_{min}) have been correlated using a power function ($y = ax^b$) to determine a maximum and minimum solubility boundary for each drug category, r^2 reported along with $P < 0.05$ for slope significantly non-zero.

3. Results and discussion

3.1. Equilibrium solubility data sets

One hundred and twenty nine literature Fa/FeHIF equilibrium

solubility values for the measured drugs (**supplementary tables 3 and 4**) are plotted in Figs. 1 and 2. The data are taken from 23 published literature studies and span a single drug value, to a maximum of eight values from four studies for a single drug. The data sets are not balanced (FaHIF 84 values vs Fa9SIF 207 (23x9), FeHIF 45 vs Fe9SIF 180 (20x9)) reflecting issues associated with Fa/FeHIF availability, study drug choices and the multiple research groups performing the research.

3.2. Human intestinal fluid experimental protocols

The Fa/FeHIF collection and solubility measurement protocols vary (**supplementary tables 3 and 4**) with potential to influence the SIF vs HIF comparison. The duodenum and jejunum predominate as a sampling location and HIF compositional data indicates minimal differences between these sites [23], although FeHIF comparisons are limited. Subject ages range from 18 to 49 in the fasted and 45 in the fed, with an average study span of 16 and 19 years respectively. Age effects on HIF solubility have been investigated [24] and although samples exhibited a high inter-individual variability, specific age-dependency was not observed. The study utilized to calculate Fa/Fe9SIF [5], sampled from the duodenum with an age range from 18 to 31, parameters consistent with the Fa/FeHIF protocols.

The average subject number per HIF measurement is 10 in the fasted state and 11 in the fed state, with a range of 4 to 20 and sample pooling in 63 % of FaHIF and 74 % of FeHIF measurements. Where Fa/FeHIF samples are not pooled there will be solubility variation due to inter and intra individual compositional variability [7]. Pooling will mitigate variability dependent upon number of samples, but pools will have an unknown composition. Fa/Fe9SIF is based on 20 volunteers and 324 samples comparable to the Fa/FeHIF protocols, but due to the variability unlikely to be identical especially for un-pooled and low number pooled measurements. Seventy nine percent of the fed state protocols use Ensure Plus™ as a standard meal with a mean collection time of 110 min starting on average 10 min after Ensure administration. This is comparable to the study utilized to calculate Fe9SIF [5], although differences in sampling duration (90 min vs 270) for some studies may have an impact [6].

Solubility measurement protocols are consistent with incubation at 37 °C, equilibration time of ≥ 24 h and separation of undissolved drug prior to analysis. In one case, room temperature was applied, which will have a minor impact on solubility [25]. Studies indicate that for slowly dissolving drugs to attain equilibrium solubility requires twelve hours [12] and only 3 h for soluble drugs. One study utilized a 3 h incubation time and it is noticeable that the poorly soluble drugs (phenytoin and itraconazole) exhibit low solubility compared with Fa/Fe9SIF, whilst the soluble (furosemide and dipyridamole) do not. Drug solid form has not been uniformly assessed, which could impact solubility; for atazanavir for example only the crystalline equilibrium solubility values have been utilized.

3.3. Summary

Since the literature Fa/FeHIF solubility data arise from 23 different studies it is inevitable that there will not be absolute consistency between experimental protocols. This will produce variability that can impact the comparison and two main sources can be identified the Fa/FeHIF sampling protocol and the solubility determination in the sampled fluid.

The Fa/Fe9SIF media were calculated to cover 95 % [7] of the compositional variability of a Fa/FeHIF data set taken from 20 volunteers [5]. Literature information on the composition of HIF samples and the impact of sample pooling is limited as well as the potential impacts of changing physiological factors such as sampling site and volunteer status. This issue is further discussed in section 3.2.1 for a Fa/FeHIF study which includes compositional data. The fasted state, as a resting state is likely to exhibit greater compositional consistency than the fed state which will be more dynamic as digestion and intestinal transit occurs [26]. With the additional complication for the fed state of the nature of the meal ingested. The solubility determination protocol is generally consistent as discussed above.

The analysis indicates that although Fa/Fe9SIF were calculated to cover 95 % of Fa/FeHIF compositional space, the solubility comparison limits should be relaxed to allow for the multiple issues discussed above. Irrespective of the comparison and variability problems, realistically the approach applied is all that is possible due to the inherent issues associated with the literature results.

3.4. Comparison of solubility data sets

Previous SIF solubilities are not normally distributed [17] therefore non-parametric statistical comparison is required. There are seventeen fasted drugs and seven fed with three or more Fa/FeHIF values; comparison of prandial groups (Wilcoxon matched by drug pairs test) calculates no significant solubility difference between FaHIF and Fa9SIF or between FeHIF and Fe9SIF (Fig. 1a and Fig. 2a). When drugs are compared individually (Mann-Whitney test) there is no significant

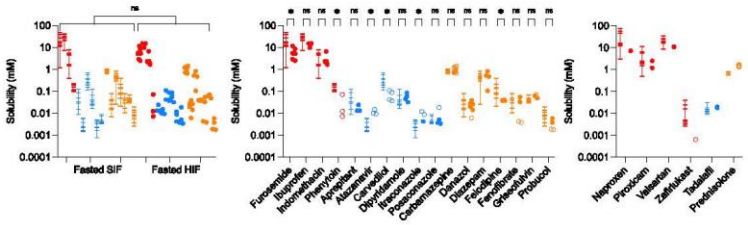

Fig. 1. Comparison plots of Fasted Equilibrium Solubility Values 9 media (Fa9SIF) and literature Fasted Human Intestinal Fluid (FaHIF). Fig. 1a. Drugs with 3 or more FaHIF solubility values. – 9 media, ●FaHIF; red acidic drugs, blue basic drugs, orange neutral drugs; ns – no significant difference between media (Wilcoxon matched pairs signed rank test, P 0.1202 (Two Tailed)(Pairing significantly effective P < 0.0001 (One Tailed) Spearman value 0.9167)); drug order as per Fig. 1b. Fig. 1b. Drugs with 3 or more FaHIF solubility values. – 9 media, ●FaHIF; red acidic drugs, blue basic drugs, orange neutral drugs; closed symbols value lies within 9 media solubility range, open symbols value lies outside range; ns no significant difference between media, * P < 0.05. Mann-Whitney comparison individual P values, furosemide 0.0079; ibuprofen 0.0636; indomethacin 0.6993; phenytoin 0.0091; aprepitant 0.0955; atazanavir 0.0091; carvedilol 0.0028; dipyridamole 0.414; itraconazole 0.0364; posaconazole 0.9399; carbamazepine 0.351; danazol 0.607; diazepam 0.7105; felodipine 0.0182; fenofibrate 0.3301; griseofulvin 0.0636; probucol 0.0503. Fig. 1c. Drugs with less than 3 FaHIF solubility values. – 9 media, ●FaHIF; red acidic drugs, blue basic drugs, orange neutral drugs; closed symbols value lies within 9 media solubility range, open symbols value lies outside range. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

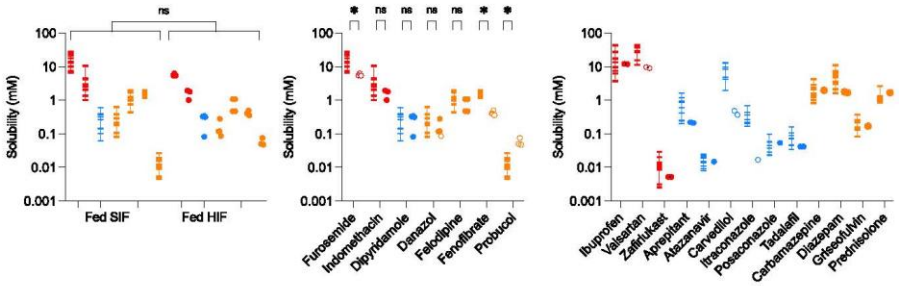

Fig. 2. Comparison plots of Fed Equilibrium Solubility Values 9 media (Fe9SIF) and literature Fed Human Intestinal Fluid (FeHIF). Fig. 2a. Drugs with 3 or more FeHIF solubility values. – 9 media, ●FeHIF; red acidic drugs, blue basic drugs, orange neutral drugs; ns – no significant difference between media (Wilcoxon matched pairs signed rank test, P 0.0781 (Two Tailed)(Pairing significantly effective P < 0.014 (One Tailed) Spearman value 0.9643)); drug order as per Fig. 2b. Fig. 2b. Drugs with 3 or more FeHIF solubility values. – 9 media, ●FeHIF; red acidic drugs, blue basic drugs, orange neutral drugs; closed symbols value lies within 9 media solubility range, open symbols value lies outside range; ns no significant difference between media, *P < 0.05. Mann-Whitney comparison individual P values, furosemide 0.0091; indomethacin 0.2091; dipyridamole > 0.9999; danazol 0.4140; felodipine 0.2601; fenofibrate 0.0028; probucol 0.0091. Fig. 2c. Drugs with less than 3 FeHIF solubility values. – 9 media, ●FaHIF; red acidic drugs, blue basic drugs, orange neutral drugs; closed symbols value lies within 9 media solubility range, open symbols value lies outside range. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

difference for 11 out of 17 drugs between FaHIF and Fa9SIF (Fig. 1b) and for 4 out of 7 drugs between FeHIF and Fe9SIF (Fig. 2b). The fasted felodipine difference is due to the narrow FaHIF solubility distribution a result possibly due to FaHIF pooling. Furosemide displays a similar behavior but this cannot be attributed to pooling.

For felodipine and furosemide the statistical difference is not significant, FaHIF values within Ha9SIF range, based on this study's aim. Therefore, for fasted 76 % (82 % if the phenytoin result is excluded due to the lower equilibration time) and in the fed 57 % of the drugs with ≥ 3 HIF solubility values there is no individual significant solubility difference between Fa/Fe9SIF and Fa/FeHIF. Comparison of individual FaHIF solubility values with the Fa9SIF range (Fig. 1b/c) indicates that 68 % are within the boundaries and in the fed state the value is 64 % (Fig. 2b/c). One study correlated ten poorly soluble drugs in three different FaSIF media and where a comparison to FaHIF is presented 48 % were within the range [11]. Fa9SIF agreement is higher, expected based on the range coverage compared to individual FaSIF media, although the difference between the studies and drugs examined impacts this comparison.

3.4.1. *Impact of composition on solubility behaviour*

Comparison of solubility behavior determined in fasted [12] and fed [13] state DoE studies reinforces the correlation discussed above. Fa9SIF media composition has minimal impact on carbamazepine solubility [21] a feature that is present for FaHIF solubility values from five studies (Fig. 1b). One study [27] analysed HIF pooled from 16 volunteers for pH, bile salt and phospholipid, three of the five Fa/Fe9SIF components or dimensions, which can be compared with Fa/Fe9SIF values [7]. The pooled fasted bile salt (FaHIF 3.52 mM vs Fa9SIF 1.06–11.45 mM)/phospholipid (0.16 mM vs 0.19–2.48 mM) ratio is low compared to Fa9SIF (Fig. 3a), whilst the bile salt/pH (6.83 vs 5.72–8.04) ratio is in the centre. The pooled fed state pH (FeHIF 5.96 vs Fe9SIF 5.86–6.59)/bile salt (8.91 mM vs 4.94–19.04 mM) ratio (Fig. 3b) is low when compared to Fe9SIF range whilst the bile salt/phospholipid (3.72 mM vs 2.07–7.94 mM) ratio is in the centre. For acidic drugs pH is the major solubility driver [12,13,21,22] hence in the fasted comparisons ibuprofen and valsartan are equivalent, zafirlukast requires bile salt and phospholipid solubilization [12], which in the pooled FaHIF are low and could be linked to the low solubility value. In the FeHIF pool this is reversed where valsartan solubility is low due to the low pH but zafirlukast is equivalent due to the "normal" bile salt phospholipid concentrations. Probucol requires monoglyceride for solubility [13] a component not in Fe9SIF but present in the pooled FeHIF [5], potentially explaining the higher solubility.

These examples illustrate the issue of reconciling different drugs' solubility behavior in media of defined against unknown composition. The results indicate that increasing the number of HIF values increases compositional coverage and provides a greater chance of agreement with Fa/Fe9SIF, multiple drugs have solubility values outside the Fa/Fe9SIF range but this is not statistically significant (Figs. 1 and 2). Highlighting that the single value comparison is a stringent test and multiple value comparisons provide greater coverage. This implies that a larger HIF composition data set is required to improve the analysis using more or different dimensions [7], and that HIF solubility measurement should be linked to chemical composition [27]. This latter modification would permit a systematic comparison of HIF and SIF solubility.

3.5. *Solubility correlation boundary*

To extend the literature Fa/FeHIF comparison for the drugs measured using Fa/Fe9SIF, upper and lower correlation boundaries have been calculated based on the minimum and maximum solubility values (x_{min},y_{max}; x_{max},y_{min} where min or max represents the Fa/Fe9SIF minimum and maximum solubilities) and plotted graphically (Fig. 4a). The acidic and basic drug correlations are statistically significant and for neutral drugs in the fed state but not the fasted, this is not critical since the relationship defines a boundary with a span equal to the average solubility range for each drug category. The boundaries shape reflects drug category solubility behavior previously determined by DoE studies [12,13]. Acidic drug solubility is pH driven and the similarity of pH ranges between Fa9SIF (5.72 – 8.04) and Fe9SIF (5.97 – 6.59) leads to contiguous boundaries with fed (lower pH range) inside the fasted. Basic and neutral drug solubility is driven by pH and total amphiphile content (pH x TAC) and the difference between Fa9SIF (15.1 – 122.4) and Fe9SIF (109.1 – 493.1) is reflected in the boundaries. The boundary changes between fasted and fed states for these drug classes is indicative of solubility changes between fasted and fed states and the presence of a food effect, see next section.

An additional literature [9] Fa/FeSIF vs Fa/FeHIF solubility data set of 66 values for 25 drugs has been plotted with the boundaries (Fig. 4b) and 95 % are inside. This is a first exploration of this relationship and reinforces the statistical conclusion that Fa/Fe9SIF provide an in vitro in

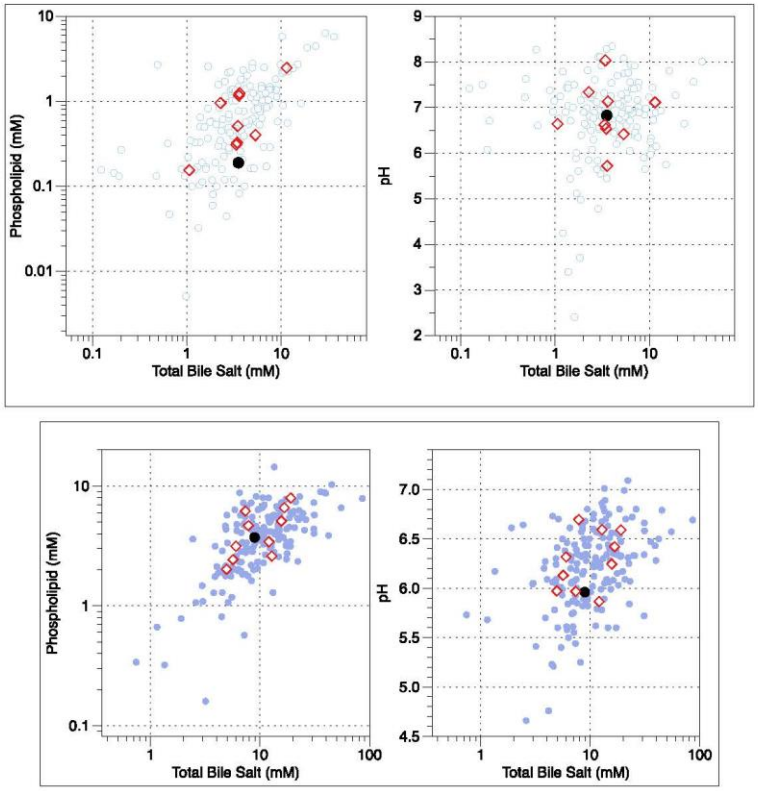

Fig. 3. Compositional comparison Fa/Fe9SIF and sampled pooled FaHIF. Fig. 3a. Dahlgren Pooled FaHIF Composition vs FaHIF Data Set and Fa9SIF Composition. ○Bile salt, phospholipid and pH individual sample values from Pyper[7]. Fa9SIF ◇nine media points; ●Dahlgren[27] pooled FaHIF values. Fig. 3b. Dahlgren Pooled FeHIF Composition vs FeHIF Data Set and Fe9SIF Composition. ●Bile salt, phospholipid and pH individual sample values from Pyper[7]. Fe9SIF ◇nine media points; ●Dahlgren[27] pooled FeHIF values.

vivo solubility correlation, but should be treated with caution. A wide enough boundary will accommodate any data, especially if centered on the equivalence line around which correlation is unavoidable. In addition, the boundary is based on the study drugs which may not be a representative sample.

3.6. Potential biopharmaceutical application

The DCS [2] applies a single poorly characterised Fa/FeHIF or Fa/FeSIF solubility measurement to evaluate a drug's potential biopharmaceutical performance. Fa/Fe9SIF is an advance by providing a bioequivalent solubility range (see above) linked to an intestinal solubility population distribution, which can be applied to provide DCS [19,20] boundary limits. Absorption depends on the solubility, intestinal permeability interplay (other issues e.g. first pass metabolism are not considered in this paper), which along with intestinal transit time and surface area can be utilized to calculate a Solubility Limited Absorbable Dose (SLAD) [2]. The utility of a bioequivalent Fa/Fe9SIF solubility range can be visualized by calculating the Dose/SLAD ratio and plotting against the intestinal solubility population distribution (Fig. 5).

Dose/SLAD < 1, indicates that intestinal equilibrium solubility, permeability and transit time is sufficient to permit complete absorption and the highest value for ibuprofen provides a > 10 fold solubility excess or safety factor. Dose/SLAD > 1, indicates that intestinal solubility, permeability and transit time is not sufficient to permit complete absorption and for griseofulvin that the maximum solubility deficit is > 10 fold. This provides a performance level, supersaturated concentration and time relationship, for formulation strategies, for example amorphous systems [28], to ensure complete absorption. The Fa9SIF, Fe9SIF griseofulvin Dose/SLAD curves, indicate that there is a fed state induced solubility difference and since the curves do not overlap is detecting in vitro the known griseofulvin food effect [29]. Other drugs also display this phenomenon (Fig. 4a, e.g dipyridamole) indicating that this result is worthy of further examination for the in vitro detection of solubility based food effects.

Fa/Fe9SIF display structured solubility distributions that permit identification of the minimum and maximum solubility media for the drug categories [21,22]. This permits a pick-n-mix, drug development

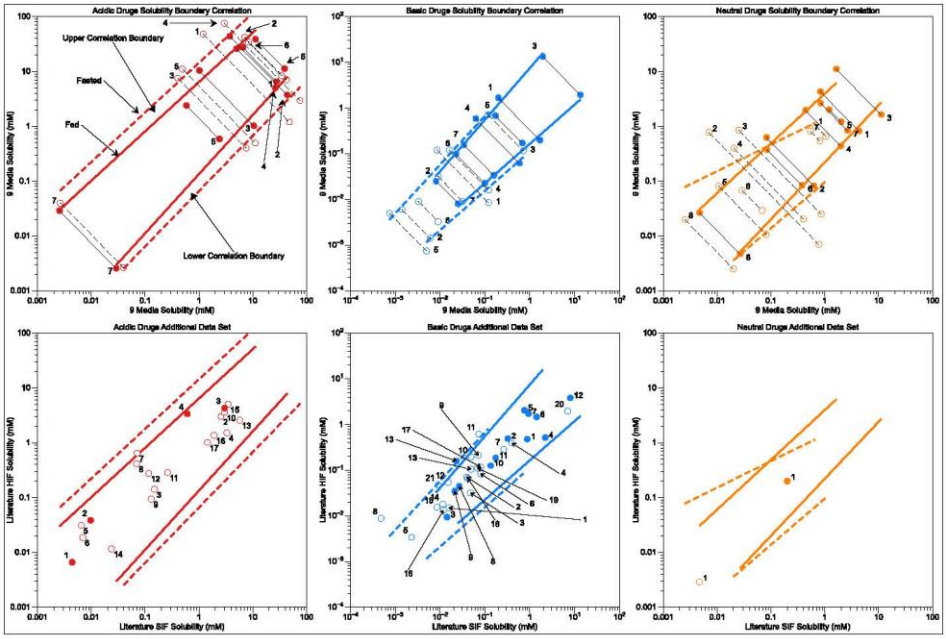

Fig. 4. Solubility Boundary Correlation. Fig. 4a. Solubility boundary correlation – Upper Panel. Acidic, basic and neutral, fasted and fed, upper and lower solubility correlation boundaries based on the minimum and maximum solubility for individual drugs (see numbers) in each Fa/Fe9SIF state plotted as x_{min},y_{max} and x_{max},y_{min} (fasted open symbol, fed closed symbol, fasted drug points connected by dashed black line, fed solid black line); best fitting power correlation line ($y = A x^{B}$) (fasted – dashed coloured line; fed – solid coloured line). Acidic Drugs 1- Furosemide, 2-Ibuprofen, 3-Indomethacin, 4-Naproxen, 5-Piroxicam, 6-Valsartan, 7-Zafirlukast. Basic Drugs 1-Aprepitant, 2-Atazanavir, 3-Carvedilol, 4-Dipyridamole, 5-Itraconazole, 6-Posaconazole, 7-Tadalafil. Neutral Drugs 1-Carbamazepine, 2-Danazol, 3-Diazepam, 4-Felodipine, 5-Fenofibrate, 6-Griseofulvin, 7-Prednisolone, 8-Probucol. Acidic Drugs Lower Correlation Boundary: Fasted y = 0.066013*x^1.009, R^2 = 0.9122, P = 0.0008; Fed y = 0.13456*x^1.0792, R^2 = 0.9707, P < 0.0001. Upper Correlation Boundary: Fasted y = 14.389*x^0.90412, R^2 = 0.9122, P = 0.0008; Fed y = 6.4331*x^0.89946, R^2 = 0.8995, P < 0.0001. Basic Drugs Lower Correlation Boundary: Fasted y = 0.11225*x^0.86083, R^2 = 0.9200, P = 0.0006; Fed y = 0.16457*x^0.8606, R^2 = 0.9716, P < 0.0001. Upper Correlation Boundary: Fasted y = 7.9449*x^1.0687, R^2 = 0.9200, P = 0.0006; Fed y = 7.4946*x^1.1289, R^2 = 0.9716, P < 0.0001. Neutral Drugs Lower Correlation Boundary: Fasted y = 0.096135*x^0.84039, R^2 = 0.4058, P = 0.0894; Fed y = 0.22133*x^1.0385, R^2 = 0.9420, P < 0.0001. Upper Correlation Boundary: Fasted y = 1.41*x^0.48289, R^2 = 0.4058, P = 0.0894; Fed y = 3.9606*x^0.90705, R^2 = 0.9420, P < 0.0001. Fig. 4b. Additional literature data comparison. Acidic, basic and neutral, fasted and fed, upper and lower solubility correlation boundaries based on the minimum and maximum solubility for individual drugs in each Fa/Fe9SIF state Fasted open symbol, fed closed symbol, Fasted – dashed coloured line; Fed – solid coloured line. Additional solubility data from [9]. Fasted – open symbols, Fed – closed symbols. Acidic Drugs Fasted 1-Atovaquone, 2-Diclofenac, 3-Diethylstilbestrol, 4-Flufenamic acid, 5&6-Glibenclamide, 7, 8&9-Glipizide, 10-Hydrochlorothiazide, 11-Irbesartan, 12-Nimesulide, 13-Probenecid, 14-Rimonabant, 15&16-Sulfasalazine, 17-Warfarin. Acidic Drug Fed 1-Glibenclamide, 2-Glipizide, 3-Hydrochlorothiazide, 4-Sulfasalazine. Basic Drugs Fasted 1&2-AZD0865, 3-Cinnarizine, 4-Darunavir, 5-Etravirine, 6-Indinavir, 7-Irbesartan, 8-Itraconazole, 9,10,11,12&13-Ketoconazole, 14,15&16-Loviride, 17,18&19-Nifedipine, 20-Quinidine, 21-Ritonavir. Basic Drug Fed 1-Cinnarizine, 2-Darunavir, 3-Etravirine, 4-Indinavir, 5,6&7-Ketoconazole, 8&9-Loviride, 10&11-Nifedipine, 12-Quinidine, 13-Ritonavir. Neutral Drugs Fasted 1-Cyclosporine. Neutral Drugs Fed 1-Cyclosporine.

stage or requirement based approach for intestinal solubility measurement [4]. A total intestinal solubility range screen can be assessed with two measurements, both prandial states with four, providing assessment of potential food effect and eighteen to provide the full assessment.

4. Conclusions

The in vitro in vivo comparison of intestinal solubility is in principle simple but confounded by multiple factors associated with HIF's natural variability and limited availability. Twenty three drugs are not a comprehensive or structured sample and arises due to published study choices, which limits comparison. This could be ameliorated by targeting additional drugs with multiple Fa/FeHIF (≥3) measurements or optimally a compositional assessment of Fa/FeHIF prior to solubility measurement.

Statistical comparison does not detect a significant solubility difference between Fa9SIF and FaHIF or Fe9SIF and FeHIF data sets. The result indicates that the Fa/Fe9SIF solubility range can be considered bioequivalent to Fa/FeHIF. A novel comparison based on solubility boundaries encompasses 95 % of an additional solubility data set, further reinforcing the statistical conclusion of in vitro in vivo correlation. Solubility differences and behavior can be linked to SIF DoE study results and the influence of media components, indicating that further intestinal fluid composition assessment can refine the approach delivering the potential to measure in vitro intestinal solubility in multiple population and patient groups or species.

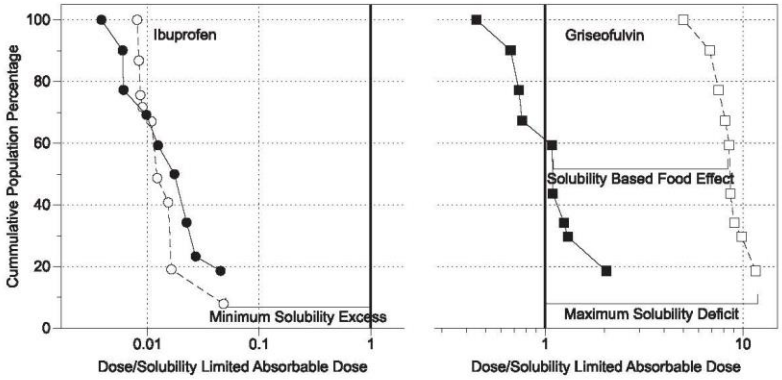

Fig. 5. Biopharmaceutical analysis. Ibuprofen Fasted ○; Fed ●Dose 400 mg: Griseofulvin Fasted□; Fed ■Dose 500 mg.

An in vitro bioequivalent solubility range measurement incorporating population distribution information [19,20] expands DCS [2] approaches to biopharmaceutical performance assessment. A novel graphical analysis utilising the administered dose divided by the solubility limited absorbable dose permits the calculation of drug and dose related solubility safety margins, formulation performance requirements and potential solubility based food effects. Since equilibrium solubility [1] is a key parameter controlling oral absorption an in vitro bioequivalent measurement can be applied to refine PBPK [30] and in silico modelling with potential to generate individual or disease related intestinal solubility profiles and reduce in vivo testing. The Fa/Fe9SIF system is therefore worthy of further investigation with linkage of system results to in vivo performance a key next stage and may also represent a methodology applicable to other multicomponent biological fluids where no single component is responsible for performance.

CRediT authorship contribution statement

Qamar Abuhassan: Writing – original draft, Data curation. **Maria Inês Silva:** Writing – original draft, Data curation. **Rana Abu-Rajab Tamimi:** Data curation. **Ibrahim Khadra:** Supervision. **Hannah K. Batchelor:** Supervision. **Kate Pyper:** Formal analysis, Conceptualization. **Gavin W. Halbert:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2024.114302.

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