Novel experimental techniques for biopharmaceutical analysis

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List of Abbreviation

4MD	4-component mixture design
API	active pharmaceutical ingredient
BA	bioavailability
BCS	Biopharmaceutical Classification Systems
BS	bile salt
DCS	Developability Classification System
DoE	design of experiment
EMA	European Medicines Agency
FaSSIF	fasted simulated intestinal fluid from Galia *et al.,* 1998
FDA	Food and Drug Administration
FeSSIF	fed simulated intestinal fluid from Galia *et al.,* 1998
FFA	free fatty acid
GI	gastrointestinal
HB	hydrogen bond
HP media	high point media
HPLC	high performance liquid chromatography
IDR	intrinsic dissolution rate
IVIVC	*in vitro – in vivo* correlation
MG	monoglyceride
MP media	median point media
MW	molecular weight
OA	sodium oleate
OrBiTo	oral biopharmaceutical tools
PBPK	Pharmacokinetic
PC	phosphatidylcholine
PL	phospholipid
PSA	polar surface are
RoB	rotatable bond
RSD	relative standard deviation
SD	standard deviation
SDDS	supersaturating drug delivery systems
SGF	simulated gastric fluid
SIF	simulated intestinal fluids
USP	United States Pharmacopeia

Abstract

Drug solubility and dissolution are important attributes controlling the bioavailability (BA) of oral dosage forms and can be determined *in vivo,* however it is expensive and can generate ethical issues. *In vitro* tests have been introduced utilising simulated gastrointestinal (GI) fluids, capturing the GI composition and conditions. However, individual variation and food-induced changes of the GI environment affecting drug BA have been recognised and in-depth knowledge is required to understand their effects on drug absorption.

A fractional factorial design of experiment (DoE) and a 4-component mixture design (4MD) were used to investigate drug equilibrium solubility in media presenting in different levels of pH, concentration of amphiphiles, buffer, salt and pancreatin. Poorly soluble acidic, basic and neutral drugs with various physiochemical properties were tested. Solubility results correlate well with literature values, indicating they explored the solubility variation in the biorelevant space. Except pancreatin, all factors showed significant impacts on drug solubility. The descending order of average effect magnitude is pH, amphiphiles, buffer and salt, some of which also displayed remarkable drug specific interactions. Changing amphiphile ratios in 4MD further indicated that solubilisation is not a simple accumulative solubilisation of individual amphiphiles, but interactions between amphiphile-amphiphile and drug-amphiphiles. Powder dissolution using various biorelevant media indicates intrinsic dissolution rates (IDR) are positively correlated with equilibrium solubility and the diffusion coefficient of drugs in different ionisation states and interactions with amphiphiles.

The above studies illustrate the convoluted nature of the GI fluids and provide a visualisation of how solubility/dissolution map varies within the ranges of GI fluid parameters. Statistical approaches can systematically detect critical factors

affecting drug solubility and dissolution, and the output can potentially be applied in physiologically based pharmacokinetic (PBPK) model to achieve better *in vivo-in vitro* correlation (IVIVC).

1. Introduction

1.1 Oral bioavailability

Drugs taken orally are by far the most inexpensive and convenient way for drug dosing; however the bioavailability (BA) is not 100%. BA is the measurement of the rate and extent of the active pharmaceutical ingredient (API) that reaches the site of action (FDA/CFR, 2015). Generally, the oral BA is expressed by percentage of the oral API dose available in the systemic circulation (F). Equation 1 indicates the components affecting BA. Fraction dose absorbed (f_a) is the fraction of drug and its metabolites that pass through the apical membrane of the gut epithelial wall. Metabolism of drugs after absorption can occur with enzymes both in enterocytes and liver (i.e. Cytochrome P450 3A4 in enterocytes, P-glycoprotein in both enterocytes and liver). E_G and E_H refer to the fraction of drugs through gastrointestinal (GI) and hepatic first-pass metabolism respectively, before reaching the systemic circulation (Dressman and Reppas, 2016). An overview of these processes is shown in Figure 1.1.

$$F = f_a*(1-E_G)*(1-E_H) \qquad \text{(Equation 1)}$$

The absorption process is affected by multiple factors, which can be grouped as the physiochemical properties of the API, drug formulation and physiological conditions in absorption sites (Yu *et al.*, 1996). For example, API structure and drug formulation are associated with drug solubility, which is a key property to affect drug absorption; GI physiology (i.e. pH, lipid composition, GI wall surface area, protein contents of the transporters and enzymes) can affect drug concentration at the absorption site and permeability through the GI wall (Washington *et al.*, 2001). These factors may interact and vary along the GI tract and lead to regional-specific drug absorption (Dressman and Reppas, 2016).

Therefore it is very important to gain knowledge of the influence of these factors to forecast and optimise oral drug absorption and BA. One aspect is to understand

the physicochemical properties of the drug candidates at the early stage of drug discovery, which has led to the development of *in silico* pre-screening and *in vitro* high throughput methodologies in industry, such as solubility, dissolution and permeability characterisation, due to the large amount of drug candidates but small available quantities (Bergstrom *et al.*, 2014). Another aspect is to provide biorelevant *in vitro* methodologies to measure these properties in order to build the *in vivo* - *in vitro* correlation (IVIVC). IVIVC describes the relationship between *in vitro* dissolution extent/rate and *in vivo* plasma drug concentration-time profile. When the correlation is established, the *in vitro* data can be used as surrogate for human studies to indicate if there are any changes of the drug absorption due to modification such as formulation and manufacturing process changes, therefore reducing the time and cost for *in vivo* studies (Guidance for industry, 1997b).

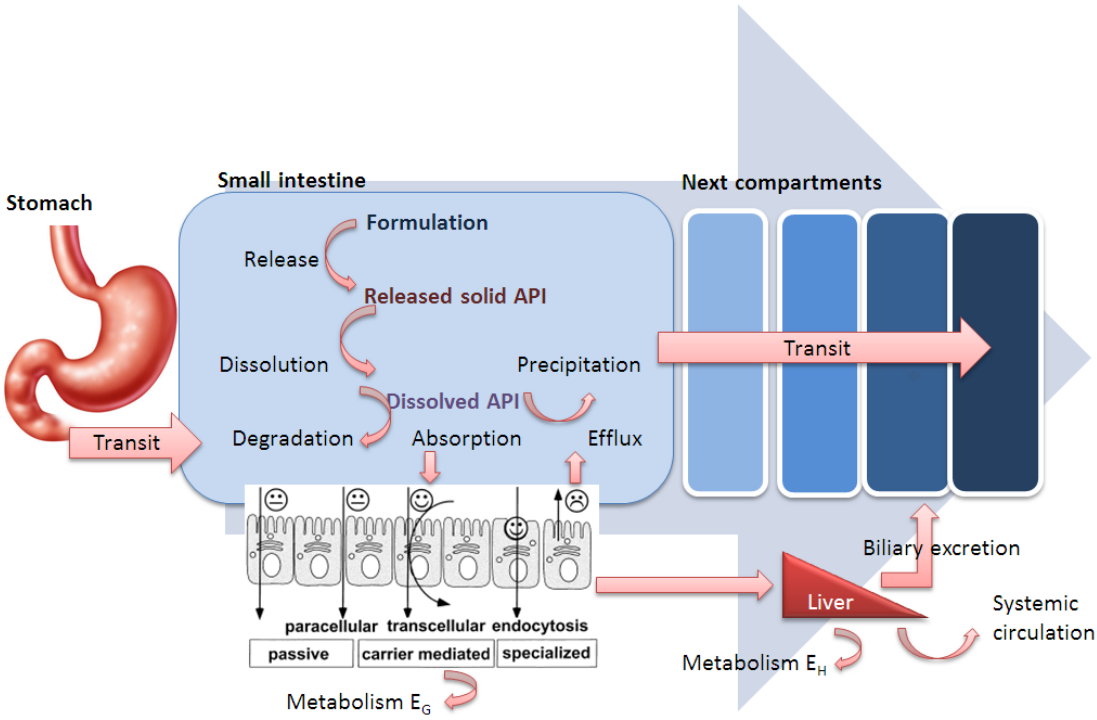


Figure 1. 1 Oral drug absorption in the GI tract (Dressman *et al.*, 1998)

1.2 Solubility

Equilibrium solubility, also regarded as the thermodynamic solubility, is the

concentration of a drug when there is excess amount of solid present in the media at a constant temperature, and the solid and solvate in the solution are at equilibrium (Ma and Hadzija, 2012). For neutral drugs or ionisable drugs in their unionised form, the equilibrium solubility is their intrinsic property and therefore is referred as intrinsic solubility (Stuart and Box, 2005). The apparent solubility is the sum of concentration of both unionised and ionised species of an ionisable compound at the media pH. Solubility of API in the GI media defines the potential concentration available for permeation and absorption in the GI tract, as in most cases the drug molecules have to be in the solution to be absorbed (Lipinski *et al.*, 2012). Currently 40% of the marketed drugs and drugs under development have a low water solubility problem (Williams *et al.*, 2013).

The solubilisation of a drug into water is mainly controlled by two forces. First, the solute molecule needs to break from the packed solid state. Melting point (T_m) provides the information about the strength of the solute-solute affinity in the solid. Crystal solids typically have stronger intermolecular force than amorphous, leading to a lower solubility. The solute molecule will then be accommodated by the solvent in a cavity, depending on how favourable it is to the solvent, which is also known as "like dissolves like" (Williams *et al.*, 2013). Several factors such as the hydrogen donor/acceptor properties can affect the solute solvation. It is highly demanding to have predicted aqueous solubility directly from molecular structures of interest while the samples are in absentia or small amount. The "General Solubility Equation" (Equation 2) is commonly used to predict the molar water solubility (S_w) based on log P and the Celsius T_m (Yang *et al.*, 2002). This equation explains that poor aqueous solubility is mainly driven by lipophilicity, however at high T_m (> 200 - 250 °C), the solubility of low lipophilic drugs is mainly a result of high crystal lattice packing rather than poor solvation (Wassvik *et al.*, 2008). T_m and log P can be obtained experimentally or predicted from *in silico* tools, however

experimental data remarkably improved the accuracy of predicted solubility (Glomme *et al.*, 2005). However, currently no available methods can predict solubility accurately (Lipinski *et al.*, 2012) and the existence of salt forms and polymorphs can also alter drug solubility significantly.

$$\text{Log } S_w = - 0.01*(T_m - 25) - \log P + 0.05 \qquad \text{(Equation 2)}$$

For ionisable compounds, apparent solubility is significantly altered by media pH because the ionised species are orders of magnitude more soluble than neutral species (Stuart and Box, 2005). The solubility equation of monoacidic or monobasic compounds can be calculated from Henderson-Hasselbalch equation:

$$\text{Monoacid: } S_a = S_0 (1+10^{pH-pKa}) \qquad \text{(Equation 3)}$$
$$\text{Monobase: } S_a = S_0 (1+10^{pKa-pH})$$

Where S_0 refers to the intrinsic solubility of the compound and S_a refers to the apparent solubility at a certain pH (Dressman *et al.*, 2007). A monoacid for example, when pH = pK_a, 50% of the compound is ionised, and this number increased to 90% and 99% with pH increase 1 and 2 above pK_a. Thus a small variation of media pH, when close to drug's pK_a, can induce a large solubility variation.

Accurate measurement of solubility profile relies on experimental approaches such as the classic shake-flask method and the potentiometric titration. Potentiometric titration has been used in semi-automated instrument to measure solubility profile in the whole pH range (Glomme *et al.*, 2005), while shake-flask only provides a determination for one pH at a time, yet generating highly reliable thermodynamic data (Avdeef, 1998). In addition, the chasing equilibrium solubility (CheqSol) technique has been deployed to measure intrinsic solubility of ionisable compounds. An appropriate amount of solid samples is first introduced at an acidic or basic pH where the sample is fully ionised and dissolved. The solution is then back titrated until sample becomes unionised and precipitates out. The assay continues by

adding acid or base to actively change the pH around drug's pK_a, which forces the neutral form to cycle between subsaturation and supersaturation, repeatedly crossing the solubility equilibrium point several times. The intrinsic solubility is determined at the equilibrium points where the rate of pH changing is zero. Therefore, CheqSol reduces the time for each measurement and provides reproducible intrinsic solubility of weak acids, bases and ampholytes (Stuart and Box, 2005).

1.3 Dissolution

The BA of the orally administered drugs relies on the release of API from the dosage form if in a formulation and the solubilisation of API into the GI tract (Guidance for industry, 1997a). This is summarised as the dissolution properties of the drugs. It has been long recognised that dissolution rate can affect the absorption rate and BA if permeation from the GI tract is rapid (Edwards, 1951). *In vitro* GI dissolution test is a sensitive, effective and straightforward tool to assess the batch-to-batch quality of drug products, predict absorption of drugs among various formulations and IVIVC (Dressman and Krämer, 2005). Because of the importance of dissolution, guidance has been established for development of dissolution tests and regulatory application mainly for marketed drugs. For the quality control purposes, an example of dissolution test employs a dissolution medium of 1000 ml pH 6.8 phosphate buffer in a paddle or basket apparatus (British Pharmacopoeia *et al.*, 2001, European Pharmacopoeia, 2001, United States Pharmacopoeia, 2011).

Since oral drug BA is closely related with dissolution of the API into the biological fluids *in vivo* (Dressman *et al.*, 2008), the *in vitro* - *in vivo* correlation (IVIVC) Guidance was introduced since 1997 (Guidance for industry, 1997b). The *in vitro* dissolution has merged from a traditional quality control test to a surrogate of *in vivo* bioequivalence (BE) test, which can be used for biowaivers and applied

together with Biopharmaceutics Classification Systems (BCS) (Food and Drug Administration, 2000, European Medicines Agency, 2010, World Health Organization, 2011). The development of reliable IVIVC can significantly reduce the time and cost of animal and clinical studies, increase product quality, and to identify drug formulations with desired BA (Dressman *et al.*, 1998). However, current dissolution media used in guidance and pharmacopoeias has not incorporated all GI fluid physiological parameters. The research group of Dressman and Reppas has initiated the development of using more biorelevant dissolution media such as simulated gastric fluid (SGF), fasted simulated intestinal fluid (FaSSIF) and fed simulated intestinal fluid (FeSSIF) (Galia *et al.*, 1998) and developed to different media recipes (Table 1.1) (Jantratid *et al.*, 2008, Kleberg *et al.*, 2010, Psachoulias *et al.*, 2012, Fuchs *et al.*, 2015). Those dissolution media are mainly used for drug development proposes and provide better prediction of *in vivo* absorption to select candidate drugs (Dressman and Krämer, 2005).

At the early stage of drug development, when sample amounts are limited, miniaturised version of rotating disk and powder dissolution apparatuses are more suitable to gain knowledge of solubility and dissolution of the pure API. Those apparatuses have been utilised and monitored in real time by using *in situ* UV dip-probe and Raman spectroscopy. Automated platforms such as Sirius T3, µDISS Profiler™ and Sirius SDI (UV surface imaging system) were used to facilitate the control of pH, stirring speed or temperature (Avdeef and Tsinman, 2008, Tsinman *et al.*, 2009, Fagerberg *et al.*, 2010, Qiao *et al.*, 2013). Recently, increasing evidence of solid state transformation during dissolution has been reported, applying together with visualisation techniques such as *in situ* Raman analysis (Savolainen *et al.*, 2009). These techniques made it possible for early stage dissolution studies utilising FaSSIF and FeSSIF with limited available compounds and providing good correlation with data in human intestinal fluid (HIF) (Persson *et al.*, 2005).

However, analysis relies on clear dissolution media for accurate measurement of the UV signals, which can cope with commercialised FaSSIF/FeSSIF media and use UV signal derivative calculation to minimise interference (Fagerberg *et al.*, 2010). However human GI fluids can be more turbid with varying levels of the GI contents such as endogenous amphiphiles and food digestive products, considering the individual variation of GI physiology and different prandial stages (Riethorst *et al.*, 2015).

1.3.1 Parameters affecting dissolution rates

Noyes and Whitney were the first to determine that the dissolution rate (dC/dt) is proportional to the difference between current concentration (C) and the saturated solubility (C_s), through a diffusion layer around the dissolving materials to the bulk solution (Noyes and Whitney, 1897). D is the diffusion coefficient of the solute in the diffusion layer. This theory was further developed by Bruner and Tolloczko (Bruner and Tolloczko, 1900) and later Nernst (Nernst, 1904), accommodating the solid surface area (A), the diffusion layer thickness (h) and the dissolution medium volume (V). The modified Noyes-Whitney equation is described below:

$$\frac{dC}{dt} = \frac{A * D}{V * h}(C_s - C) \qquad \text{(Equation 4)}$$

Saturated solubility

The gradient concentration across the boundary layer is the major driving force for dissolution. According to Equation 4, the gradient concentration is linearly correlated with drug's dissolution rate, provided same surface area and dissolution medium (volume, diffusion layer thickness and diffusion coefficient are constant). The predominant factors to increase the dissolution rate of insoluble drugs are the media solubilisation capabilities with the presence of endogenous amphiphiles in GI tract that affect saturated solubility (Porter and Charman, 2001). In a dissolution

test measured in a non-saturated “sink condition”, the media volume should be at least 3 - 10 times more than the volume for drug saturation ($C<<C_s$) (British Pharmacopoeia *et al.*, 2001), therefore the dissolution rate is closely related with drug solubility (C_s). Additionally, for ionisable compounds and salt forms, the diffusion layer can also create a pH gradient and according to Henderson-Hasselbalch equation (Equation 3), this in turn can significantly influence the solubility of the compounds and alter the gradient concentration.

Surface area & wetting

Particle size reduction can increase the surface area, thus affect the dissolution rate. However, techniques such as micronisation can also cause particle agglomeration. Therefore, effective surface area is actually determined by the wetting of compounds in the dissolution media (Weintraub and Gibaldi, 1969). Wetting means that the liquid media maintain contact with the solid drug, with contact angle approaches to zero. Good wetting is achieved when both liquid surface tension and solid-liquid interfacial tension are small (Adamson and Gast, 1967). This ideally can be accomplished by endogenous amphiphiles in GI tract which can reduce the interfacial tension. Studies have shown that both media composition and amphiphile concentration can affect surface tension and wetting activity. Surface tension is lower in fed than fasted state due to a higher amphiphile and lipid concentration. Interestingly, pH can also affect the wetting by changing the ionisation of free fatty acids (FFA) in the dissolution media (Luner and Kamp, 2001), which emphasises the importance of considering both pH and amphiphiles in biorelevant media when simulating the wetting properties of GI fluids.

Diffusion coefficient

The Stokes-Einstein equation (Equation 5) explains what factors influence diffusion coefficient, where D is the diffusion coefficient, k is the Boltzmann constant, T is the

absolute temperature, η is the diffusion medium viscosity and r is the radius of the drug molecule (Dresse *et al.*, 1978).

$$D = \frac{k \cdot T}{6\pi \cdot \eta \cdot r} \qquad \text{(Equation 5)}$$

Both k and T are constant at body temperature, thus D depends on the viscosity of the diffusion layer and the molecular size of the solvate. Viscosity is inversely proportional to the drug diffusion coefficient thus the dissolution rate (Braun and Parrott, 1972). While the drug molecules are solubilised by secretion of biorelevant amphiphiles and the food intake, which then facilitate dissolution, high concentration of these contents may also increase viscosity, therefore decreases diffusion coefficient. The diffusion coefficient (D) also decreases due to large size micelle formation and is further reduced in mixed micelles. For example, diffusion coefficient of hydrocortisone in a mixture of 15 mM sodium taurocholate (NaTC) and 3.75 mM lecithin is only $2x10^{-8}$ cm^2/s, 100-fold smaller than in solution with only NaTC (Naylor *et al.*, 1993). A combination result of increased solubilisation capabilities and decreased diffusion coefficient introduced by amphiphiles is that the dissolution rate does not change to the same extent as solubility.

Drug lipophilicity

Drug lipophilicity can affect the equilibrium of free drug and drug partitioned in micelles in dissolution media, therefore changing the drug solubility and diffusion coefficient, both of which can influence dissolution (Persson *et al.*, 2005, Gamsiz *et al.*, 2010). The dissolution of danazol was mainly mediated through enhanced solubilisation by NaTC, while the solubilisation and diffusivity change were minimal for a series of steroids (log P 1.01 - 1.94) at NaTC concentration up to 30 mM, since they were hardly incorporated into micelles (Bakatselou *et al.*, 1991). At low NaTC concentration covering the typical range of fasted GI fluid, dissolution rate is mainly enhanced by the wetting of NaTC, whilst in the fed state, it can also benefit from

increased solubilisation capabilities of micelles, which is advantageous to more hydrophobic compounds.

1.4 Permeation

Oral drugs have to permeate the cellular membrane to reach the systemic circulation. Drug permeation can occur through several routes: transcellular, paracellular, endocytosis and transporter mediated efflux (Figure 1.1). Permeation of most drugs is driven by passive transcellular or paracellular transport. Drug molecular properties such as lipophilicity, polar surface area (PSA) and number of hydrogen bond donors and acceptors are important factors to affect passive transport, as this usually involves lipid bilayer membrane partition (Lipinski *et al.*, 2012). Caco-2 cell or artificial membranes are commonly used to assess the drug apparent permeability (P_{app}) from a donor chamber to a receiver chamber (Dressman and Reppas, 2016). Alternatively, *in situ* rat jejunal perfusion assays can be used to calculate effective permeability (P_{eff}) and results indicated good correlation with human *in vivo* permeability for passively absorbed compounds (Fagerholm *et al.*, 1996).

The drug P_{app} (unit cm/s) is calculated according to the following equation, where V is the volume in the donor chamber; (dC/dt) is the concentration transported per unit time in the receiver chamber; A is the surface area of the permeation surface (i.e. tissue, monolayer or membrane); C_0 is the starting drug concentration in the donor chamber (Rubas *et al.*, 1993).

$$P = \frac{V \cdot dC}{A \cdot C_0 \cdot dt} \qquad \text{(Equation 6)}$$

Positive correlation can be seen between permeability and percentage of drug absorbed in human. Drugs with high permeability are correlated with more than 85% of oral absorption (European Medicines Agency, 2010). Permeability > $1x10^{-6}$

cm/s was reported in an unstirred system using Caco-2 (Artursson and Karlsson, 1991), while in stirred system the value is approximately one order of magnitude higher (~ $10x10^{-6}$ cm/s) (Rubas *et al.*, 1993, Pade and Stavchansky, 1998). The P_{eff} is calculated from the solute concentration difference between entering and leaving the cannulated jejunal region (Volpe, 2010).

1.5 Biopharmaceutics Classification System

1.5.1 Early Biopharmaceutics Classification System

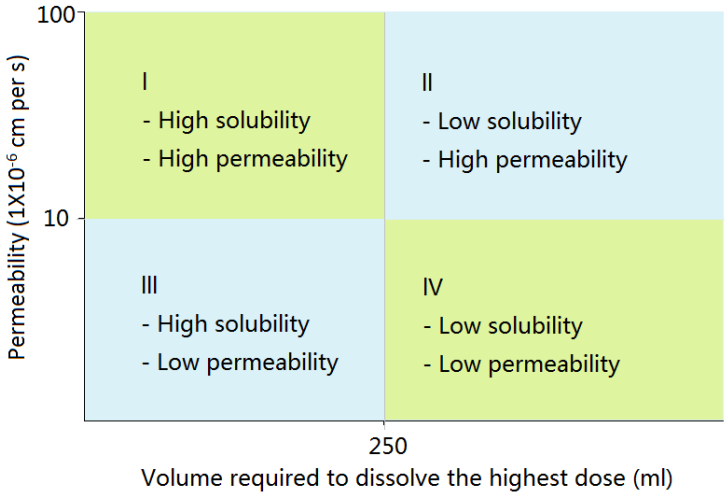


Figure 1. 2 Biopharmaceutical classification systems (BCS).
A drug is considered highly soluble when dose to solubility ratio is over 250 ml water at the pH of 1 - 7.5 at 37 ^{O}C, while a permeable drug can reach ≥ 85% of oral BA by *in vivo* data, with permeability > $10x10^{-6}$ cm/s. Adapted from (Rautio *et al.*, 2008, European Medicines Agency, 2010).

Dressman *et al.* (1998) summarised the rate-limiting steps affecting oral drug absorption from solid dosage form:

i. **Dissolution limitation**: the drug is not released from its formulation and dissolved into solution form in a limited period in the well-absorbed sites of GI tract.
ii. **Permeability limitation**: the drug cannot permeate through the gastric mucosa and intestinal wall effectively after it dissolves in solution form.

iii. **Stability**: the drug is not stable in the GI tract or it forms a complex which cannot be absorbed.

iv. **First pass metabolism**: the drug is metabolised or eliminated by intestine or liver before reaching the blood circulation system.

Both the available drug intraluminal concentration and permeability determine rate of dissolution and absorption at the specific location. The Biopharmaceutics Classification System (BCS) (Fig. 1.2) was introduced in the 1990s to place drugs into four categories, based on dissolution and permeability limitation factors (Amidon *et al.*, 1995a). According to the FDA guidelines, solubility and permeability constitute the two criteria to classify a drug in BCS (FDA, 2016). Particularly, drug solubility takes account of the drug dose and calculates the minimum volume required to dissolve the dose, therefore comparable for drugs with different doses. The drug solubility is defined as high when the maximum oral dose is soluble in 250 ml water at 37 ^{O}C at pH 1 - 7.5. High permeability leads to 85% or more of the drug administered dose being absorbed in GI tract, a literature boundary value is > $10x10^{-6}$ cm/s for high permeability from *in vitro* test (Pade and Stavchansky, 1998).

1.5.2 Biorelevant and holistic consideration of solubility, dissolution and permeation

Poor solubility is the major obstacle for efficient drug dissolution and absorption. With the simplicity of BCS, drug solubility is considered as a function of pH in aqueous buffer, yet many GI parameters are not accurately simulated in the testing media, such as endogenous bile, lipids, enzymes and food contents. These parameters can largely affect solubility and dissolution of insoluble, lipophilic drugs (i.e. BCS II and IV drugs) and therefore important in *in vitro* tests to assess oral absorption and forecast food effects (Dressman and Reppas, 2000). Therefore, biorelevant media such as SGF, FaSSIF and FeSSIF were designed based on

physiological data such as pH, osmolality and bile contents and introduced in dissolution test of API and formulations (Galia et al., 1998, Dressman et al., 1998, Vertzoni et al., 2004a, Jantratid et al., 2008). Fagerberg *et al.* investigated ten BCS II compounds and observed solubility increased when biorelevant media were employed, with some of the drugs shifted to BCS I (Figure 1.3) (Fagerberg *et al.*, 2010). Those drugs tend to be in low oral dose and "grease ball" molecules, which possess the characteristics of moderate to high lipophilicity, high molecular weight (MW) and high molecular flexibility (Fagerberg *et al.*, 2010, Zaki *et al.*, 2010a).

	FaSSIF blank	FeSSIF blank	FaSSIF	FeSSIF
BCS I		Astermizole Carvedilol	Astermizole Indomethacin Tamoxifen	Aastermizole Carvedilol Felodipine Tamoxifen
BCS II	Albendazole Astermizole Carvedilol Cinnarizine Danazol Felodipine Glybyride Indomethacin Tamoxifen Tolfenamic acid	Albendazole Cinnarizine Danazol Felodipine Glybyride Indomethacin Tamoxifen Tolfenamic acid	Albendazole Carvedilol Cinnarizine Danazol Felodipine Glybyride Tolfenamic acid	Albendazole Cinnarizine Danazol Glybyride Indomethacin Tolfenamic acid

Figure 1. 3 Modified BCS of ten BCS II compounds in FaSSIF, FeSSIF and their corresponding blank buffer.

FaSSIF blank and FeSSIF blank were phosphate buffer with pH adjusted to 6.5 and 5 respectively. For FaSSIF, FeSSIF composition, see Table 1.1. Solubility was obtained experimentally and permeability was taken from literature (Fagerberg *et al.*, 2010).

Biorelevant media potentially increase the solubility of drugs, which is primarily related to the concentration of dietary lipids and bile salts, which can consequently

affect their dissolution rates *in vitro*. For BCS I drugs, the API dissolution profiles were similar in simple buffer and biorelevant media due to high solubility even in aqueous media (Galia *et al.*, 1998). For formulated drug, rapid and complete dissolution was also possible with fast disintegration and well dispersed API particles by using aqueous buffer (Galia *et al.*, 1998). Whilst for BCS II drugs, dissolution becomes the rate limiting step due to the poor solubility, thus biorelevant media such as FaSSIF and FeSSIF were more suitable to assess their dissolution (Galia *et al.*, 1998). According to Noyes-Whitney equation, there is a linear relationship between solubility and dissolution under "sink conditions" (Equation 4). Solubility and dissolution can both be increased in biorelevant media compared with aqueous buffer, however the experimental data showed they varied to different extent (Horter and Dressman, 2001, Kossena *et al.*, 2004, Persson *et al.*, 2005). One reason is the diffusion coefficient changes with free drug and drug partitioned in micelles, the latter can be the predominant solubilised form in these media for lipophilic drugs. The ratio of each form relies on the composition of the micelles (Naylor *et al.*, 1995) and drug lipophilicity, therefore making the real scenario more complicated than the standard Noyes-Whitney equation. Another reason is inadequate simulation of the GI flow pattern which allows for efficient mixing and wetting by using instruments *in vitro* (Fagerberg *et al.*, 2010). Further studies are required to improve simulation of these physiological conditions to better understand drug absorption and build IVIVC.

High permeability can minimise the negative effect of low solubility as permeation allows removal of drugs from GI and facilitate further dissolution (Fagerholm, 2007). For example, BCS II acidic drugs with low gastric solubility, can still exhibit high absorption in the intestine and associated increased BA *in vivo*, due to both increased solubility at intestinal pH and high permeability (Yazdanian *et al.*, 2004, Fagerholm and Bjornsson, 2005, Potthast *et al.*, 2005). Moreover, a lipophilic drug,

with high permeability, can have a greater BA in fed state with an oily meal (Castro *et al.*, 2000, Zimmermann *et al.*, 1994). Recently, incorporating simultaneous dissolution and permeability apparatus in combination with biorelevant media has become a new strategy to improve IVIVC (Kobayashi *et al.*, 2001, Kataoka *et al.*, 2003), which enables the dissolution evaluation dynamically with the absorption. The dissolution/permeation (D/P) system consists of two chambers connected by either a pump or a Caco-2 cell monolayer. The data obtained from *in vitro* D/P of six fenofibrate formulations were used to calculate predicted absorption, which correlated well with the *in vivo* plasma concentration area under the curve (AUC) in rats using both fasted and fed SIF, which indicated the potential of D/P system to holistically evaluate formulation and food effects on both dissolution and permeation (Buch *et al.*, 2009). Kataoka *et al.* used the D/P system to define a few water insoluble drugs in terms of their solubility or dissolution limitation. The system identified the oral absorption rate limiting step related with solubility- or dissolution-limited. In addition it demonstrated provided that the drug has sufficient permeability, the F_a would not be influenced by the limited solubility and became dose-dependent (Kataoka et al., 2013).

Biorelevant media can increase or decrease the permeability of poorly soluble drugs (Kleberg *et al.*, 2010, Lind *et al.*, 2007, Patel *et al.*, 2006, Miller *et al.*, 2011). If the solubility is simply enhanced by ionisation in the physiological pH, the ionised molecules are less lipophilic thus less permeable than neutral compounds through passive diffusion in epithelium (Kerns and Di, 2008). Palm *et al.* evaluated the permeability coefficient of two weak bases in Caco-2 cells, and demonstrated that the unionised form can transport 150-fold (alfentanil) and 30-fold (cimetidine) quicker than ionised form. However, the contribution of ionised form in drug permeation through intestinal epithelium was not negligible when this form was more than 90%. This included but not limited to paracelluar pathway (Palm *et al.*, 1999). NaTC can reduce the absorption rate of lipophilic drugs griseofulvin and

ketoconazole. The reduction is correlated with the decreased free drug fraction in the solution when NaTC concentration is above critical micelle concentration (CMC) (Poelma *et al.*, 1990), and the solubility-permeability interplay introduced is a trade-off between solubility increase and permeability decrease by amphiphiles (Miller *et al.*, 2011). Based on these examples above, the application of GI biorelevant media is essential to comprehensively understand the inter-correlation of solubility, dissolution, permeability and integrate them into IVIVC.

1.5.3 Developability Classification System

Recently, a Developability Classification System (DCS) (Figure 1.4) was developed from the BCS based on the rate limiting factors of oral absorption (Butler and Dressman, 2010). The volume used to dissolve the maximum dose was extended from 250 to 500 ml, which was a compensation for the pH-dependent solubility and the fluid contribution from GI secretion and food intake. Additionally, BCS II was divided into two groups: the dissolution-rate limited (IIa) and the solubility limited drugs (IIb). Provided that the high permeability are compensatory for low solubility, the main issue for formulation development is to identify whether it is the particle size and wetting for improved dissolution or a specialised solubilisation form is required (Butler and Dressman, 2010). This system tries to further explore the risks for *in vivo* drug performance and define strategies for formulation optimisation. Another sub-classification of BCS was proposed to divide BCS II and IV into acid, base and neutral groups according to their pK_a, addressing the ionisation-induced solubility/dissolution changes and potential effects to absorption/supersaturation in stomach and intestine. This classification aims to provide guidance on *in vivo* predictive dissolution tools to establish quality by design (QbD) specifications and IVIVC (Tsume *et al.*, 2014).

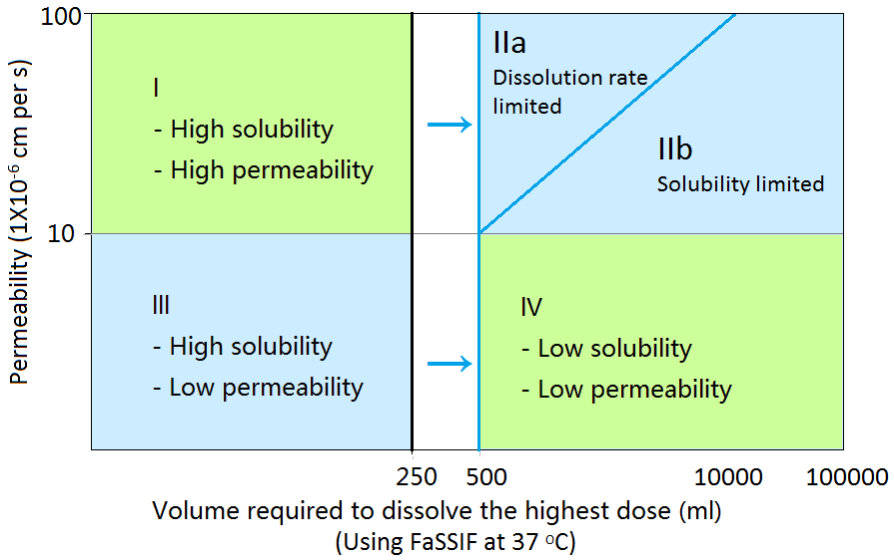


Figure 1. 4 Developability Classification System (DCS)

The transit time of a drug formulation from the stomach to the proximal small intestine is around 2 - 3 h with meals, while it can take more than 20 hours to pass through the colon (Washington *et al.*, 2001). Due to the pH changes along the GI tract, the favourable adsorption sites vary according to the properties of the drugs and for poorly soluble neutral and acidic drugs, a pH >5 (duodenum pH) is critical to evaluate the solubility as the small intestine is the main adsorption region (Yazdanian *et al.*, 2004). Poorly soluble weak basic drugs (BCS IIb and IV) would have a higher dependency on the pH and residence time in stomach due to their improved solubility at low pH. However, information on solubility within the intestinal pH range may still be very useful considering the retention time in stomach is comparatively shorter than in intestine even after food intake (Dressman *et al.*, 1998), and also indicative to forecast degree of supersaturation and precipitation that might occur in the intestine. Therefore using main absorption sites to address the solubility information and place drug in the DCS would be helpful to identify developability issues and choose formulation strategies. The transit time should be tailored according to the diet conditions and site specific active transportation of drugs in GI tract (Butler and Dressman, 2010).

1.5.4 Biowaivers

BCS provides guidelines to improve the drug development process with approval of the efficacy and the safety of the drug based on *in vitro* dissolution data to establish BE, reducing the expenditure for assessing *in vivo* BE. These drugs are termed the biowaivers. FDA first exempts BCS I drugs as BCS-based biowaivers and the drugs in IR (immediate release) formulations have to perform rapid dissolution (> 85% of the drug dissolved in 30 min in 900 ml buffer solutions) (Guidance for industry, 2000). The European Medicines Agency (EMA) in 2008, the World Health Organization (WHO) in 2015 and the FDA guidelines in 2015 harmonised and extended the biowaiver to the BCS III drugs in IR formulations with very rapid dissolution (> 85% of the drug dissolved in 15 min) (Committee for Medicinal Products for Human Use, 2010, Guidance for industry, 2015, World Health, 2015), similar to BCS I drugs, BCS III drugs have high solubility, thus dissolution is usually not the rate limiting step for absorption. However, biowaivers are not applicable to drugs with narrow therapeutic window and the effect of the excipients on the drug adsorption must be well recognised. For BCS III drugs, the excipients have to be quantitatively and qualitatively the same (Davit *et al.*, 2016). These criteria are still conservative and suggestions to revise BCS boundaries and extend biowaiver categories to some BCS II drugs have been proposed in several studies (Yu *et al.*, 2002, Tubic-Grozdanis *et al.*, 2008, Tsume *et al.*, 2012). Fagerholm also suggested that the rate-limiting step should be identified and the BCS could be reduced to two categories: permeation-rate (dissolution kinetics > permeation kinetics *in vivo*) and dissolution-rate limited absorption (dissolution kinetics < permeation kinetics *in vivo*), with the former class suitable for biowaivers (Fagerholm, 2007).

The extension of biowaivers to BCS III was discussed in several papers (Yu *et al.*, 2002, Vogelpoel *et al.*, 2004, Kortejarvi *et al.*, 2007), which include the redefining of permeability from high (currently 85%) to lower values, because even though permeability is the rate limiting step for BCS III drugs, it is less critical when

combined with a slow elimination rate. In the modelling examples, highly permeable drugs in two formulations leading to rapid and slow dissolution rates, the maximum drug concentration in plasma (C_{max}) varies significantly with drugs of a very high permeability (10 x 10^{-4} cm/s) and a fast elimination (1/2 h^{-1}), while with drugs of a high permeability (1.5 x 10^{-4} cm/s) and a slow elimination (1/20 h^{-1}), the pharmacokinetic maintained consistent. Therefore, ignoring individual elimination rate may restrict the solubility and dissolution criteria of BE and biowaiver justification (Fagerholm, 2007). Kortejarvi *et al.* used STELLA® compartmental models to simulate the adsorption of solid BCS I and BCS II drugs. They used a series of elimination rates (0.014 – 0.9 h^{-1}) and absorption rates (0.1 – 8 h^{-1}) in GI tract in the simulation and compared the C_{max} and AUC of different formulations with the data generated from oral solution. Results showed that BCS III drugs may be better qualified for biowaivers since passive permeability is the rate-limiting step of absorption, and they are less sensitive to changes of dissolution rate, gastric emptying rate and elimination rate, while BCS I drugs with fast absorption and elimination rate failed in BE studies with 10 - 25 % difference in C_{max} and AUC (Kortejarvi *et al.*, 2007).

1.6 Design of simulated gastrointestinal fluids

The earliest dissolution tests utilised water and surfactants such as sodium lauryl sulfate (SLS) to improve dissolution of poorly water soluble drugs (Shah *et al.*, 1989). Buffer and 0.1 M hydrochloric acid were also used to provide a pH simulating gastric and intestinal pH (Cohen *et al.*, 1990, Stolk *et al.*, 1990, Gray and Dressman, 1996). However, *in vivo* dissolution media have more various and complex composition including bile salts, electrolytes and a wide range of lipids. The simulated gastric and intestinal fluids were proposed, based on *in vivo* data of a range of GI factors at fasted and fed states (Dressman et al., 1998, Galia et al., 1998, Vertzoni et al., 2004a, Jantratid et al., 2008). This section summarises the characteristics of GI fluids from

human samples and development of biorelevant media of the following aspect: pH, bile salt, phospholipid, lipids and osmolality. Table 1.1 summarises some commonly used biorelevant media in the literature. Table 1.2 summarises the level of parameters in human gastric fluids (HGF), duodenum and jejunum fluids under fasted and fed states and details are described in the following sections.

1.6.1 pH

Gastric fluid pH

The gastric pH is below 3 in fasted state and a range of 1.7 - 3 is representative of most conditions (Lindahl *et al.*, 1997, Pedersen *et al.*, 2000b, Kalantzi *et al.*, 2006a, Pedersen *et al.*, 2013), but variability can be very high (pH 1.2 - 7.4) (Kalantzi *et al.*, 2006a). A higher pH can be observed with patients who have hypochlorhydria, under antacid therapy or in geriatric groups who are less capable of producing gastric acid (Dressman *et al.*, 2007). In a fed state, the digested food buffers the gastric fluids to a less acidic state with typical pH in the range from 3 to 7. With the secretion of more acid, the pH level returns to the fasted value within 2 to 3 h, depending on the meal size. Thus only the drugs taken immediately after the meal will be affected by the increased gastric pH (Dressman *et al.*, 1998). The weak basic drug dipyridamole (pK_a 6.4) became less soluble and had a slower absorption, with 29% reduction of C_{max} after food intake. However, it still had a 12% increase of AUC due to the compensatory longer gastric residence time (Kostewicz *et al.*, 2002).

Intestinal fluid pH

In the fasted state, a pH value of 5.6 - 7.0 is typical for the upper small intestine (duodenum) and the pH in the proximal (pH 6.5 - 7.8 jejunum) and distal regions (pH 6.5 - 8.0 ileum) is slightly higher than neutral (Kalantzi *et al.*, 2006a, Dressman *et al.*, 2007, Clarysse *et al.*, 2009b, Bergstrom *et al.*, 2014). Large individual variability

was observed both inter-subject and intra-subject, with the lowest pH found to be 3 - 4.5, and highest close to 8 (Kalantzi *et al.*, 2006a, Clarysse *et al.*, 2009a). In the fed state, pH is relatively lower in the upper small intestine than distal as it is more affected by the chyme from stomach, while the pH in the distal part appears to remain stable at pH 7 - 7.5 (Dressman *et al.*, 1998). Compared with fasted state, pH is less variable and this stability could last around 2 h (Clarysse *et al.*, 2009a). Figure 1.5 illustratively indicates the pH change with time and intra-subject variability.

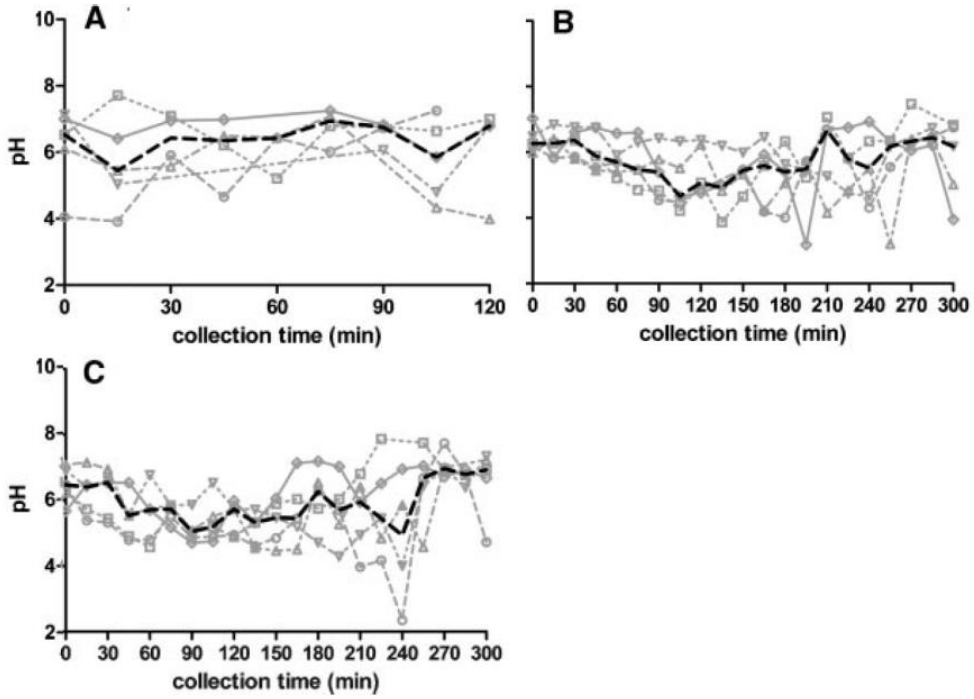


Figure 1. 5 Individual (grey dash) and median (dark dash) pH in duodenum as a function of time in fasted (A), fed (B), and fat-enriched fed (C) state HIF for five healthy subjects (Clarysse *et al.*, 2009a).

1.6.2 Buffer

A biorelevant fluid should provide the physiological pH and buffer capacity. Dissolution of ionisable drug can alter the bulk media pH and the pH of the solid API boundary layer, both of which depend on the buffer capacity of the media and apparent solubility of the drug, hence the dissolution will be influenced in a weakly

buffered simulated fluid (Horter and Dressman, 2001). Most researchers choose phosphate as the buffer, while a few used maleate (Jantratid *et al.*, 2008, Marques *et al.*, 2011). Maleic acid can slow down the rancidity of fats and oils (Jantratid *et al.*, 2008, Zughaid *et al.*, 2012). However, maleate was reported to potentially accelerate the oxidation of the drug troglitazone (Vertzoni et al., 2004b). Buffer anion may affect the solubility and dissolution profiles of the drug due to the different salting in and salting out properties of the anions. Salting out anions decrease the solubility of nonpolar molecules by dehydration at the interface, whilst salting in anions help solvate molecules (Leontidis, 2002). Therefore, the choice of buffer system becomes important especially for highly lipophilic drugs or formulation with high hydration demand (Vertzoni et al., 2004b). On the other hand, maleic acid can interfere in high performance liquid chromatography (HPLC) analysis if drug has a short retention time and low detection wavelength, while for phosphate buffer, this is not a problem. Nevertheless, current studies have observed no significant buffer effects towards solubility of a series of neutral and ionisable poorly soluble drugs (Ottaviani *et al.*, 2010, Fuchs *et al.*, 2015).

Maleic acid has a pK_{a2} = 6.27 (pK_{a1} = 1.92, irrelevant for buffering at intestinal pH), whilst phosphoric acid has a pK_{a2} = 7.21 (pK_{a1} = 2.15, pK_{a3} = 12.32). Therefore maleic acid optimally falls in the physiological intestinal pH (5 - 7) in a concentration to reach required buffer capacity without exceeding osmolality (Jantratid *et al.*, 2008). The osmolality and buffer capacity of phosphate buffer in FaSSIF were 270 mOsm/kg and 12 mmol/L·ΔpH, and for maleic buffer in FaSSIF-V2 were 180 mOsm/kg and 10 mmol/L·ΔpH (Vertzoni et al., 2004a, Jantratid et al., 2008). However, experimental buffer capacity of FaSSIF were 16.4 mmol/L·ΔpH, slightly higher than the fasted duodenum HIF buffer capacity (4 - 13 mmol/L·ΔpH) (Moreno *et al.*, 2006), and another reported jejunum value 2.4 – 2.8 mmol/L·ΔpH (Persson *et al.*, 2005). This measured buffer capacity might be underestimated due to the loss

of carbon dioxide (CO_2) in extracted HIF. Literature on *in situ* measurement of CO_2 concentration in small intestine is rare. An early *in situ* study reported hydrogen carbonate (HCO_3^-) concentration in fasted duodenum was 4 - 21 mM, equivalent to buffer capacity of 2 - 12 mmol/L·ΔpH at pH 6.8 (Karr and Abbott, 1935). It should be mentioned that buffer capacity depends on both the pH and concentration of the buffer systems and reaches the highest when local pH equals buffer pK_a, thus both local pH and individual CO_2 concentration can cause the variability (Figure 1.6).

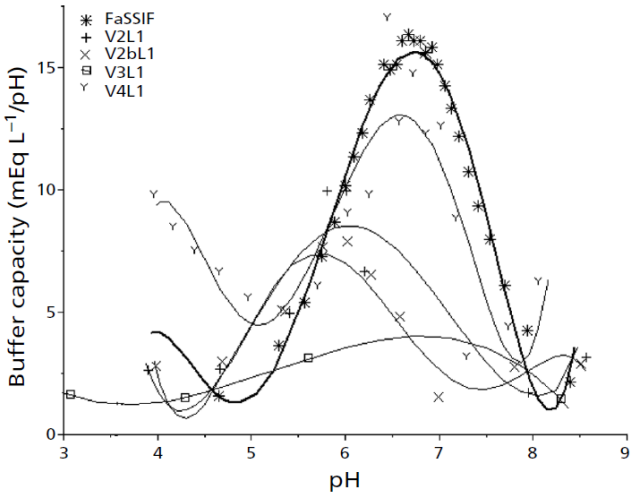


Figure 1. 6 Experimental data of buffer capacity versus pH (Galia *et al.*, 1998). V2L1, V2bL1, V3L1, V4L1 were HIF samples from different volunteers (Moreno *et al.*, 2006).

The biological bicarbonate buffer is a dynamic equilibrium of carbonic acid, hydrogen carbonate, dissolved CO_2 and the ambient pressure of CO_2, thus the multi-step equilibrium and thermodynamic instability make it difficult to simulate *in vitro*. Biorelevant media utilising bicarbonate buffer was reported recently in dissolution studies of BCS II weak acids and bases (Krieg *et al.*, 2015). Results indicated the difference of intrinsic dissolution rate (IDR) between systems using phosphate buffer and bicarbonate, mainly related to the drug's pK_a and intrinsic solubility (Sheng *et al.*, 2009). Another study investigated different dissolution profiles of various mesalazine in enteric coating formulations using phosphate and bicarbonate buffer, and dissolution profiles in bicarbonate system provided improved correlation with pharmacokinetic data *in vivo* (Fadda *et al.*, 2009). It is

advantageous yet time-consuming to use bicarbonate buffer in combination with simulated GI fluids in the United States Pharmacopeial (USP) apparatus with additional CO_2 pumping system (i.e. Auto pH System™ and pHysio-stat®) (Merchant *et al.*, 2014, Garbacz *et al.*, 2014); additionally, IVIVC and effect of hydrodynamics changes (gas bubble, instrument insert) still need to be verified. In a fed state, food digestion also plays an important role in pH adjusting, using bicarbonate system alone may not meet the buffer capacity required, making the situation more convoluted to model on a physiological basis. The current practical solution is employing an appropriate buffer of less interference with drug dissolution and good reproducibility, or alternatively monitoring and adjusting pH by adding acid/base in an automated system to achieve and maintain the required biorelevant pH.

1.6.3 Amphiphiles and digestion products

Bile salts

Bile salts (BS, abbreviation refers to all compounds from human bile salt category) have a hydrophobic rigid steroidal skeleton and a hydrophilic polar side composed of hydrogen bonds and an ionisable amine tail, which allows them to form mixed micelles and play crucial part to the solubilisation and absorption of dietary lipids *in vivo* (Hjelm *et al.*, 2000). Human bile is a combination of several bile salts (Figure 1.7), differentiated by a combination of their amine conjugation (taurocholate and glycholate) and hydroxylation status, both of which increase polarity. BS can be divided into three groups: the trihydroxy conjugated (e.g. taurocholate, glycocholate), the dihydroxy conjugated (e.g. glycodeoxycholate, taurodeoxycholate) and the unconjugated (e.g. deoxycholate, cholate) (Hofmann and Mysels, 1987).

For economic consideration, most of the *in vitro* dissolution and solubility studies were conducted using a single BS species, commonly TC (Galia et al., 1998, Vertzoni et al., 2004a, Kleberg et al., 2010), even though two thirds of the BS are conjugated

with glycine (Wiedmann and Kamel, 2002). The pK_a of TC and GC are 1.5 and 3.7 respectively, hence in the small intestine volume and pH, the salt form NaTC is less likely to precipitate (Kleberg *et al.*, 2010). In addition, application of pure BS products is suggested than crude BS for convenient standardisation of BS amount (Vertzoni et al., 2004b).

BS	R1	R2	R3
Cholic acid	OH	OH	OH
Chenodeoxycholic acid	OH	H	OH
Deoxycholic acid	H	OH	OH
Glycocholate (GC)	OH	OH	$NHCH_2COO^-$
Taurocholate (TC)	OH	OH	$NHCH_2CH_2SO_3^-$
Glycodeoxycholate	H	OH	$NHCH_2COO^-$
Taurodeoxycholate	H	OH	$NHCH_2CH_2SO_3^-$

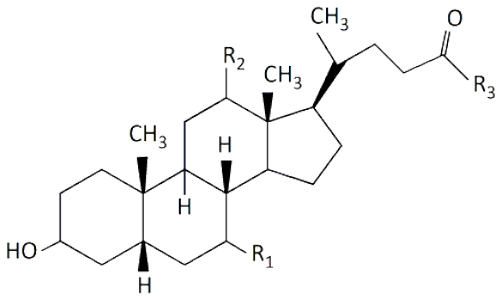


Figure 1. 7 Chemical structures of various BS

BS can remarkably enhance the solubility of a range of poorly soluble drugs (Pedersen *et al.*, 2000a, Zughaid *et al.*, 2012). Reports showed that changing the combination of two or more trihydroxy BS had less influence on drug solubility compared with factors such as the concentration of BS, lecithin and pH (Soderlind *et al.*, 2010, Zughaid *et al.*, 2012). For example, no significant difference was found between TC and GC to affect the solubility of hydrocortisone (Pedersen *et al.*, 2000a) and eight insoluble neutral compounds (Söderlind *et al.*, 2010) at both fasted and fed state concentration. Although conjugated amino acid has little effect on drug

solubility, the hydroxylation of the BS may make minimal differences on the solubilisation (Wiedmann and Kamel, 2002, Soderlind *et al.*, 2010). Difference of drug-bile interaction existed between non-conjugated and conjugated BS for negatively charged drug nitrazepam (de Castro *et al.*, 2001), and also relies on the lipophilicity and ionisation of the drugs (Schwarz *et al.*, 1996). Zughaid *et al.* (Zughaid *et al.*, 2012) tried to find a correlation between the hydrophobicity of different BS and the solubility of hydrophobic drugs, by applying the hydrophobic index (HI) of BS. HI indicates the hydrophobicity of BS according to their retention factors in HPLC (Heuman, 1989). Correlation was not significant due to other variable factors, such as BS concentration and solution osmolality. In spite of the research above, no systematic study reports the solubilisation effects provided by different BS composition. The current single bile system tends to be simplistic in nature but more investigation is required.

BS are derived from cholesterol in liver and stored in gallbladder at high concentration. The chyme initiates the secretion mainly into the duodenum (Björkhem, 1985). In fasted state, the level varies individually. However the average total concentrations of BS in duodenum and jejunum are similar, with jejunum slightly lower (Dressman *et al.*, 1998). Ranges of 1.5 - 5.9 mM (Kleberg *et al.*, 2010, Porter *et al.*, 2007), 2 - 6.4 mM (Holm *et al.*, 2013) and 1.4 - 5.9 mM (Bergstrom *et al.*, 2014) were summarised by reviews, with typical value of 3 mM recommended according to the literature data (Kleberg *et al.*, 2010). In fed state, the peak level appears within 30 min of meal intake (average 15 mM) but gradually reduces thereafter. The concentration is more variable (4 - 24 mM duodenum) in fed state depending on the measuring protocols used, such as meal type, quantification methods and sampling time (30 – 240 min) (Dressman *et al.*, 1998, Kalantzi *et al.*, 2006a, Vertzoni *et al.*, 2012, Holm *et al.*, 2013).

Phospholipids

Phospholipid (PL, abbreviation refers to common phospholipids such as phophatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinostitol and hydrolysis product such as lyso-phosphatidylcholine) is a classical head-and-tail amphiphile composed of a choline and glycerophosphoric acid head with two fatty acid tails. The most common PL present in bile is phosphatidylcholine (PC) and it converts to lyso-phosphatidylcholine (lyso-PC) in small intestine. Its low water dispersability leads to the physical instability and cloudiness of the biorelevant media and requires to be solubilised by other amphiphiles in the media. Larger micelles and vesicles can form when mixing PL with BS (Birru *et al.*, 2014) and potentially alter the solubility of certain drugs (Zughaid *et al.*, 2012).

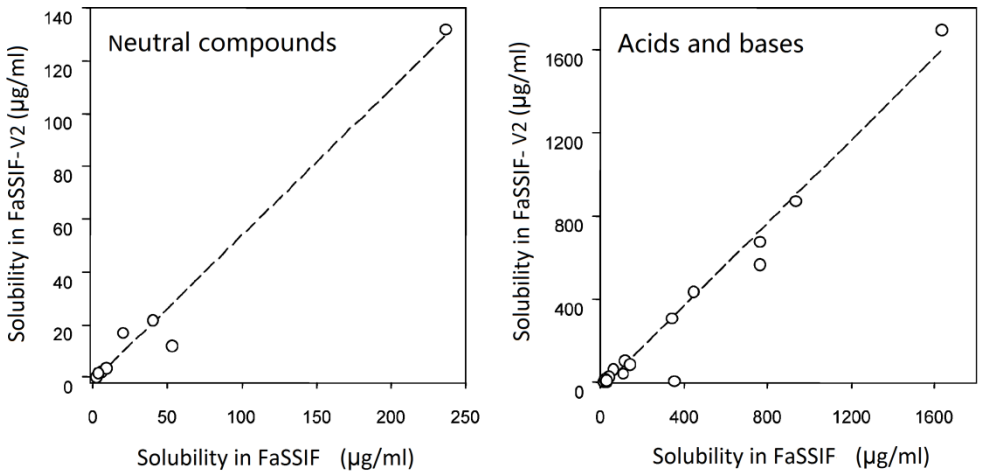


Figure 1. 8 Correlation of the solubility of different drug in FaSSIF and FaSSIF-V2.
It shows that for neutral drugs, the solubility is doubled in FaSSIF compared with FaSSIF-V2, while for acidic and basic drugs, the solubility in two media is similar. Each point represents a poorly soluble compound (Soderlind *et al.*, 2010).

The concentration of PL in fasted duodenum and jejunum on average is 0.2 mM (Bergstrom *et al.*, 2014). The original level of PL in biorelevant media was revised from 0.75 mM (FaSSIF) to 0.2 mM (FaSSIF-V2) to match the *in vivo* osmolality and

maintain stability over 72 h (Jantratid *et al.*, 2008), and validated by comparing solubility data with fasted HIF (Soderlind *et al.*, 2010). The results indicated that for neutral compound, FaSSIF with an excessive amount of PL, tends to over-predict solubility, while solubility in FaSSIF V2 shows good agreement with HIF, even though the contribution of PL for ionisable drugs was marginal (Fig. 1.8) (Soderlind *et al.*, 2010). The reported PL concentration in fed duodenal fluid was between 1.2 to 6 mM and affected by the meal composition (Clarysse *et al.*, 2009b, Vertzoni *et al.*, 2012).

Lipid digestion products: monoglyceride, free fatty acids and cholesterol

Several studies have reported the solubilisation effects of lipid digestion products such as monoglyceride (MG) and free fatty acids (FFA), both of which are formed during enzymatic hydrolysis of triglycerides. For instance, the *in vitro* solubility of seocalcitol was increased in a fasted state intestinal biorelevant media (5 mM NaTC and 1.25 mM PL), with addition of either medium or long chain lipolytic composites (Grove *et al.*, 2005), but the enhancement was not obvious in fed state as it was disguised by the much higher concentration of NaTC and PL present (20 mM NaTC and 5 mM PL). However, in another study of five lipophilic drugs, the solubilisation capabilities of long and medium-chain FFA was enormous in both fasted and fed states. Interestingly, the medium chain and long chain triglycerides had similar solubilisation capacities towards the drugs on the same molar ratio (Kaukonen *et al.*, 2004). Solubility of danazol has better correlation with the total concentration of BS + MG + FFA than BS alone, and IVIVC can only be obtained with the presence of MG and FFA (Zangenberg *et al.*, 2001, Sunesen *et al.*, 2005). In fed state, the triglycerides from food are digested to MG and FFA by lipases, which can then interact with BS and PL to form mixed micelles. Studies have shown it would be worthwhile to take into account the dynamic process of lipolysis of triglycerides in the system, as the change of the triglyceride oil phase may significantly change the partition of drugs depending on its log P (Zangenberg *et al.*, 2001).

Very few studies reported the levels of these two parameters *in vivo*, especially in fasted state as they are digestion products (Kleberg *et al.*, 2010). Low level (0.1 mM) of neutral lipids including FFA and cholesterol were found in the fasted HIF (Persson *et al.*, 2005). In fed state, a variety of esters such as tri-, di- and mono-glycerides were present and the levels depended on the meal composition (Persson *et al.*, 2005). The total neutral lipid amount reported in jejunum is 22 mM, with FFA at 13.2 mM (60%) and MG at 2.2 mM (10%) (Persson *et al.*, 2005). In duodenum, reported values are higher, for FFA are 39 mM and 52 mM and for MG are 5.9 mM and 8.1 mM after 30 min of meal intake in two papers (Kalantzi *et al.*, 2006b, Vertzoni *et al.*, 2012). In both FeSSIF-V2 and Copenhagen Fed medium, lipid digestion products, such as FFA and MG, were introduced as a revision of Galia's recipes (Kleberg *et al.*, 2010) (Table 1.1). Cholesterol in the intestine is derived either from the diet or biliary secretion (Cohn *et al.*, 2010), and it is an enzyme substrate to synthesise BS (Cohen, 2008). In fasted HIF, the concentration is very low (0 - 0.48 mM), while in fed HIF, both level and variability increased (0 - 3.29 mM), with mean value of 0.7 mM (Riethorst *et al.*, 2015) and 1.5 mM (Vertzoni *et al.*, 2012) reported.

BS/PL ratio and other amphiphile ratios:

The original intestinal biorelevant media by Galia *et al.* used BS/PL ratio of 4 in both FaSSIF and FeSSIF, with the BS concentration based on *in vivo* conditions (Galia *et al.*, 1998). The most commonly used FaSSIF from Galia *et al.*, is composed of 3 mM BS (NaTC) and 0.75 mM PL (egg PC) (Galia *et al.*, 1998). Later, Jantratid *et al.* updated the recipe to FaSSIF-V2 by reducing the concentration of PL to 0.2 mM, in order to comply with the osmolality *in vivo* (Jantratid *et al.*, 2008), and leading to the ratio of BS/PL increasing to 15. The ratio in fed state was still 5, with a substantial increase in the concentration of both ingredients. The pH was maintained, but the buffer was changed from phosphate to maleate. The most recent modification from

Kleberg *et al.* brought back the BS/PL ratio to 4 and used a Trizma maleate buffer, termed Copenhagen fluids (Kleberg *et al.*, 2010). Both FeSSIF-V2 and Copenhagen Fed considered the lipid digestion products (FFA and MG) for further adaption. However, the data from HIF suggest that the BS/PL ratio can be quite variable among individuals (Kleberg *et al.*, 2010). In fasted state, the ratio is 5:1 - 15:1 (Persson *et al.*, 2005, Riethorst *et al.*, 2015). After food intake reported typical values are 2:1 - 4:1 (n=5) (Clarysse *et al.*, 2009a) and 1:1 - 2.5:1 (n=12) (Persson *et al.*, 2005), hence in general the ratio in fed state is rather constant and remarkably lower than fasted state (Riethorst *et al.*, 2015).

Unlike FaSSIF and FaSSIF-V2, with only two amphiphiles present, FaSSIF-V2 Plus provides FFA and cholesterol, while FaSSIF-V3 also provides the PC hydrolysis products (lyso-PC), to better mimic the composition and surface tension in the upper small intestine (Psachoulias *et al.*, 2012, Fuchs *et al.*, 2015). FaSSIF-V3 has multiple recipes, with FaSSIF-V3-GC/TC-Chol as the lead prototype. Table 1.1 displays details of these various recipes.

In general, variation of solubilisation effects exhibited among different media (FaSSIF, FaSSIF-V2 plus and FaSSIF-V3), but within difference of one log unit (<10 times). Solubility exponential correlation between fasted HIF and FaSSIF is $y=2.2x^{0.89}$ ($r^2=0.85$, $p<0.0001$), and exponential correlation between fed HIF and FeSSIF or crude BS is $y=5.0x^{0.81}$ ($r^2=0.83$, $p<0.0001$), which are relatively strong correlation. Correlation is better at high solubility than low solubility (<10 μM) and better for neutral than ionisable compounds, which is due to pH-dependent solubility of ionisable compounds if intestinal pH varies (Augustijns *et al.*, 2014). For poorly soluble drugs, it provide better correlation with HIF than data generated by compendial media such as the underestimated aqueous buffer or overestimated 0.5% SDS solution suggested in the USP. Thus biorelevant media are good surrogates for biological samples such as HIF for routine laboratory practice. The adjustment of

amphiphile levels and addition of FFA and cholesterol better mirror the physiological parameters, however validation data are still limited (Koumandrakis *et al.*, 2014, Fuchs *et al.*, 2015). Moreover, most of these studies focus on modification of content concentration in a fixed value based on the average data or pooled aspirates *in vivo*. However, which recipe has reflected the *in vivo* solubility is different from compound to compound (Kleberg *et al.*, 2010). This indicated that currently no single medium is sufficiently "biorelevant" for all compounds, which address the importance to understand the variation and what causes the difference. One has to consider if a value generated from an average GI condition is enough for drug solubility/dissolution studies or a more comprehensive variability map is required to understand the potential drug absorption changes from different individual GI conditions at pre-prandial and postprandial stages.

Table 1. 1 Composition of the commonly used fasted and fed intestinal biorelevant media

	FaSSIF	FaSSIF-V2	FaSSIF-V2 Plus	FaSSIF-V3	FeSSIF	FeSSIF-V2	Copen. Fasted	Copen. Fed
BS (mM)	3	3	3	TC/GC 1.4/1.4	15	10	2.5	5-20
PL (mM)	0.75	0.2	0.2	PC/lyso-PC 0.035/0.315	3.75	2	PC/lyso-PC 2.5/0.625	1.25-5
BS/PL	4	15	15	9	4	5	4	4
MO (mM)	-	-	-		-	5	-	0-10
OA (mM)	-	-	0.5	0.315	-	0.8	-	2-7.5
Cholesterol	-	-	0.2	0.2	-	-	-	
Buffer	Phosphate	Maleate	Maleate	Maleate	Acetate	Maleate	Trizma maleate	
Salt (mM)	KCl 103	NaCl 69	NaCl 69	NaCl 93.3	KCl 204	NaCl 125	NaCl	NaCl
pH	6.5	6.5	6.5	6.7	5	5.8	6.5	6.5
Osmolality (mOsmol/kg)	270	180	180	220	635	390	270	Varying
Surface tension (mN/m)	54	54		35	48	40		
Buffer capacity (mmol/L·pH)	12	10		5.6	76	25		
Reference	1	2	3	4	1	2	5	5

BS, bile salt; PL, phospholipid; lyso-PC, lysophosphatidylcholine (lysolecithin); MO, monooleate; OA, sodium oleate; Copen., Copenhagen.

Reference: 1. Galia *et al.*, 1998; 2. Jantratid *et al.*, 2008; 3. Psachoulias *et al.*, 2012; 4. Fuchs *et al.*, 2015; 5. Kleberg *et al.*, 2010

Table 1. 2 Physiological parameters in fasted and fed human gastric and intestinal fluids

	Fasted stomach	Fasted duodenum	Fasted jejunum	Fed stomach	Fed duodenum	Fed jejunum
BS (mM)	0.0 - 0.8 (0.28)*	1.4 - 6.4 (3)	1.4 - 5.5 (2.5)	0.051 - 0.31*	4 - 24 (11.8)	4.5, 8.0 *
PL (mM)		0.26	0.19	0.022*	1.2 - 6	2.0 - 3.0*
BS/PL		5:1 - 15:1			1:1 - 4:1	
OA (mM)		0.1			39, 52*	13.2*
MO (mM)					5.9, 8.1*	2.2*
Cholesterol (mM)		0 - 0.48*			0.7, 1.5*	
Buffer			Bicarbonate			
Salt (mM)	Na^+ 19 - 122 (68)		Na^+ 111 - 165 (142)			
pH	1.7 - 3 (2.5)	5.6 - 7.0 (6.3)	6.5 - 7.8 (6.9)	4.5-6.7	5.4 - 6.5 (6)	6.1*
Osmolality (mOsmol/kg)	190 - 220 (202)	130 - 240	200 - 280	388*	400 - 600	400 - 600
Surface tension (mN/m)	30 - 45 (36.8)	30 - 40	30 - 35	30	25 - 35 (30)	25 - 35 (30)
Buffer capacity (mmol/L·pH)	13.3 - 19.0 (14.3)	5.6 - 8.5	4	14 - 28*	24 - 30*	13.9*

Data were provided in a range and a suggestive median or mean value in brackets if available. *Literature sparse.

Refer to Section 1.6 for detailed references.

1.6.4 Proteins

Proteins in GI fluids include the enzymes (i.e. lipase, pepsin) excreted to the GI tract and the dietary proteins ingested from the meal. Armand *et al.* reported the activity of gastric lipase was 44 U/ml in fasted state and dropped to 10 U/ml one hour after meal intake. The pancreatic lipase on the other hand was 400 - 600 U/ml and increased up to 1400 U/ml in fed state (Armand *et al.*, 1996). Lipase is an enzyme that hydrolyses dietary fat in the human digestive system, the triglyceride is digested into MG and FFA (Washington *et al.*, 2001). A few drug-protein interactions have been reported. For example, dalcetrapid, a thioester compound, can experience a rapid hydrolysis with the presence of lipases in FeSSIF (Gross *et al.*, 2012). This interaction can be site specific due to the pH variation in stomach and intestine. Ghazal *et al.* investigated the effect of four different proteins (albumin, casein, gluten and gelatine) towards the solubility and dissolution rate of itraconazole, a highly lipophilic weak base. A quantitative correlation was found between the albumin concentration and drug solubility as well as dissolution in the range of 0.3% - 2% (w/v) albumin. This was attributed to the hydrophobic and electrostatic interaction between albumin and itraconazole. Slight increase of itraoconazole solubility was also obtained in casein, gluten and gelatine, which might due to the reduced surface tension induced by proteins (Ghazal *et al.*, 2009). A series of drugs were reported to have much higher solubility in milk than normal aqueous buffer, which is not only attributed to the drug binding to milk components and implies a different solubilisation mechanism (Macheras *et al.*, 1989, Macheras *et al.*, 1990).

1.6.5 Surface tension

Surface tension is a parameter reflecting the wetting property of the media that can affect drug dissolution. BS, PL, FFA, cholesterol and protein can reduce the surface tension of blank buffer. The gastric surface tension reported is between 30 - 45

mN/m in fasted state, mainly due to the presence of pepsin (0.1 - 0.2 mg/ml) and reduced to 30 mN/m in fed state with increased pepsin concentration (0.3 - 0.6 mg/ml) (Efentakis and Dressman, 1998, Kalantzi *et al.*, 2006a, Pedersen *et al.*, 2013). Surface tension shows least variation among subjects in fasted and fed states in intestine, with ranges of 30 - 40 mN/m, and the value slightly lower in fed state (Kleberg *et al.*, 2010). The value in jejunum is slightly lower (25 - 35 mN/m) than duodenum and much lower than that of water (72 mN/m) due to the presence of surface active materials such as pancreatin, BS and PL (Kalantzi *et al.*, 2006a, Bergstrom *et al.*, 2014).

1.6.6 Osmolality

The predominant cation and anion of extracellular fluids for human body is sodium (Na^+) and chloride (Cl^-). In fasted gastric and jejunal fluids, the reported concentration of Na^+ is 68 ± 29 mM and 142 ± 13 mM respectively. The concentration of potassium (K^+) is 13 ± 3 mM in stomach and 5 ± 2 mM in jejunum (Banwell *et al.*, 1971, Lindahl *et al.*, 1997). Thus a more typical biorelevant media is recommended to be composed of sodium buffer rather than potassium. Potassium dihydrogen phosphate and sodium hydroxide are recommended in Pharmacopeia for dissolution media (British Pharmacopoeia *et al.*, 2001), even though no practical difference on drug dissolution was observed (Vertzoni, Fotaki *et al.* 2004). Cl^- is the main anion, with 102 ± 28 mM in stomach and 126 ± 19 mM in jejunum (Lindahl *et al.*, 1997). Cl^- can slow down the dissolution rates of haloperidol in hydrochloride salt form (Li, Doyle *et al.* 2005), which may address an issue on the effect of salt concentration especially on insoluble drugs in specific salt forms. The ion concentration of various intestinal biorelevant media is shown in Table 1.1, which is in line with their *in vivo* concentration.

Osmolality is the measure of ratio of fluid solute and water, and the intake and

excretion of Na^+ regulate the extracellular fluid osmolality in a tight range. The normal osmolality of plasma is 280 - 300 mOsmol/kg. The osmolality in fasted state was reported statistically higher for aspirates collected from the jejunum than stomach (Lindahl *et al.*, 1997), while no significant difference between jejunum and duodenum was found because of the large variability among subjects (Moreno *et al.*, 2006) (Table 1.2 and 1.3). The osmolality showed more variation during fed state, but with a noticeable increase to 400 - 600 mOsmol/kg, which was the result of the intrinsic high osmolality of the food (i.e. Ensure Plus®) (Kalantzi *et al.*, 2006a). Dissolution media of phosphate buffer in USP (2000) and International Pharmacopeia (1994) have the same osmolality (140 mOsmol/kg), significantly lower than physiological data. The biorelevant media in Table 1.1 adjust this parameter.

Table 1. 3 Osmolality (mOsmol/kg) of healthy human stomach and small intestine in fasted state

Stomach	Jejunum	Duodenum	References
191 ± 36	271 ± 15		(Lindahl *et al.*, 1997)
	200 ± 68	137 ± 54	(Moreno *et al.*, 2006)
		81 - 306	(Clarysse *et al.*, 2009b)
221 ± 15	278 ± 16		(Pedersen *et al.*, 2000b)
		236 ± 40	(Deferme *et al.*, 2003)
		178	(Kalantzi *et al.*, 2006a)

1.6.7 Simulated gastrointestinal fluids in different stages

Practically, the variation of composition and concentrations in intestinal fluids are usually not considered in terms of time and location as food passes through the intestine. It is believed the dominant absorption site is the upper small intestine during the first 2 - 3 h, therefore employing the average physiological data to design a consistent biorelevant medium can be a more applicable choice. Jantratid *et al.* first employed the "snapshot" to divide both gastric and intestinal fluids into three stages (Jantratid *et al.*, 2008). The gastric media reflected the pH and osmolality of

gastric aspirates for the early (75 min), middle (75 - 165 min) and late (after 165 min) stages. FeSSIF was divided into early (30 min), middle (130 min) and late (200 min) stages by adjusting pH, osmolality and composition of biorelevant amphiphiles. Clarysse *et al.* further supported the division as they found the solubility of drugs varies in fed state HIF at different time after meal. Despite the complexity of dividing FeSSIF into three stages, the results were still not consistent with HIF, especially the early FeSSIF overestimated the solubility of all tested drugs, which addressed the demand to analysing the colloidal structures and identify the interplay of multi kinds of components in biorelevant media (Clarysse *et al.*, 2009a).

Despite the large amount of studies on biorelevant media simulating the upper GI tract, the information on distal regions (especially ascending colon) is quite limited. Since BS are reabsorbed in the ileum, most of the lipolysis occurs in the jejunum (Dressman *et al.*, 2007). Therefore most of the current studies focus on the solubilisation effects mainly in the upper small intestine. However, further studies are required to validate the media representing the distal small intestine and colon as prolonged adsorption may arise in these regions where the longest residence time occurs. Details of biorelevant media of ileum and colon were summarised in a recent review by Markopoulos *et al.* (Markopoulos *et al.*, 2015).

Table 1. 4 Factors considered in various levels of biorelevant media

	Factors and composition
Level 0	pH
Level I	pH + buffer capacity
Level II	pH + buffer capacity + bile + lipids + lipid digestion + osmolality
Level III	pH + buffer capacity + bile + lipids + lipid digestion + osmolality + protein + enzymes + viscosity

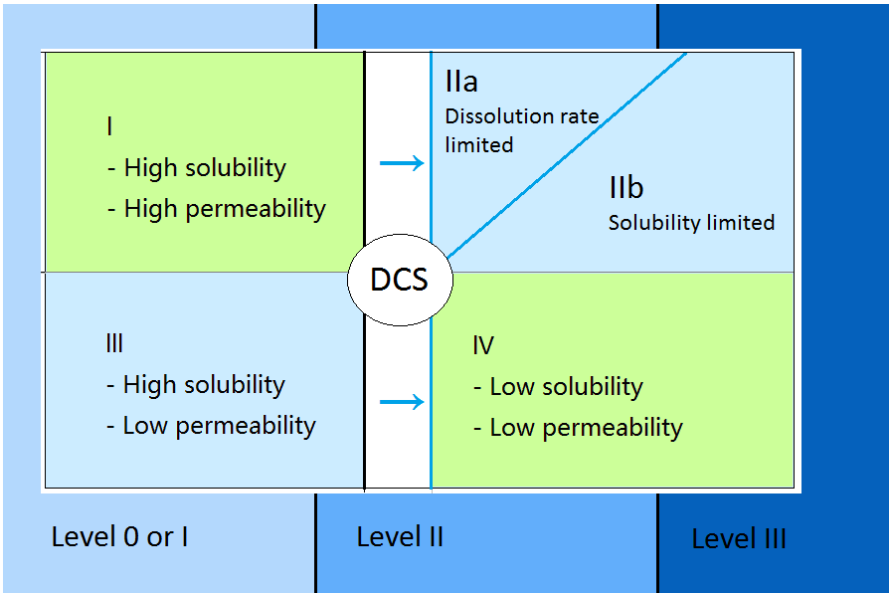


Figure 1. 9 A pragmatic selection of appropriate biorelevant media based on DCS. DCS I and III drugs are recommended to be tested in level 0 or I media, DCS II and IV drugs and those stay at the solubility borderline are recommended to be tested in level II. Studies on Level III media are limited as they are not easy to prepare for routine formulation evaluation and are only suggested for drugs with special interactions to intestinal conditions (Markopoulos *et al.*, 2015).

This review also suggests four levels of biorelevant media to evaluate performance of formulated drugs (Table 1.4). Level 0 media with only pH reflecting the intestinal environment, is suitable for DCS I and III drugs with high solubility and rapid dissolution. In level I media, both pH and buffer capacity are adjusted and useful to detect potential pH related food effects for DCS I and III. A rational approach to choose buffer species (phosphate, maleates and bicarbonate) is required to validate IVIVC. Level II media are commercialised media such as FaSSGF, FaSSIF, FaSSIF V2 and FeSSIF (Biorelevant, 2016) that are commonly used in recent literature, in order to facilitate laboratory practice and reproducibility. This level is suitable for DCS II and IV drugs, which are highly lipophilic and the solubility is likely to be affected by the intestinal contents before and after food intake. BS, PL, and currently cholesterol and lyso-PL are all proposed to be used in Level II media. Level III media has addition of dietary proteins, dietary lipids and increased

viscosity, and they are suitable for lipid-based formulation, formulations with potential wetting or intestinal stability issues. However, *in vivo* validation data is still limited and more IVIVC studies are required (Figure 1.9) (Markopoulos *et al.*, 2015).

1.7 Supersaturation and precipitation

Supersaturation describes the situation when drug concentration is above the equilibrium solubility, before it establishes the solid-solution equilibrium. The solubility at this state is kinetic solubility, which can be generated from multiple routes, such as the dissolution from an amorphous form, pH shift leading to ionisable compounds from high to low solubility. The equilibrium is reached eventually by precipitation and the supersaturating process has its pharmaceutical importance for drug absorption (Ma and Hadzija, 2012). The supersaturating drug delivery systems (SDDS) were introduced to circumvent the limitation of low aqueous solubility and dissolution rate, with modification on drug solid forms or formulation (Brouwers *et al.*, 2009). A high energy solid form (cocrystal, crystalline salt forms, amorphous forms or prodrug) or solubilised drug solution (cosolvent, lipid formulation) provides rapid dissolution, which is known as the "spring" (Figure 1.10), and creates a supersaturated solution of drug at the administration site, allowing for significant absorption and BA. However, fast recrystallisation back to equilibrium solubility and thermodynamically stable solid forms can limit these benefits. Therefore modification of the second stage of SDDS such as addition of precipitation inhibitors can prolong the metastable supersaturated state, providing a parachute to retain drug in solution for a longer period (Figure 1.10). These inhibitors can be either blended with the API in advance (excipients i.e. Pluronics) (Guzman *et al.*, 2007) or intrinsically present in the media (i.e. BS, PL) (Kostewicz *et al.*, 2004). It is challenging to develop a robust system to dynamically detect the supersaturation and precipitation of a drug under various biorelevant conditions.

The associated characterisation instruments (i.e. spectroscopic techniques) are also expected to detect and discriminate different solid states before and after recrystallisation and the underlying mechanism of the inhibitors (nucleation/crystal growth inhibitors) (Lindfors *et al.*, 2008) on-line or off-line.

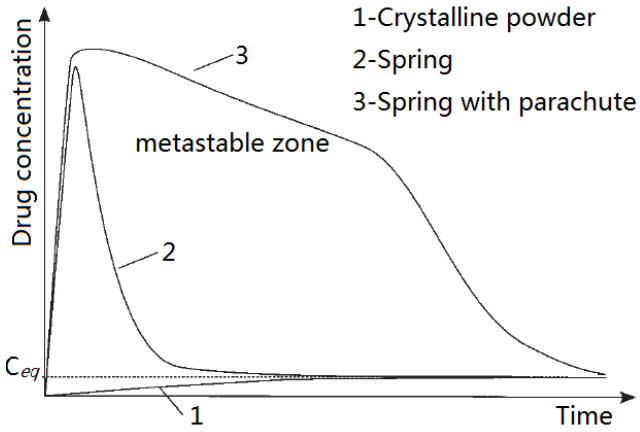


Figure 1. 10 The spring and parachute graph displays the drug concentration-time profile during supersaturation.
1: Predicted dissolution profile of the most stable solid form; 2: Rapid dissolution of the high energy form, followed by a rapid precipitation; 3: Rapid dissolution of the high energy form with precipitation inhibitors, which stabilise the metastable supersaturation for a longer period. C_{eq}, equilibrium solubility (Brouwers *et al.*, 2009)

Unintentional supersaturation - Weak basic drugs

The ionised form of weak basic drugs contributes to the high gastric solubility, while after transferring to the neutral pH intestine this concentration will exceed its solubility as unionised form according to Henderson-Hasselbalch equation (Equation 3) and lead to supersaturation and precipitation. However, since the preferential form for mucosa permeation is the unionised form, which facilitates the absorption in the small intestine and removes the drugs during supersaturation. Together with the endogenous precipitation inhibitors, the period of metastable state can be maximised and rapid precipitation can be avoided. Similar supersaturation scenario also occurs with the soluble salt form of weak acidic drugs in the stomach. Thus a drug delivery system capturing the drug transformation

and change of both pH and biorelevant composite from stomach to intestine can reveal information about these effects on API or a given formulation *in vitro*. Kostewicz *et al.* (Kostewicz *et al.*, 2004) used a transfer model to observe the precipitation of three weak basic drugs in the intestine. This *in vitro* system dynamically simulated the change of pH and media composition by using fasted SGF (pH 2), FaSSIF (pH 6.5) and FeSSIF (pH 5). A supersaturation ratio of 3 - 8 was observed in FaSSIF media compared with the equilibrium solubility in SGF. The precipitation and the time to reach the highest concentration (t_{max}) were closely related with the transfer rate from SGF to FaSSIF, while no precipitation was observed for two drugs in fed state due to multiple reasons such as the decreased pH, increased micelle concentration and media volume after meal intake and potential solid state transformation (Kostewicz *et al.*, 2004). This clearly indicates that GI physiology can alter the precipitation potential of supersaturated state. However, comparison with *in vivo* pharmacokinetic studies showed that this method still overestimated the risk of precipitation that could affect BA, as the influence of drug permeability, time-dependent HIF composition and lumen hydrodynamics were not considered (Carlert *et al.*, 2010). Similar models have been developed with additional absorption channels to include drug permeation, and better predict the precipitation potential (Sugawara *et al.*, 2005, Gu *et al.*, 2005).

1.8 Physicochemical properties of API

1.8.1 Chemical informatic tools to identify "drug-like" properties

High throughput screening for compounds targeting the biological receptors, provides no bias of favourable chemical properties (Lipinski, 2000). However it may pose risks of candidates with poor solubility and oral absorption. A wider range of "drug-like" properties (Lipinski, 2000) including compounds with sufficiently acceptable absorption, distribution, metabolism, excretion (ADME) and

toxicity, pose more questions to scientists about the criteria to identify the optimal leading candidates in the early stages and choose formulation strategies. Several classification and scoring systems have been developed, examples include the BCS (Amidon *et al.*, 1995b), the DCS (Butler and Dressman, 2010), Lipinski's rule of five (Lipinski *et al.*, 2001) and physiochemical scoring system (Lobell *et al.*, 2006) (Table 1.5), which have been discussed and applied in practical work (Bergstrom *et al.*, 2014).

The influence of the drug physiochemical attributes towards solubility, dissolution and permeability makes it important to identity the criteria for suitable candidates with good absorption. The Lipinski's rule of five describes that poor drug absorption is usually correlated with MW over 500 Da, partition coefficient (log P) more than 5, hydrogen bond acceptor more than 5 and hydrogen bond donor more than 10 (Lipinski *et al.*, 1997). High MW can lead to low oral absorption due to poor intestinal permeability and risk of high clearance (Navia and Chaturvedi, 1996). Log P is the logarithm value of the concentration ratio of the compound neutral species dissolved in the organic solvent (octanol) and the aqueous (water), which quantitatively describes lipophilicity. Log P between 0 - 3 is a good indicator for optimal drug absorption. A too low log P (< -3) leads to poor membrane permeation in GI tract, while a too high log P (> 6) is associated with low aqueous solubility (Navia and Chaturvedi, 1996). However, carrier-mediated transport can still facilitate molecules with negative log P to achieve high BA. Despite its simple conceptual definition, the accuracy of a series of log P predictive tools is still quite low, therefore experimental log P or predictive values based on log P of analogues are highly recommended (Mannhold *et al.*, 2009).

Ritchie *et al.* analysed 280 compounds to find the relationship between the number/type of aromatic rings and the drug developability (Ritchie and Macdonald,

2009, Ritchie *et al.*, 2011). An aromatic ring number more than two tends to decrease drug developability with poor solubility, higher lipophilicity, high protein binding and low oral BA. Despite the enormous choices of tools and principles for predicting the probability of successful leading molecules, *in silico* models still have their limitations and outliers. One problem is if using over strict criteria, it may simply mislead the results and lose potential candidates. A trend classification (low/medium/high) or fine grading (1 to 10) may be a better option than a hard cut-off value (i.e. log P < 5) to soften the boundaries.

Table 1. 5 Traffic light (TL) model of physiochemical scoring system.
The total TL value of the five components ranges from 0 to 10. The lower the value, the better the compound can be as a leading candidate for oral administered drugs. Adapted from Lobell *et al.*(2006).

TL Colour	TL Value	Solubility (mg/L)	Log P	MW	PSA [Å2]	RoB
Green	0	≥ 50	≤ 3	≤ 400	≤ 120	≤ 7
Amber	1	10 - 50	3 - 5	400 - 500	120 - 140	8 - 10
Red	2	< 10	> 5	> 500	> 140	> 10

MW, molecular weight; PSA, polar surface area; RoB, rotatable bonds

1.8.2 *In silico* tools to support pharmacokinetic studies

Recently, evaluating the *in vivo* drug behaviour with simulated physiologically based PBPK models has become a prosperous area for IVIVC (Harwood *et al.*, 2013). PBPK modelling tools such as GastroPlus™ (Okumu *et al.*, 2009), Simcyp® (Shaffer *et al.*, 2012) and STELLA® (Fei *et al.*, 2013, Shono *et al.*, 2009) have been employed to provide supplementary information for preclinical *in vivo* studies. These models are based on mechanistic approaches and provide a holistic assessment of multiple processes in drug absorption, utilising well-defined input data from different sources at all stages of drug development, such as experimental solubility, dissolution and permeation data generated from simulated physiological conditions *in vitro*, drug properties (i.e. pK_a, log P, solubility) and formulations (i.e. particle size).

Input data affects the performance of the model significantly, and solubility in biorelevant media and Caco-2 permeability are usually of better quality than *in silico* predicted data to obtain more accurate results (Sjogren *et al.*, 2016).

European project oral biopharmaceutical tools (OrBiTo) supported by Innovative Medicines Initiative (IMI) is trying to gain new knowledge of oral drug absorption and develop predictive biopharmaceutical tools that can be used in laboratory (*in vitro*) and on computer (*in silico*) for oral drug delivery system (IMI/EFPIA, 2016). Four work packages are divided among collaborators, focusing on different areas: investigating a set of biorelevant media on solubility and dissolution of API and formulated drugs, improvement of *in silico* and PBPK models, validated by clinical data such as new knowledge of HIF characteristics, drug solubility in HIF and *in vivo* drug absorption data. The expectation is to better understand factors affecting GI drug absorption and find indicative guide for efficient candidate selection, formulation development and establish IVIVC based on the physiochemical properties of the drugs.

1.9 Statistical experimental design

Factorial design of experiment (DoE) is widely used in industrial chemical process to simultaneously observe the effects of individual factors (pressure, materials and temperature etc.) and factor interactions on the properties of resultant products and optimise the conditions for a desired result in terms of time, cost etc. Factors can be quantitative (i.e. pH) or qualitative (i.e. base/acid) and the most commonly used design has two levels of each factor, low and high. In a full factorial design, all the possible combinations of each factor level are tested and a statistical analysis usually requires replication. Replication means an independent repeat of each factor combination. Replication allows for estimation of experimental error and also help to infer an estimation of the sample mean more precise to the true mean

(Montgomery, 2008). However, the scale of a DoE can be quite large and labour intensive to accomplish when the factors are numerous and replicates are needed, in this case a fractional factorial design is a more practical choice (Myers *et al.*, 2009). Instead of testing all the factor combinations, fractional factorial design selects only a fraction of the full design. A direct result is the loss of information such as aliasing among factors, however, by identifying critical factors and carefully select the fraction of DoE, informed and useful information can be gain with limited time and resources (Gunst and Mason, 2009).

Another type of design, termed mixture design (MD), focuses on modifying the ratio of the mixture composition and analyses the resulting products from the mixture. The basic feature of a mixture design is that the sum of all components is held at 100%, therefore component levels are not entirely independent of each other as is the case in a factorial DoE (Eriksson *et al.*, 1998). A mixture contour plot can visualise the change in the mixture region. With n as number of mixture components, the geometry of the mixture region has a dimensionality of n-1.

1.10 Aims and objectives

Despite the abundant availability of information on GI fluids, the variability of different components in the media still give rise to issues on how they can affect drug solubility, dissolution and consequently absorption. Simulated biorelevant media build the bridges to establish IVIVC, however the various choices pose questions to scientists and regulatory parties on the validation of different recipes and suitability of them. Currently, researches have investigated the effects of various GI factors, however experiments still lack of systematic design and comparison, therefore effects from various factors and the extent of the effects are difficult to determine. The aim of this work was to understand the influence of simulated GI fluid composition towards drug equilibrium solubility and identifying

key factors from a statistical point of view. The findings were also used to correlate with drug physicochemical properties, in order to explore potential methodologies to develop drug solubility evaluation tools to a better predictive level. In summary, the objectives was to investigate the solubility of a list of acidic, basic and neutral BCS II drugs using shake flask method in a series of fasted simulated intestinal fluids (SIF). Those fasted SIF were designed by fractional factorial DoE, investigating the influence of seven factors (pH, sodium taurocholate, phosphatidylcholine, sodium oleate, phosphate buffer, sodium salt and pancreatin) at a low and high level on the drug equilibrium solubility systematically and simultaneously. Secondly, based on the Noyes-Whitney equation, dissolution tests were performed to assess the relationship between equilibrium solubility and dissolution utilising fasted SIF recipes selected from the fractional factor DoE. Thirdly, a 4-component mixture design was conducted to specifically investigate the ratio effects of four amphiphiles (sodium taurocholate, phosphatidylcholine, sodium oleate, monoglyceride) on drug solubility. This gave a closer look on the amphiphile solubilisation capabilities on BCS II drugs whose solubility may especially sensitive to the composition of the amphiphiles in fasted SIF.

2. Design of experiment of fasted simulated intestinal fluids

2.1 Materials and method

2.1.1 Materials

Hydrochloric acid (HCl), potassium hydroxide (KOH), sodium taurocholate (NaTC), sodium chloride (NaCl), monosodium phosphate (NaH_2PO_4), naproxen, fenofibrate, griseofulvin, cinnarizine, ciprofloxacin, phenytoin, spironolactone, dipyridamole, proxicam, indomethacin, probucol, pancreatin from porcine were purchased from Sigma-Aldrich® (Poole, Dorset, UK). Ibuprofen, valsartan, aprepitant, carvedilol, zafirlukast, felodipine were kindly provided through OrBiTo by Dr. R. Holm, head of Preformulation, Lundbeck, Denmark. Sodium oleate (OA) was from BDH Chemical Ltd., Poole England. Phosphatidylcholine (PC) from soybean (98%) was gifted from Lipoid, Germany. All water used was ultrapure deionised Milli-Q® water. Methanol, acetonitrile were of HPLC grade (VWR, UK). Other chemicals used in HPLC including acetic acid (Sigma-Aldrich®, Poole, Dorset, UK), diethanolamine and ammonium acetate (Merck, Germany).

2.1.2 Design of experiment

Various recipes for fasted simulated intestinal fluids (fasted SIF) were designed by fractional factorial design. The quarter factional factorial design was constructed using MiniTab® 16.0, with 7 factors and 2 levels based on Table 2.1. This DoE has 66 different runs (32 runs in duplicate by various combinations of the 7 factors in their low/high levels and 1 centre point, see Table 2.2 for detailed media recipes). The measured solubility of each drug was analysed in MiniTab® 16.0 and significant factors and interactions were determined (interactions are denoted with "*"). A standardised effect value was calculated for each factor of each drug. The standardised effect equals the coefficient divided by standard error. Coefficient is the mean solubility at the high level minus the overall average solubility. Effects are statistically significant when the P-value < 0.05.

Table 2. 1 Composition and concentration of fasted SIF employed in DoE

Parameter	Substance	Low	Centre	High	Stock solution
BS (mM)	NaTC	1.5	3.7	5.9	88.5
PL (mM)	PC	0.2	0.6	1	15
Buffer (mM)	NaH_2PO_4	15	30	45	675
Salt (mM)	NaCl	68	87	106	530
pH	NaOH/HCl	5	6	7	-
Enzyme (U/ml)	Pancreatin	270	465	660	9900
FFA (mM)	OA	0.5	5.25	10	150

When only a fraction of the full factorial DoE is conducted, some of the effects will be confounded. For a quarter fractional factorial design, 2-way interactions may be confounded with another 2-way interactions. When two or more effects are confounded, the effects cannot be estimated separately. Therefore, the fraction must be chosen carefully and the conclusion of those confounded results should be carefully considered. A few assumptions were made before data was analysed:

1> Only main effects and 2-way interactions were considered in the analysis, 3-way (or more) interactions are not determined.

2> All the main effects were only confounded with 3-way/4-way interactions, which were neglected. There were three pairs of confounded 2-way interactions:

- Buffer*pH and pancreatin*OA
- Buffer*pancreatin and pH*OA
- Buffer*OA and pH*pancreatin

For example, if the results indicate that buffer*pH is a significant effect, this effect might be caused by buffer*pH or pancreatin*OA or both, in these cases conclusions have to be drawn with caution.

3> The main effect can be positive (+) or negative (−), but when it is involved in interactions, the conclusion should be considered with the interactions.

Table 2. 2 DoE detailed media composition

	pH	OA (mM)	NaTC (mM)	PC (mM)	Buffer (mM)	Salt (mM)	Pancreatin (U/ml)
recipe 1	5	10	1.5	0.2	15	68	660
recipe 2	5	0.5	5.9	0.2	15	68	270
recipe 3	5	0.5	1.5	1	15	68	270
recipe 4	5	10	5.9	1	15	68	660
recipe 5	5	10	1.5	0.2	45	68	270
recipe 6	5	0.5	5.9	0.2	45	68	660
recipe 7	5	0.5	1.5	1	45	68	660
recipe 8	5	10	5.9	1	45	68	270
recipe 9	5	0.5	1.5	0.2	15	106	270
recipe 10	5	10	5.9	0.2	15	106	660
recipe 11	5	10	1.5	1	15	106	660
recipe 12	5	0.5	5.9	1	15	106	270
recipe 13	5	0.5	1.5	0.2	45	106	660
recipe 14	5	10	5.9	0.2	45	106	270
recipe 15	5	10	1.5	1	45	106	270
recipe 16	5	0.5	5.9	1	45	106	660
recipe 17	7	0.5	1.5	0.2	15	68	660
recipe 18	7	10	5.9	0.2	15	68	270
recipe 19	7	10	1.5	1	15	68	270
recipe 20	7	0.5	5.9	1	15	68	660
recipe 21	7	0.5	1.5	0.2	45	68	270
recipe 22	7	10	5.9	0.2	45	68	660
recipe 23	7	10	1.5	1	45	68	660
recipe 24	7	0.5	5.9	1	45	68	270
recipe 25	7	10	1.5	0.2	15	106	270
recipe 26	7	0.5	5.9	0.2	15	106	660
recipe 27	7	0.5	1.5	1	15	106	660
recipe 28	7	10	5.9	1	15	106	270
recipe 29	7	10	1.5	0.2	45	106	660
recipe 30	7	0.5	5.9	0.2	45	106	270
recipe 31	7	0.5	1.5	1	45	106	270
recipe 32	7	10	5.9	1	45	106	660
recipe 33	6	5.25	3.7	0.6	30	87	465

2.1.3 Stock solution preparation and equilibrium solubility measurement

DoE stock solutions for each drug were freshly prepared from solids dissolved in deionised water according to concentration in Table 2.1. PC solid was dissolved in 1 - 2 ml of chloroform and chloroform was removed in a stream of nitrogen gas until a dry film was produced, which was then reconstituted with water as PC stock solution. Stock solution concentration was 15 times of the high level of each component. Volume of 4 ml medium was required for each recipe, so calculated volumes of stock solutions were mixed and diluted with water to 4 ml in the 15 ml Corning® centrifuge tubes. The pH was adjusted using 0.5 M HCl or 0.5 M KOH. A visual excess amount of solid drugs were added into each tube. The tubes were capped and placed into a shaker (OS 5 basic Yellowline, IKA, Germany) for 1 h, the pH was measured again and if required re-adjusted using 0.5 M HCl or 0.5 M KOH. The tubes were then rotated at 12 rpm for 24 h at 37 °C. Following 24 h incubation, 1 ml of the upper solution in each tube was transferred to a 1.5 ml Eppendorf® tube and centrifuged at 13,000 rpm for 5 min. The supernatant of 500 µl was transferred to HPLC vials for drug content analysis by HPLC. Due to the large sample scale, for each drug, this part of the work was assisted by a technical contribution from Dr. Ibrahim Khadra, Dr. Clair Dunn or Ms Jennifer Seaton.

2.1.4 HPLC methods

Agilent Technologies 1260 Series Liquid Chromatography system controlled by Clarity Chromatography software was used and HPLC conditions for each drug are presented in Table 2.3.

Table 2. 3 HPLC Assay Conditions

Column	Drug	Mobile phase	Flow rate (ml/min)	Injection volume (µL)	Detection (nm)	Retention time (min)	r^2	RSD
1	Aprepitant	ACN : 20 mM phosphate buffer (50 :50 v/v) pH 3	1	10	220	3	1.000	1.9%
1	Carvedilol	ACN : 10 mM ammonium acetate (40 :60 v/v) pH 4.5	1	10	243	3	0.999	0.2%
2	Cinnarizine	ACN : water : Diethanolamine (90 :10 :0.2 v/v)	1	10	250	4.1	1.000	0.2%
2	Ciprofloxacin	ACN :water : Diethanolamine (50 :50 :0.2 v/v)	1.5	5	280	1.6	0.999	0.2%
2	Dipyridamole	ACN: 50 mM ammonium acetate (55 :45 v/v) pH 4.0	1	10	280	2.2	0.999	0.2%
1	Felodipine	Methanol :water(75:25, v/v)	1	20	260	2.7	0.999	0.4%
1	Fenofibrate	ACN : water (70 : 30 v/v)	1	100	291	3	1.000	0.4%
2	Griseofulvin	ACN : water (50:50 v/v)	0.5	10	291	3.7	0.997	0.4%
2	Indomethacin	ACN:50 mM ammonium acetate (60:40 v/v) pH 4.5	1	10	254	4	0.999	0.1%
2	Naproxen	ACN: 50 mM ammonium acetate (60:40 v/v) pH 4.5	1	10	254	4	1.000	0.2%
2	Phenytoin	Methanol:20 mM phosphate buffer (55:45 v/v) pH 6	0.8	5 / 10	205	4	0.997	0.3%
1	Probucol	MeOH : ACN :water (45:45:10)	1	100	220	3.5	0.999	0.7%
2	Proxicam	Methanol:20 mM phosphate buffer (55:45 v/v) pH 6	1	10	254	2.2	0.999	0.2%
1	Spironolactone	ACN : water (50:50 v/v)	1	10	238	3	0.999	0.2%
2	Tadalafil	ACN: 20 mM phosphate buffer (70:30 v/v) pH 7	1	10 / 50	290	2	0.999	0.9%
1	Zafirlukast	ACN: 10 mM phosphate buffer (50:50 v/v) pH 6	1	10	245	2.2	0.998	1.6%

Column 1 Speck & Burke ODS-H optimal 150x30 mm id 5 µm; Column 2 Agilent Polaris 5 C18-A 150x4.6 mm id 5 µm

r^2, linear regression coefficient of calibration curve base on 6 concentrations. RSD, average relative standard deviation based on 3 replicate injections of the standard samples. Methods modified from literatures (Soderlind *et al.*, 2010, Clarysse *et al.*, 2011).

ACN: Acetonitrile

2.1.5 Validation of equilibrium solubility at centre point

Experiment was conducted at the DoE centre point (Recipe 33) in both plastic 15 ml Corning® tubes and 20 ml clear neutral squat form glass vials, media were prepared same as described above and incubated at 37 °C. 1 ml of suspension was extracted at 2h, 4 h, 24 h and 48 h, centrifuged and analysed by HPLC as before. Each sample was performed in duplicate to confirm the equilibrium was achieved in 24 h and examined the absorption effect of plastic materials.

2.1.6 Principle component analysis in SPSS

The physiochemical variables and DoE variables were analysed by principal component analysis (PCA) with SPSS® 16.0. Only the first three principle components were extracted and assessed further.

2.2 Results and discussion

2.2.1 Levels of fasted simulated intestinal fluids

The levels of each component in Table 2.1 were chosen either by covering the literature range (NaTC, PC, salt, pH) or a most commonly used level (median value) with a plus/minus variation (buffer) (Dressman et al., 1998, Vertzoni et al., 2004a, Jantratid et al., 2008, Kleberg et al., 2010). Although the presence of FFA is not suggested in most fasted state SIF as it is a food digestion product and have very few reported data in fasted HIF, a level of 0.1 mM (including FFA and cholesterol) was reported by Persson's group (Persson *et al.*, 2005). Hence a comparatively low concentration and a high level were set to investigate its influence towards drug solubility. Pancreatin levels were based on Armand *et al.* (Armand *et al.*, 1996).

2.2.2 Centre point equilibrium solubility

The comparison between glass and plastic materials was performed at the centre point of DoE (recipe 33). Figure 2.1 shows that for ten of the twelve drugs tested,

difference of solubility were less than 15% (n=2) in two materials after 24 h. For carvedilol, solubility was lower in glass container than plastic (difference 22%); while for fenofibrate, solubility was lower in plastic container than glass (difference 42%). Additionally, the difference was less than 20% in plastic after 24 h and 48 h except zafirlukast (29%), probucol (50%) and fenofibrate (37%). The reason for zafirlukast and probucol was related with their initial low solubility (< 2 μg/ml) and increased signal-to-noise ratios. For fenofibrate, the difference was potentially due to the absorption of fenofibrate or media contents that could facilitate solubilisation of fenofibrate (discussion of media contents see following sections). The latter might even be the major reason as the turbid media became clearer as the incubation increased from 24 h to 48 h, indicating the amphiphile contents (PC, OA associated with the cloudiness of the media) might be gradually adsorbed to the plastics, which affected the resulting solubility. For carvedilol, the absence of NaTC can be related with the increased solubility in plastic tubes (refer to next section for NaTC effect of carvedilol), and for fenofibrate, the absorption of amphiphiles on plastic tubes may decrease the solubility in the media. Subtle changes can also be seen in the data of aprepitant and felodipine, both of which has the equilibrium solubility lower in plastic than glass containers, and with the difference started to enlarge after 24 h and increased after 48 h. Fenofibrate, aprepitant and felodipine were all seen to be significantly and positively affected by amphiphile contents (see next section). Zafirlukast underwent a supersaturation and started to decline after 2 h and the kinetic solubility was 50 times of the equilibrium solubility. This data is also correlated with the dissolution test in Section 4.

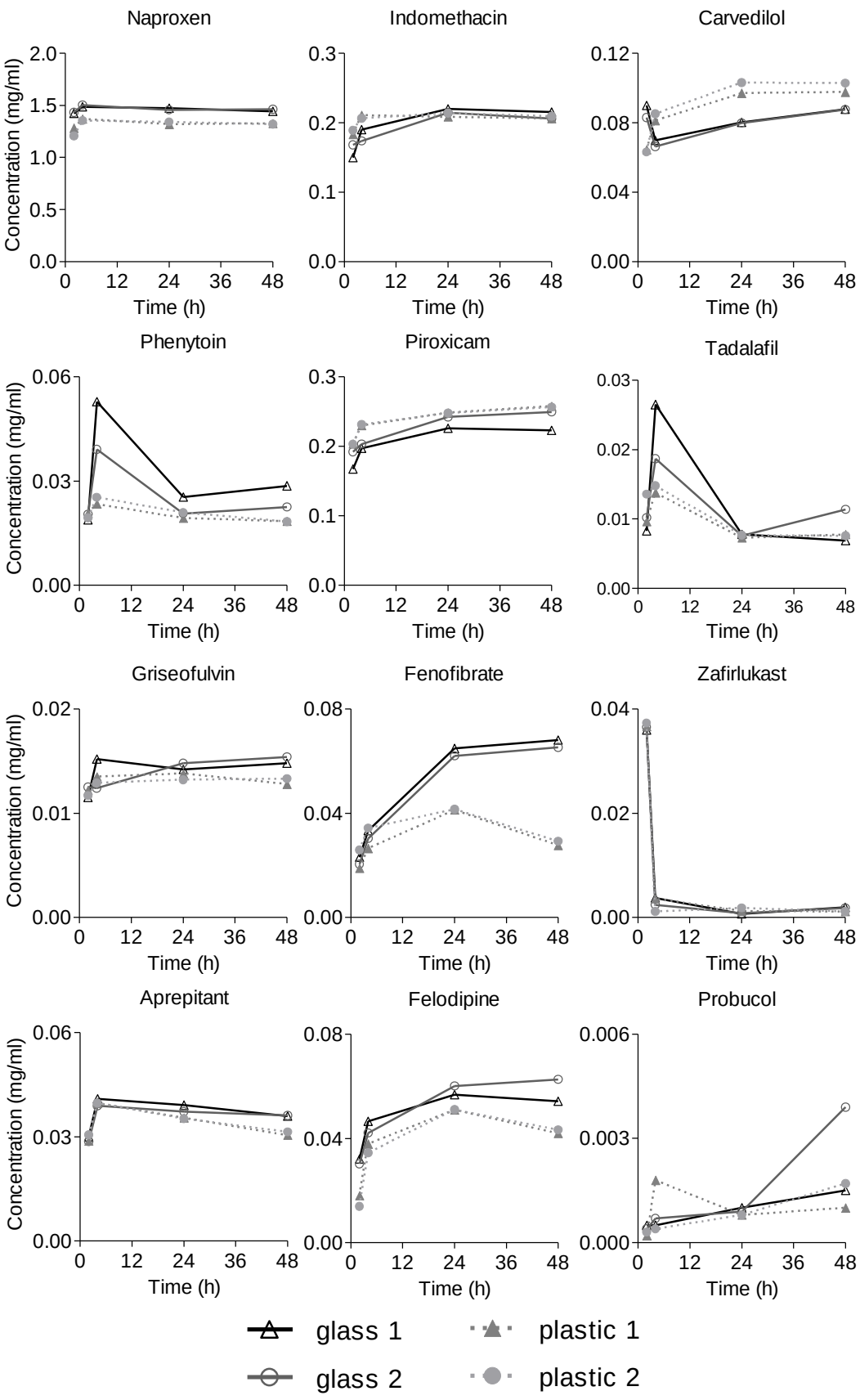

Naproxen
Indomethacin
Carvedilol
Phenytoin
Piroxicam
Tadalafil
Griseofulvin
Fenofibrate
Zafirlukast
Aprepitant
Felodipine
Probucol
Concentration (mg/ml)
Time (h)
glass 1
glass 2
plastic 1
plastic 2

Figure 2. 1 Concentration of drugs at 2h, 4 h, 24h and 48 h.
Concentration measured in both glass vails (solid line) and plastic tubes (dash line). DoE media used was Recipe 33 and performed in duplicate.

This equilibrium validation experiment indicated that 24 h in plastic tubes was sufficient to provide equilibrium within acceptable variation. It has been reported that poorly soluble drugs can reach equilibrium solubility in 24 h (Clarysse *et al.*, 2009a, Kalantzi *et al.*, 2006a). Augustijns *et al.* listed a table of methodologies used to determine equilibrium solubility, with 24 h incubation and separation by centrifugation as the most commonly employed literature protocols (Augustijns *et al.*, 2014), however the measuring container was not discussed in this review, similarly the container for shake flask method was not clearly stated in some literature (Kossena *et al.*, 2003, Soderlind *et al.*, 2010). Small scale glass vials were commonly used (Pedersen *et al.*, 2000b, Kostewicz *et al.*, 2002, Sunesen *et al.*, 2005) than plastic vials such as Eppendorf tubes (Clarysse *et al.*, 2009a) or polypropylene vials (Koumandrakis *et al.*, 2014), instead of the literal “flask” (Kalantzi *et al.*, 2006b), which required more biorelevant media and drugs. High throughput methods also utilised 96-well plates (Heikkila *et al.*, 2011) or glass UniPrep® filter chamber (Glomme *et al.*, 2005). However, the effect of container materials was not studied in the literature.

2.2.2 Equilibrium solubility

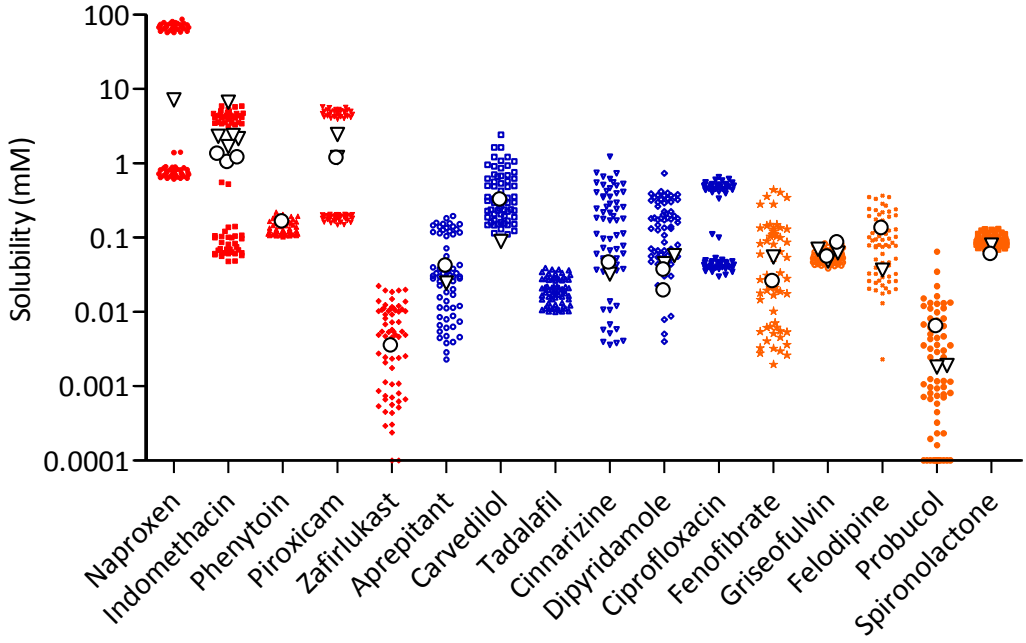


Figure 2. 2a Equilibrium solubility of 16 drugs in 66 fasted SIF

Equilibrium solubility measurements (n=66) for each drug measured in DoE media, composition detailed in Table 2.2. Acidic compounds in red, basic compounds in blue and neutral compounds in amber. ○ reported solubility values for individual drugs in FaSSIF or FaSSIF-V2 media, ∇ reported solubility values in HIF, all values from (Augustijns *et al.*, 2014, Fagerberg *et al.*, 2015). For individual solubility patterns refer to section 2.2.5.

All the equilibrium solubility of 16 drugs in DoE is summarised in Figure 2.2a. For references and discussion in the several following sections, the chemical structures are shown in Table 2.4 with pK_a and log P values. The solubility of acidic drugs naproxen, indomethacin and piroxicam is distinctively separated into two groups based on the pH of the media (details in Figure 2.2b), solubility difference in 1- 100 times, while phenytoin has a tight solubility range and zafirlukast has a distribution over 3-fold of logarithmic range, though not determined by pH. Phenytoin has a pK_a of 8.1 (Schwartz *et al.*, 1977) and zafirlukast has a pK_a of 3.68 (in house data). Therefore ionisation percentage of both drugs was marginally affected in the DoE pH range. Dissolution of phenytoin from both pure API and formulation were

reported to be independent of pH (Klein, 2010). The solubility of one basic drug ciprofloxacin is also divided into two groups according to pH (Figure 2.2b). For the other basic and neutral compounds, no single dominating factor can be identified from the figure and the solubility can be scattered in a distribution of two to three fold of logarithmic magnitude, implying a wide range of variation. Meanwhile, there are four drugs, phenytoin, tadalafil, spironolactone, and griseofulvin showing great consistency in various media (Figure 2.2b). Agreed with literature that solubility of spironolactone has showed only 1 - 2 fold variation comparing solubility in buffer and in FaSSIF or FeSSIF (Zaki *et al.*, 2010b). Griseofulvin had low inter-individual variability in HIF and no increase comparing in buffer and in FaSSIF (Annaert *et al.*, 2010). The literature data of 14 drugs are also superimposed in Figure 2.2a and the solubility is inside the DoE solubility space (Augustijns *et al.*, 2014), indicating that DoE reflects the parameters and drug solubility in both HIF and FaSSIF, and may also potentially cover the solubility space that is physiologically relevant but has not been measured if only average HIF component levels are studied.

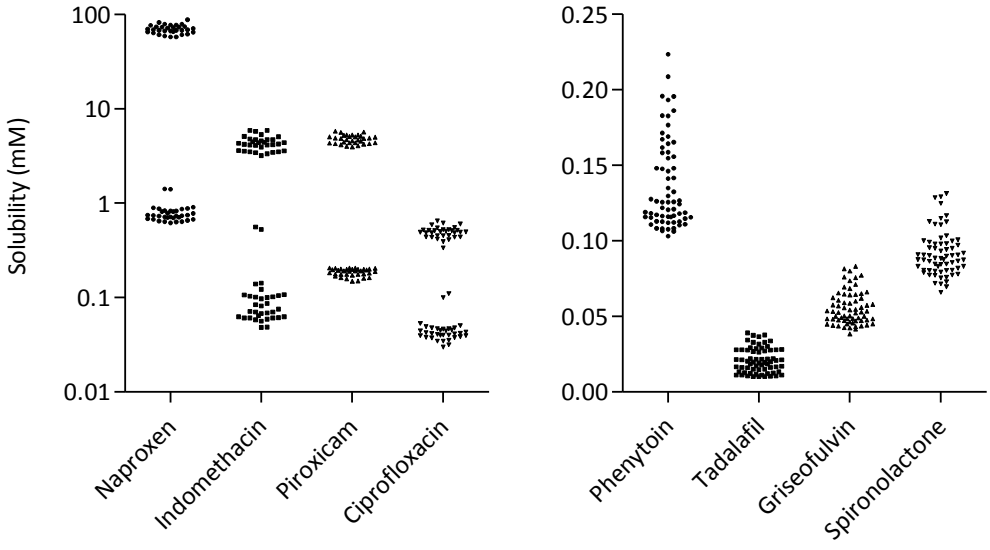
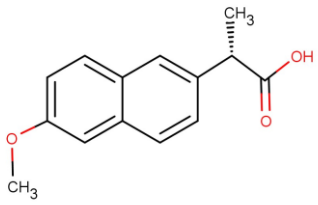
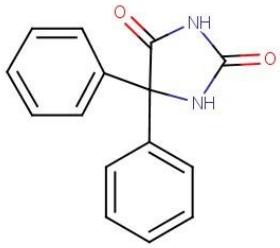
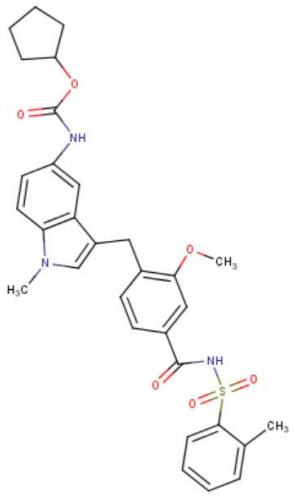
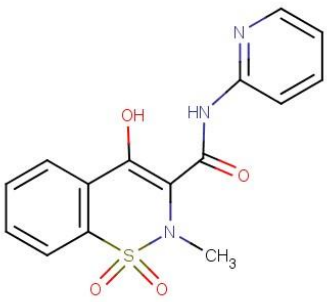
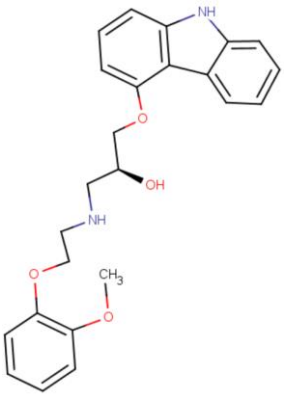
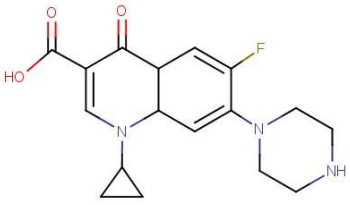
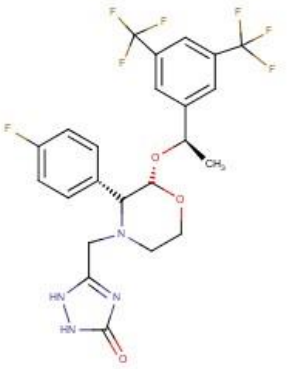
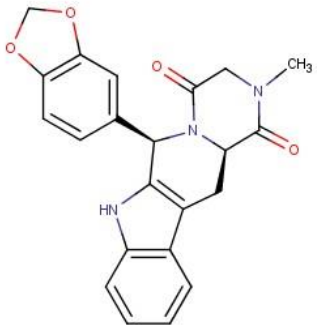
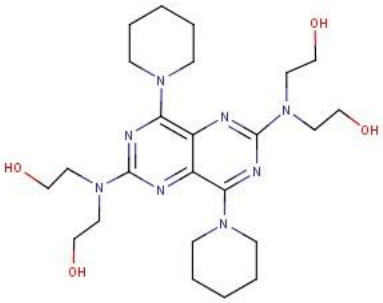
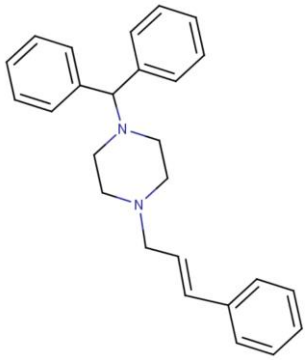
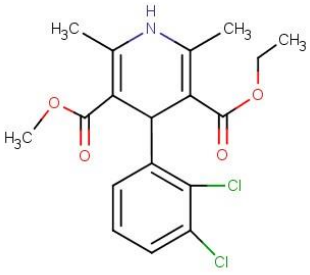
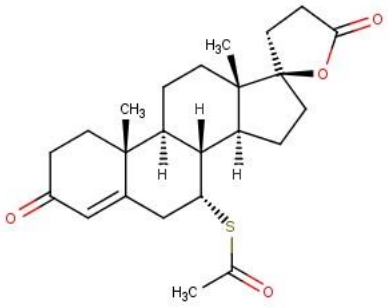
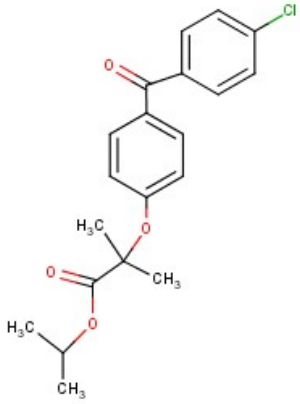
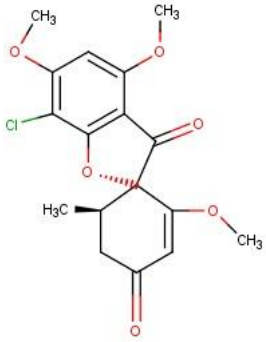
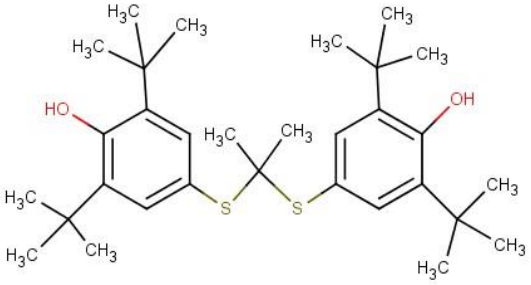


Figure 2. 2b Equilibrium solubility of four drugs influenced by pH (left) and four drugs with tight solubility range (right). N.B. solubility scales are different from Figure 2.2a.

Table 2. 4 Chemical structures, pK_a and log P of drugs

Naproxen pK_a = 4.3 log P = 3.24 	Indomethacin pK_a = 4.5 log P = 3.8 	Phenytoin pK_a = 8.1 log P = 2.29 
Zafirlukast pK_a = 3.68 log P = 6.4 	Piroxicam pK_a = 5.5 log P = 3.06 	
Carvedilol pK_a = 7.8 log P = 3.91 	Ciprofloxacin pK_a = 6.2 log P = -1.08 	Aprepitant pK_a = 9.7 log P = 4.8 

Tadalafil pK_a = 15.17 log P = 1.64 	Dipyridamole pK_a = 6.4 log P = 3.95 	Cinnarizine pK_a = 7.4 log P = 5.77 
Felodipine log P = 3.86 	Spironolactone log P = 2.78 	Fenofibrate log P = 5.24 
Griseofulvin log P = 2 	Probucol log P = 10 	

Log P, intrinsic solubility and pK_a are experimental data from literature where available (Cordero *et al.*, 1997, Wassvik *et al.*, 2006, Völgyi *et al.*, 2007, Llinas *et al.*, 2008, Mehanna *et al.*, 2010, Planinsek *et al.*, 2011), other information from DrugBank.

2.2.3 Solubility influence of individual DoE factors

The standardised effects of individual factors and factor interactions were calculated based on the equilibrium solubility (Section 2.1.2). Results indicate the magnitude and directions of the effects, and this allows for comparison among different factors and drugs. Figure 2.3a - g compares the standardised effects by factors, and Figure 2.4 compares the standardised effects in ranking orders by drugs.

Figure 2.3a and Figure 2.4 show pH as a predominant positive factor for all acidic drugs and the magnitude of NaTC, PC and OA is one tenth or less of the pH effect. pH had the highest magnitude among all significant factors and interactions (if any) for all acidic drugs, and pH was the only significant factor for naproxen and piroxicam (Figure 2.4). Besides, for zafirlukast and phenytoin, the pH effect values were one third of the other three acidic drugs and NaTC, PC, OA exhibit a moderate level of influence. The primary role of pH has been previously reported for piroxicam (Soderlind *et al.*, 2010), indomethacin and a series of other weak acidic drugs (Clarysse *et al.*, 2009a). Additionally, pH was a predominant effect for another two basic drugs dipyridamole and ciprofloxacin (Figure 2.4). In contrast to acidic drugs, pH was a negative effect, which was the solubility decreased when pH increased from 5 to 7. Ciprofloxacin was less affected by the amphiphile contents but remarkably sensitive to pH, while dipyridamole was also positively affected by NaTC but not PC and in return reduced the magnitude of pH effect (standardised effect less than ciprofloxacin). The phenomenon of dipyridamole agreed with literature reporting that solubility of dipyridamole was similar in FaSSIF and FaSSIF II (37.6 and 35.7 µM) where only PC level changed and solubility in both media were slightly higher than pH 6.5 buffer (10.7 µM) (Soderlind *et al.*, 2010). On the other hand, for aprepitant, pH showed positive significance due to its synergistic interaction with other factors such as pH*OA, which might overweigh ionisation introduced by negative pH effect on basic drugs. Similarly, pH also played a major and positive effect for four neutral drugs (griseofulvin, spironolactone, felodipine

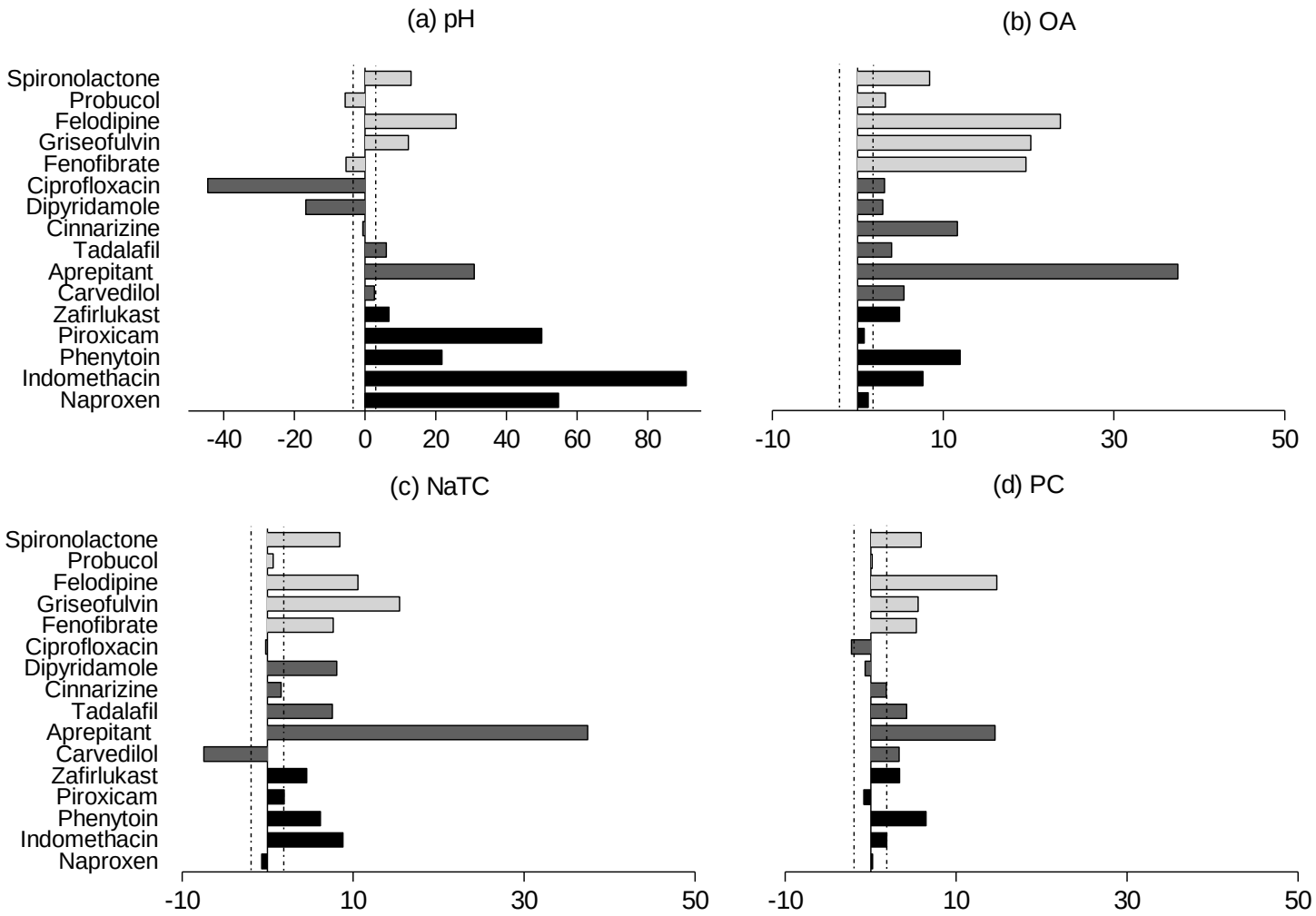
and probucol). Theoretically according to Henderson-Hasselbalch equation, pH should negatively affect the solubility of basic drugs and have no effects to neutral compounds. Therefore the pH effect was generated from interactions with other factors in the media.

In general the three amphiphiles OA, NaTC and PC showed significant positive effects to three drug categories (Figure 2.3b - d), with the descending magnitude order of neutral, basic and acidic drugs. Carvedilol showed an unusual NaTC negative effect, which was in accordance with the finding that anionic surfactants may retard solubility (Chakraborty *et al.*, 2009). The amphiphile effect value can be up to one tenth of pH effect for acidic drugs, which indicates that the solubilisation capacity of biorelevant amphiphiles is much smaller than the ionisation due to logarithmic effect of pK_a to local pH for acidic drugs (Henderson-Hasselbalch equation). Clarysse *et al.* reported that pH was more dominant than amphiphile solubilisation effects for acid drugs due to pK_a and low log P of the drugs, the solubilisation of BS and PL are more effective for lipophilic drugs (Clarysse *et al.*, 2009a), while phenytoin and zafirlukast, with ionisation not affected at the pH range and the latter also with high log P, are therefore outliers in the acidic group. Similarly basic drugs such as aprepitant, have very weak alkalinity but high lipophilicity and consequently they behave like neutral drugs in the biorelevant pH environment, which means that their “ionisation state unchanged” identity is more of a concern than their “ionisable” basic/acidic identity.

Eight drugs showed significant buffer effect and eight drugs showed salt effects out of sixteen drugs respectively (Figure 2.3e and f). The effect magnitude of buffer and salt are half of the amphiphile effects and mainly negative, except a positive buffer effect of indomethacin. With regards to Henderson-Hasselbalch equation, the solubility should not depend on the buffer used; rather it is pH that determines

the solubility. However, phosphate buffer usually exhibits lowering effect to drug solubility (Bergstrom *et al.*, 2004). Solubility enhancing and decreasing effects have also been reported for citric, lactic and phosphoric acids (Al Omari *et al.*, 2006, Shoghi *et al.*, 2013). Salt showed negative effect on one acidic drug, three basic drugs and three neutral drugs. Common ion effects are related with the buffer and salt presented, and interact with solubilised drug, NaTC and OA in the media. However, ion effect is usually substance specific and difficult to generalised (Bergstrom *et al.*, 2004). Pancreatin only showed significance for phenytoin and spironolactone out of sixteen drugs with low rank of magnitude, thus it is regarded as an insignificant factor (Figure 2.3g).

Figure 2.3h summarises the absolute value of seven factors from three drug categories (acid/base/neutral drugs). Besides the insignificance of pancreatin, salt and buffer have marginal significance to base and neutral categories, whilst buffer have a higher significance and salt had no effect in the acid category. Acid drugs have pH as the predominant factor, while for basic it is also the most significant among seven factors but less dominant. The ranking and magnitude of factors for basic and neutral drugs were quite similar, though the effects of OA, NaTC and PC on neutral drugs were slightly higher than basic drugs. However, which amphiphile(s) were more remarkable than the others and to what extent on individual drugs should be examined on a case-by-case basis (Figure 2.4). With only five acidic drugs, six basic drugs and five neutral drugs, Figure 2.3h only provides a gross mean analysis of the effects and any interpretation has to be cautious and refer to individual drugs.


(a) pH
(b) OA
(c) NaTC
(d) PC
Spironolactone
Probucol
Felodipine
Griseofulvin
Fenofibrate
Ciprofloxacin
Dipyridamole
Cinnarizine
Tadalafil
Aprepitant
Carvedilol
Zafirlukast
Piroxicam
Phenytoin
Indomethacin
Naproxen
-40
-20
0
20
40
60
80
-10
10
30
50

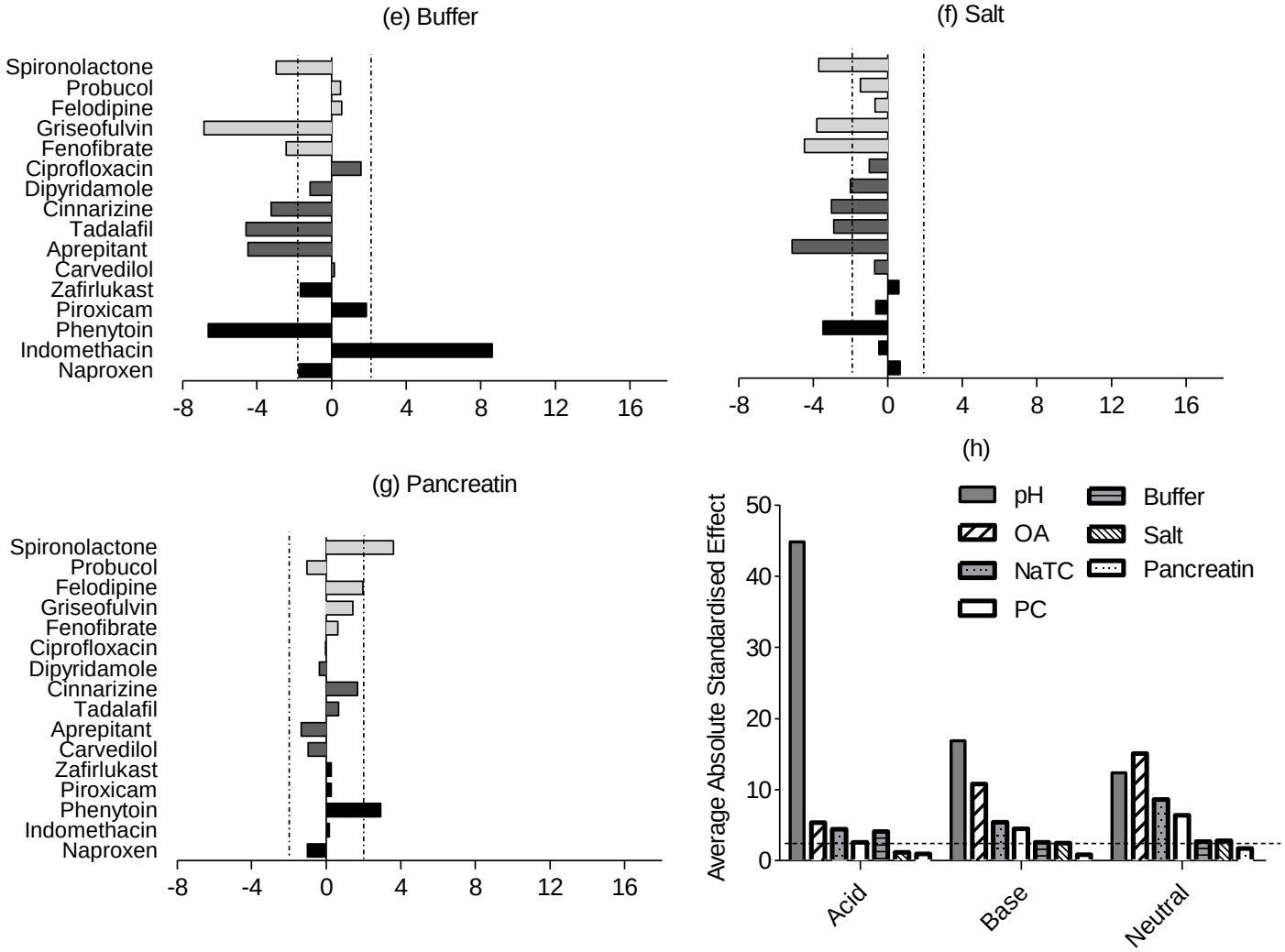
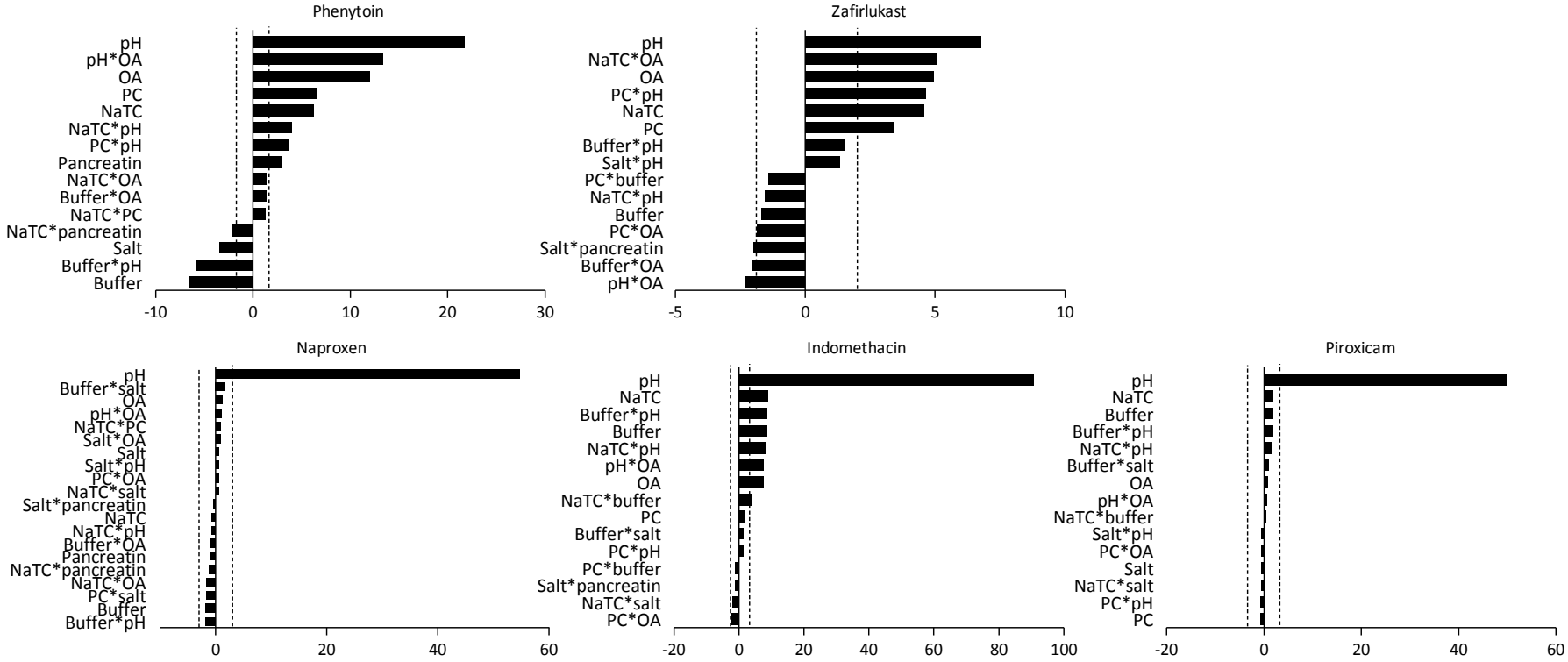
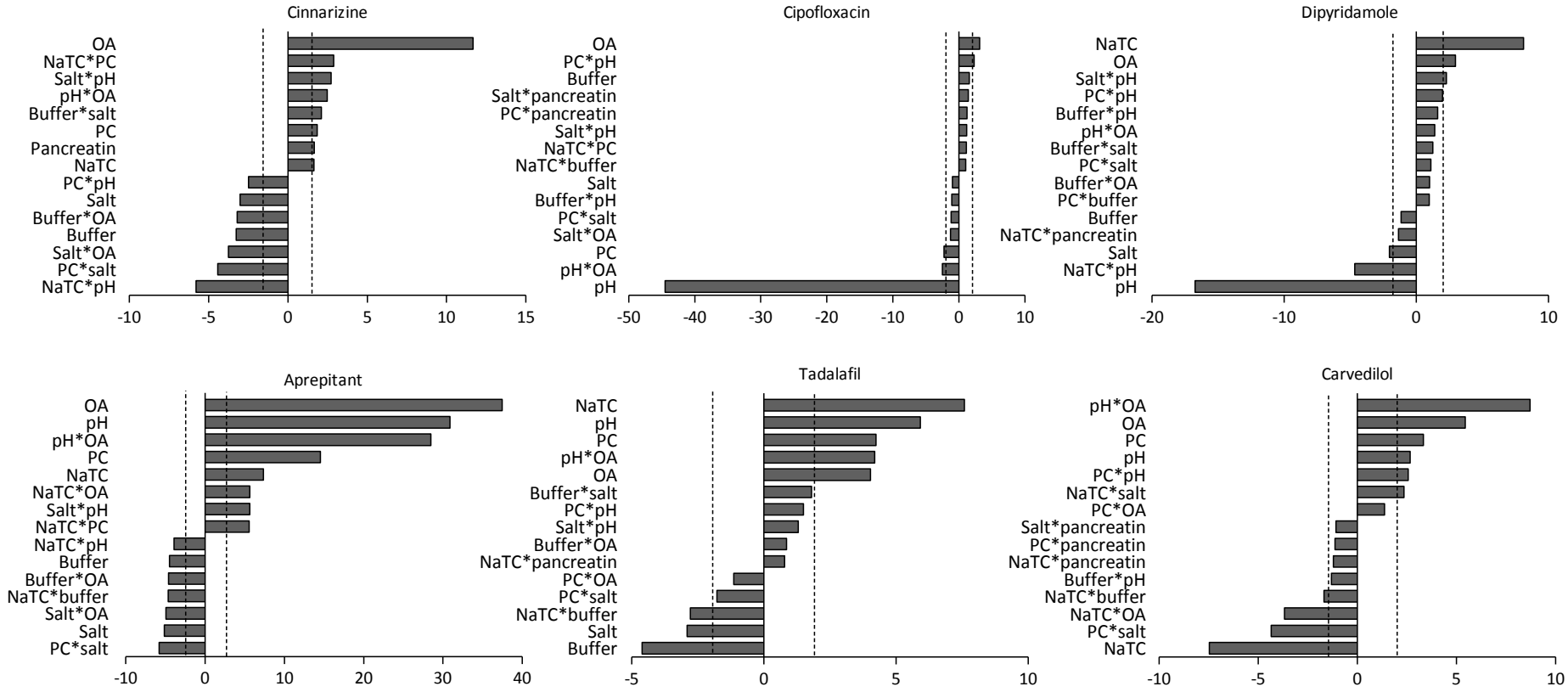

(e) Buffer
(f) Salt
(g) Pancreatin
(h)
Spironolactone
Probucol
Felodipine
Griseofulvin
Fenofibrate
Ciprofloxacin
Dipyridamole
Cinnarizine
Tadalafil
Aprepitant
Carvedilol
Zafirlukast
Piroxicam
Phenytoin
Indomethacin
Naproxen
-8
-4
0
4
8
12
16
pH
OA
NaTC
PC
Buffer
Salt
Pancreatin
Average Absolute Standardised Effect
50
40
30
20
10
0
Acid
Base
Neutral

Figure 2. 3 Standardised effect values (x-axis) of 7 main factors of each drug (Figure 2.3 a - g).

NB. Some have different scales. Bars extending beyond the dashed lines indicate the corresponding factor is significant ($P < 0.05$). Bar length indicates the effect magnitude and bar direction indicates positive or negative effects on equilibrium solubility. Figure 2.3 h, the average value of the absolute standardised effect for each factor from three drug categories. For details of calculating absolute standardised effect, see Section 2.1.2.




Cinnarizine
OA
NaTC*PC
Salt*pH
pH*OA
Buffer*salt
PC
Pancreatin
NaTC
PC*pH
Salt
Buffer*OA
Buffer
Salt*OA
PC*salt
NaTC*pH
-10
-5
0
5
10
15
Cipofloxacin
OA
PC*pH
Buffer
Salt*pancreatin
PC*pancreatin
Salt*pH
NaTC*PC
NaTC*buffer
Salt
Buffer*pH
PC*salt
Salt*OA
PC
pH*OA
pH
-50
-40
-30
-20
-10
0
10
Dipyridamole
NaTC
OA
Salt*pH
PC*pH
Buffer*pH
pH*OA
Buffer*salt
PC*salt
Buffer*OA
PC*buffer
Buffer
NaTC*pancreatin
Salt
NaTC*pH
pH
-20
-10
0
10
Aprepitant
OA
pH
pH*OA
PC
NaTC
NaTC*OA
Salt*pH
NaTC*PC
NaTC*pH
Buffer
Buffer*OA
NaTC*buffer
Salt*OA
Salt
PC*salt
-10
0
10
20
30
40
Tadalafil
NaTC
pH
PC
pH*OA
OA
Buffer*salt
PC*pH
Salt*pH
Buffer*OA
NaTC*pancreatin
PC*OA
PC*salt
NaTC*buffer
Salt
Buffer
-5
0
5
10
Carvedilol
pH*OA
OA
PC
pH
PC*pH
NaTC*salt
PC*OA
Salt*pancreatin
PC*pancreatin
NaTC*pancreatin
Buffer*pH
NaTC*buffer
NaTC*OA
PC*salt
NaTC
-10
-5
0
5
10

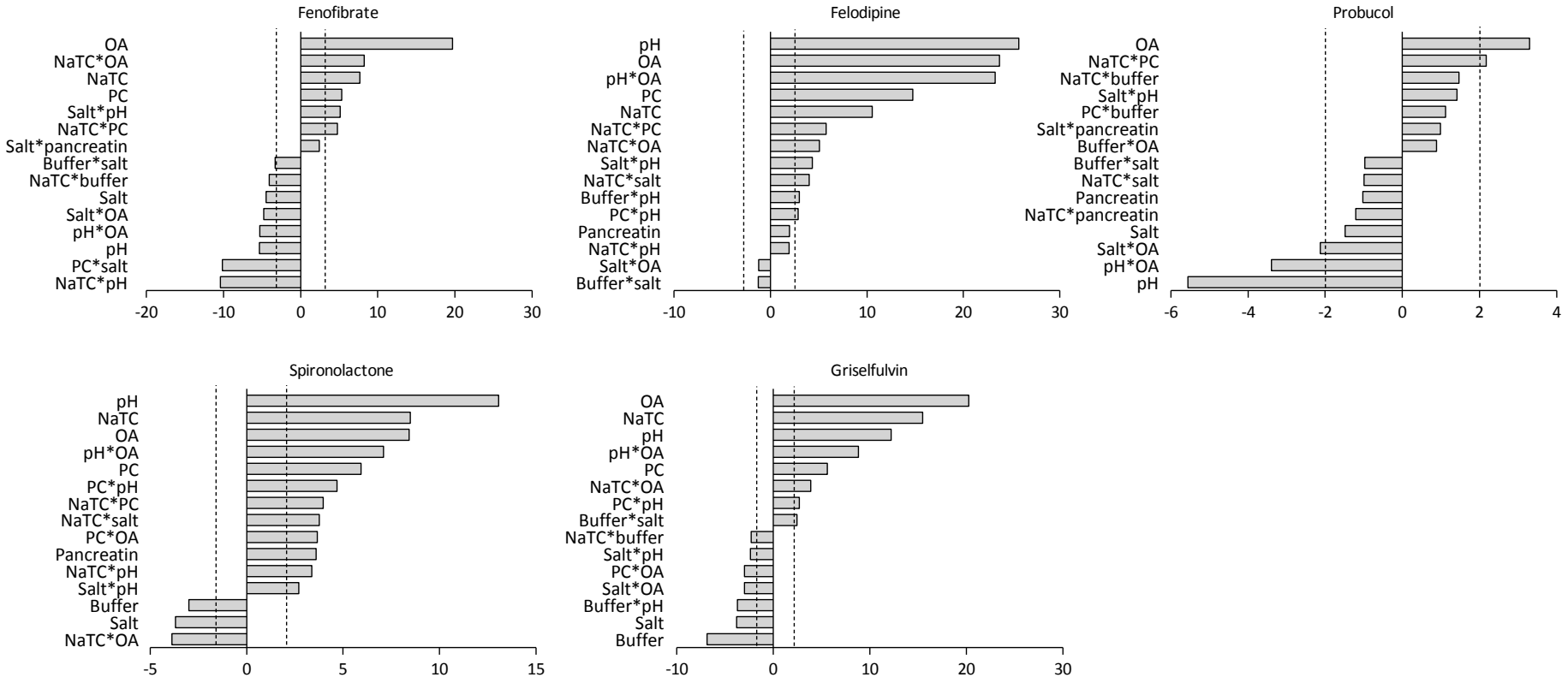


Figure 2. 4 Standardised effect values (x-axis) of 16 drugs. Standardised effects in descending order and graphs only show the top 15 single and interactions of the absolute values. Bars extending beyond the dashed lines indicate the corresponding factor is significant ($P < 0.05$).

2.2.4 Solubility influence of DoE factor interactions

The standardised effect and statistical significance of two-way interactions for each drug are shown in Figure 2.5a - r. Figure 2.5s summarises the mean of the absolute value of the standardised effect of acidic, basic and neutral drugs.

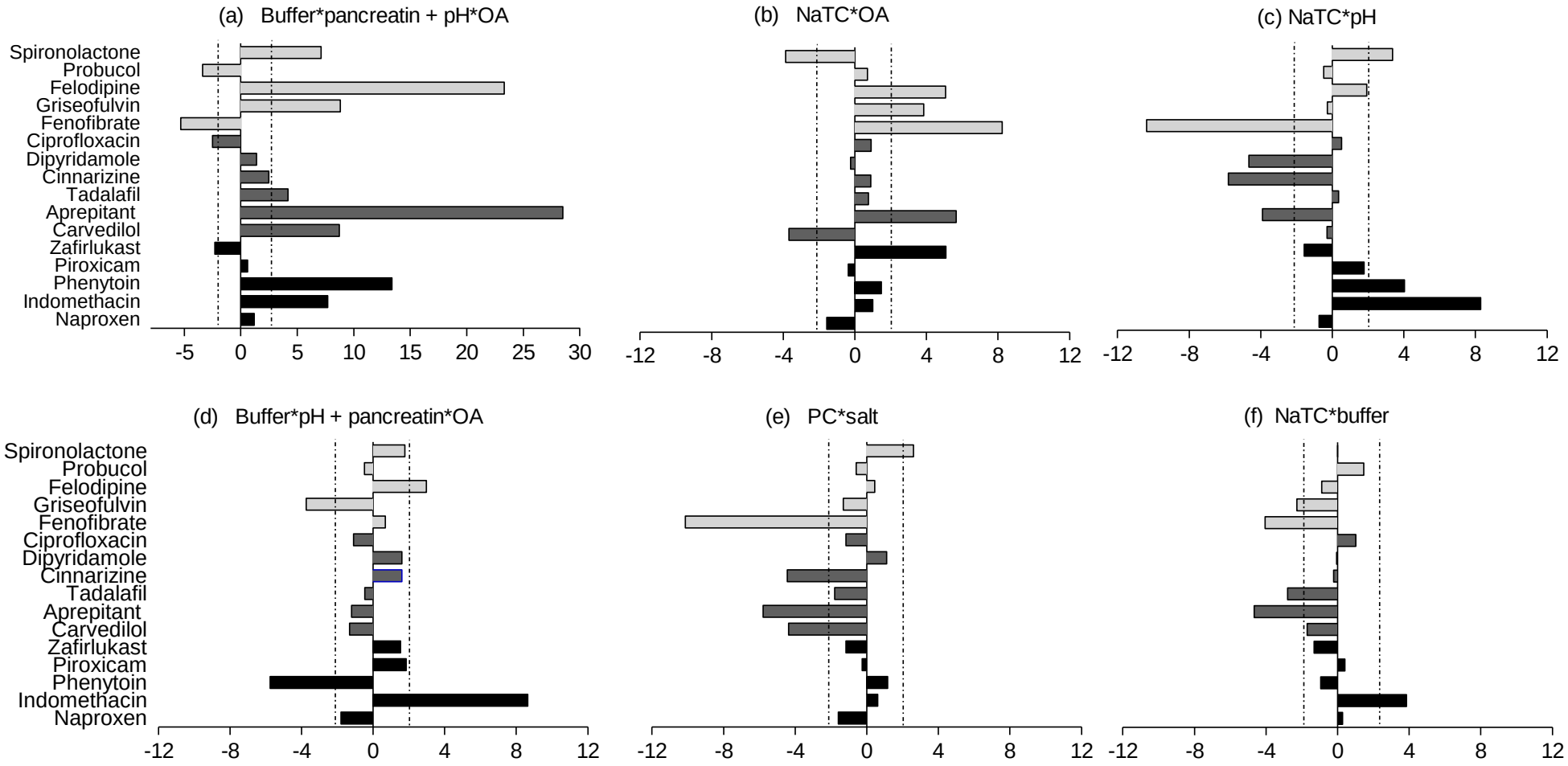
In general, the total magnitude of each interaction effect was smaller than corresponding single factors (Figure 2.3h and Figure 2.5s). Only one third of the 18 possible interactions display significance. Due to the fractional design of DoE, possible interactions between pH, OA, buffer and pancreatin were confounded. For example in the interaction effect of buffer*pancreatin and pH*OA, the effect could not be differentiated if it comes from buffer*pancreatin or pH*OA or both. Since pancreatin did not show any significance as a single factor and also exhibited no significant interactions with other non-confounded factors (NaTC, PC or salt), it was less likely to generate interaction effect with buffer, OA and pH. Therefore it was ignored and in these confounded interactions, effects was assigned to the pair without pancreatin, such as the pH*OA in the above example, and it also applies to other confounded interactions shown the Figure 2.5.

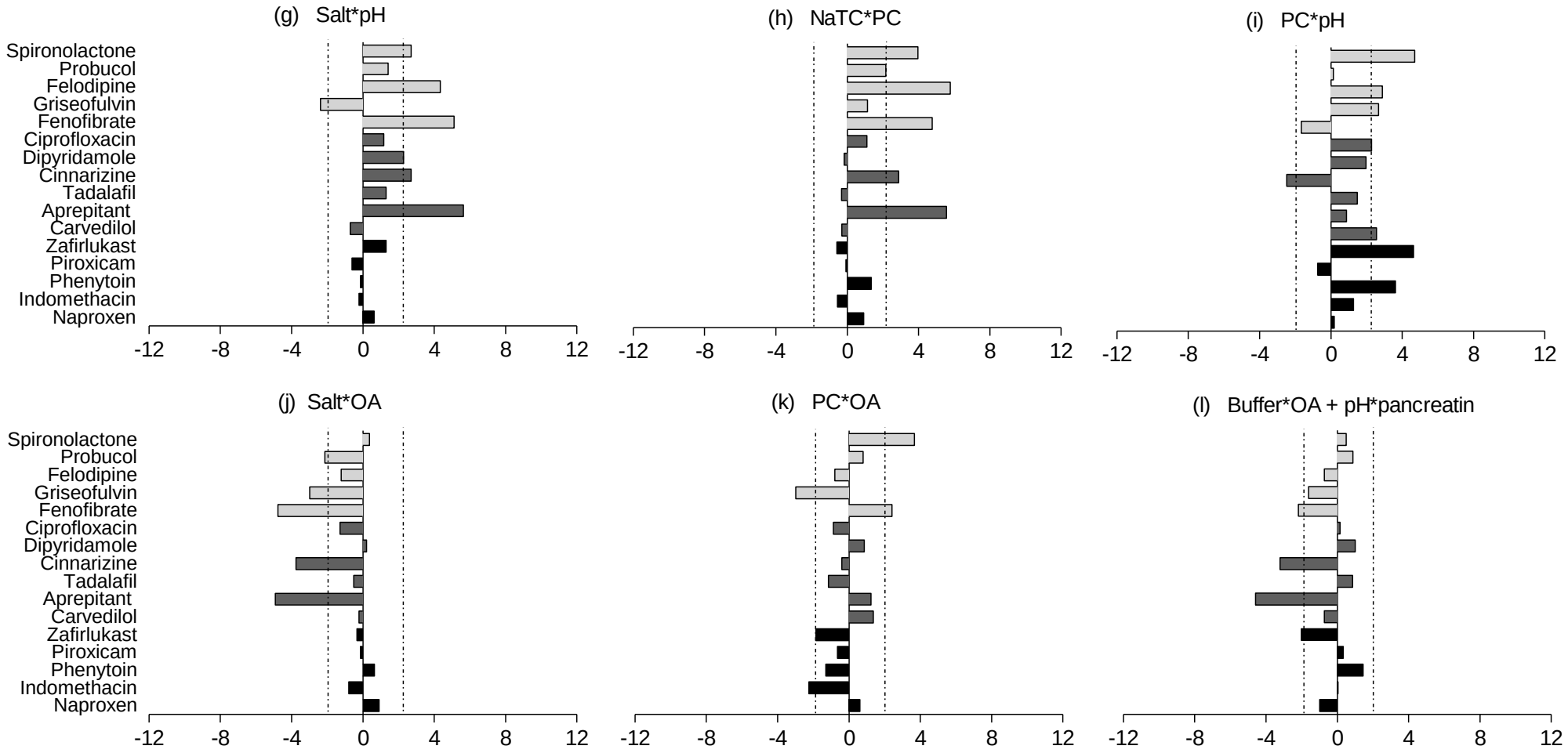
Overall, the interaction of pH and OA (pH*OA) has the highest magnitude (2 - 5 times higher) among all significant interactions. Acidic compounds were less involved in interactions due to the masking of dominating pH effect. On average, only three interactions pH*OA, NaTC*pH and buffer*pH were significant for acidic drugs (Figure 2.5s). More subtle interactions were present among basic and neutral drugs. On average, they both have the pH*OA as the most significant interaction (Figure 2.5s). For weak basic and neutral drugs, as the lipophilicity increased, the solubilisation effect of amphiphiles became more important and any factors affecting the amphiphiles can also generate significant interactions (Persson *et al.*, 2005, Clarysse *et al.*, 2009a). Literature has shown behaviour of OA in the

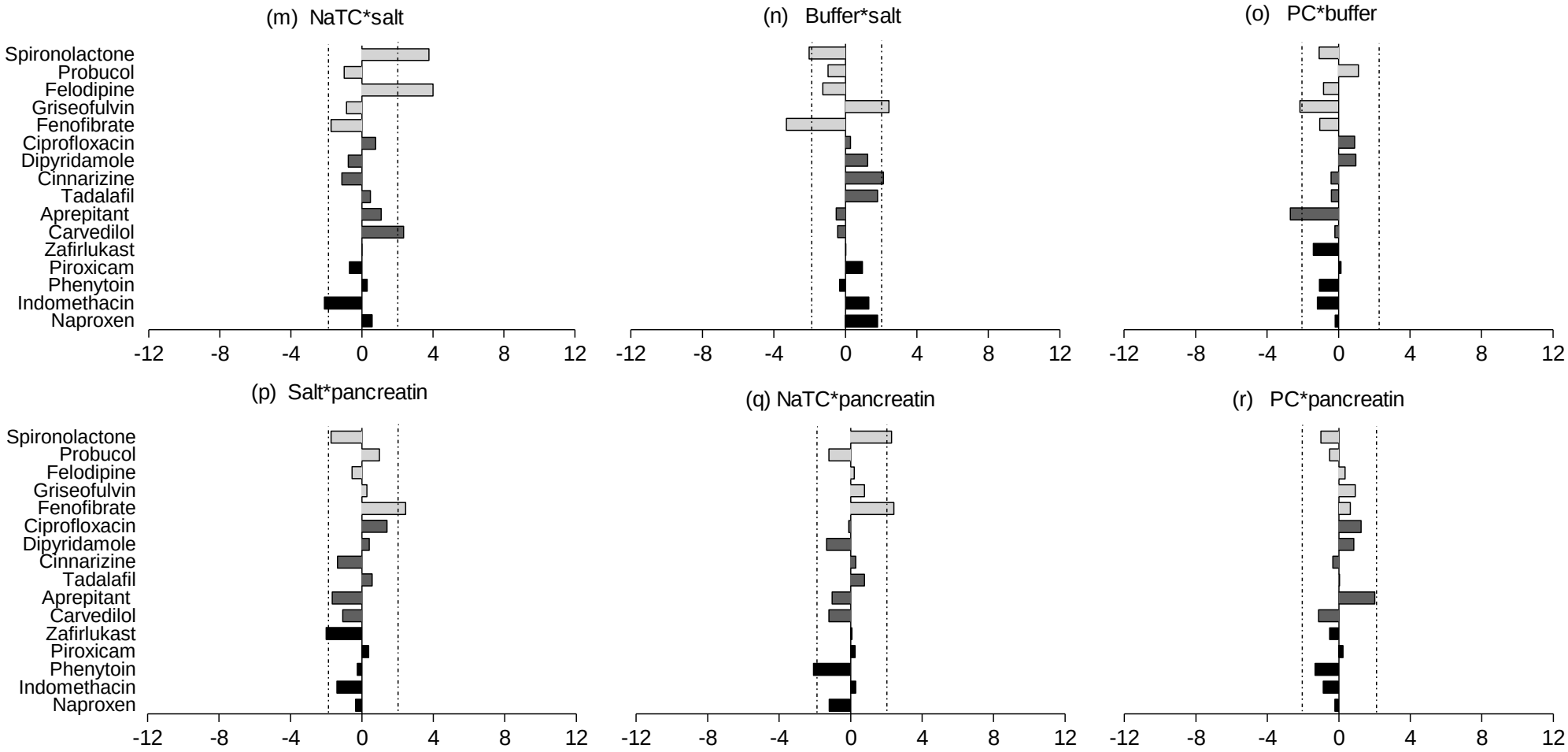
phase diagram with PC and lyso-PC was highly dependent on the pH of the solution (Bergstrand and Edwards, 2001). The pK_a of the carboxylate group of FFA has been reported to be around 5 at physiological salt concentration, but the apparent pK_a may rise to around 7 or above when FFA coexisted with PL bilayers (Ptak *et al.*, 1980). Therefore the pK_a of OA was within the DoE pH range and triggered changes in the percentage ionisation of OA, a higher pH resulted in more OA ionised and increased its aqueous solubility. This can have an influence on its solubilisation capacity for lipophilic drugs and interaction with other amphiphiles, the latter can induce higher levels of interactions. It also indicates how pH positively affected basic compounds (i.e. aprepitant, pK_a 9.7), mostly attributed to the synergic interaction of pH and OA rather than ionisation effect due to drug pK_a alone (pH negative effect). NaTC was fully ionised at pH 5 - 7 (Kleberg *et al.*, 2010) as anion, while pH can affect the ionisation stage of basic drugs if pK_a is close to 5 - 7 (i.e. dipyridamole pK_a 6.4). Therefore, pH-induced ion pair formation (Song *et al.*, 2013) between NaTC and the drug can lead to the significant positive pH* NaTC effect. However also notice that two neutral drugs (probucol and fenofibrate) showed negative pH*OA and pH*NaTC effects (Figure 2.5a and 2.5c), which cannot be explained with the above reasons, indicating in some cases a complex response of individual drug towards the pH*amphiphile, or even higher levels of interactions (i.e. NaTC*OA*pH) not considered in the DoE. For example, when pH affects the ionisation of the FFA, the characterisation of the micelle with NaTC and PC, and the solid-liquid interface may also be altered (Luner and Vander Kamp, 2001), the interaction can be complex and difficult to resolve based on the solubility data alone. The effect of pH to neutral drug has been observed with hydrocortisone (Pedersen *et al.*, 2000a) but pH interaction with media contents was difficult to identify, as tested media were not systematically designed and balanced, for example using FaSSIF and FeSSIF with pH at 6.5 and 5, but also altering the level of biorelevant amphiphiles (BS, PL, FFA etc.) at the same time (Soderlind *et al.*, 2010, Clarysse *et al.*, 2009a), and therefore subtle

effects and interactions maybe easily overlooked.

For amphiphile interactions, the NaTC*OA and NaTC*PC were mainly significant among basic and neutral drugs (7 drugs had significant NaTC*OA, 5 drugs had significant NaTC*PC), while PC*OA were significant for only 3 drugs with low ranking of magnitude (Figure 2.5b, h, k). The NaTC *OA and NaTC *PC were mainly synergistic (positive and significant) except for aprepitant and spironolactone which exhibited a negative NaTC*OA. NaTC and PC were reported as the classical solubilising micelle system in intestine to reduce CMC and increase drug solubility in lipid aggregates (Pedersen *et al.*, 2000a), and solubilisation and micelle sizes were more complicated in the system with additional FFA (Ilardia-Arana *et al.*, 2006). Figure 2.5s shows the magnitudes of amphiphile interactions were only half or even less than pH*OA. The different magnitudes may be correlated with drug-amphiphile specific affinity but also due to different concentration ranges used for seven factors in DoE, that is, the intrinsic variation of parameters in HIF itself might partially contribute to the results that some factors or interactions were more predominant than the others. Though DoE provides comprehensive information on effect significance, it relies on the factors and ranges chosen in the design and information extracted have to be interpreted in the light of these limitations. The amphiphile effects were equalised in the mixture design in section 3.


(a) Buffer*pancreatin + pH*OA
(b) NaTC*OA
(c) NaTC*pH
(d) Buffer*pH + pancreatin*OA
(e) PC*salt
(f) NaTC*buffer
Spironolactone
Probucol
Felodipine
Griseofulvin
Fenofibrate
Ciprofloxacin
Dipyridamole
Cinnarizine
Tadalafil
Aprepitant
Carvedilol
Zafirlukast
Piroxicam
Phenytoin
Indomethacin
Naproxen
-5 0 5 10 15 20 25 30
-12 -8 -4 0 4 8 12


(g) Salt*pH
(h) NaTC*PC
(i) PC*pH
(j) Salt*OA
(k) PC*OA
(l) Buffer*OA + pH*pancreatin
Spironolactone
Probucol
Felodipine
Griseofulvin
Fenofibrate
Ciprofloxacin
Dipyridamole
Cinnarizine
Tadalafil
Aprepitant
Carvedilol
Zafirlukast
Piroxicam
Phenytoin
Indomethacin
Naproxen
-12
-8
-4
0
4
8
12


(m) NaTC*salt
(n) Buffer*salt
(o) PC*buffer
(p) Salt*pancreatin
(q) NaTC*pancreatin
(r) PC*pancreatin
Spironolactone
Probucol
Felodipine
Griseofulvin
Fenofibrate
Ciprofloxacin
Dipyridamole
Cinnarizine
Tadalafil
Aprepitant
Carvedilol
Zafirlukast
Piroxicam
Phenytoin
Indomethacin
Naproxen
-12
-8
-4
0
4
8
12

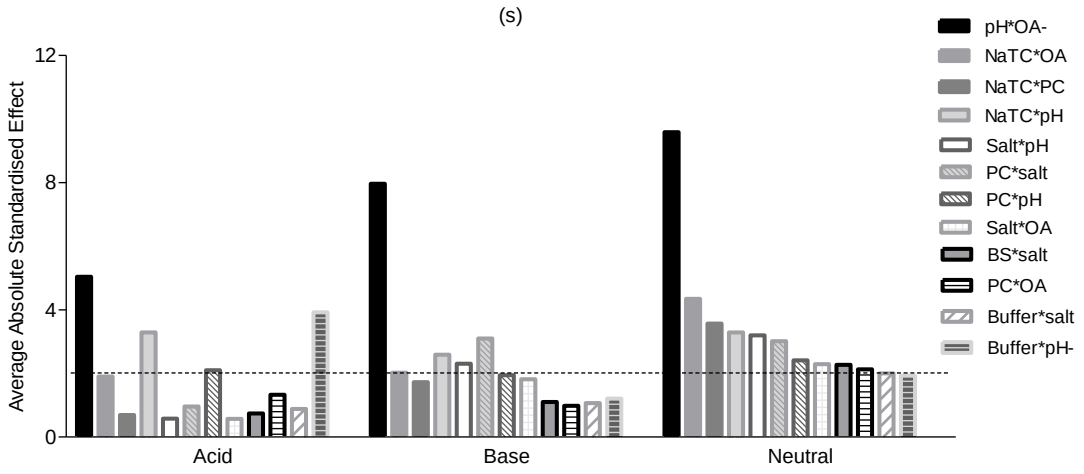
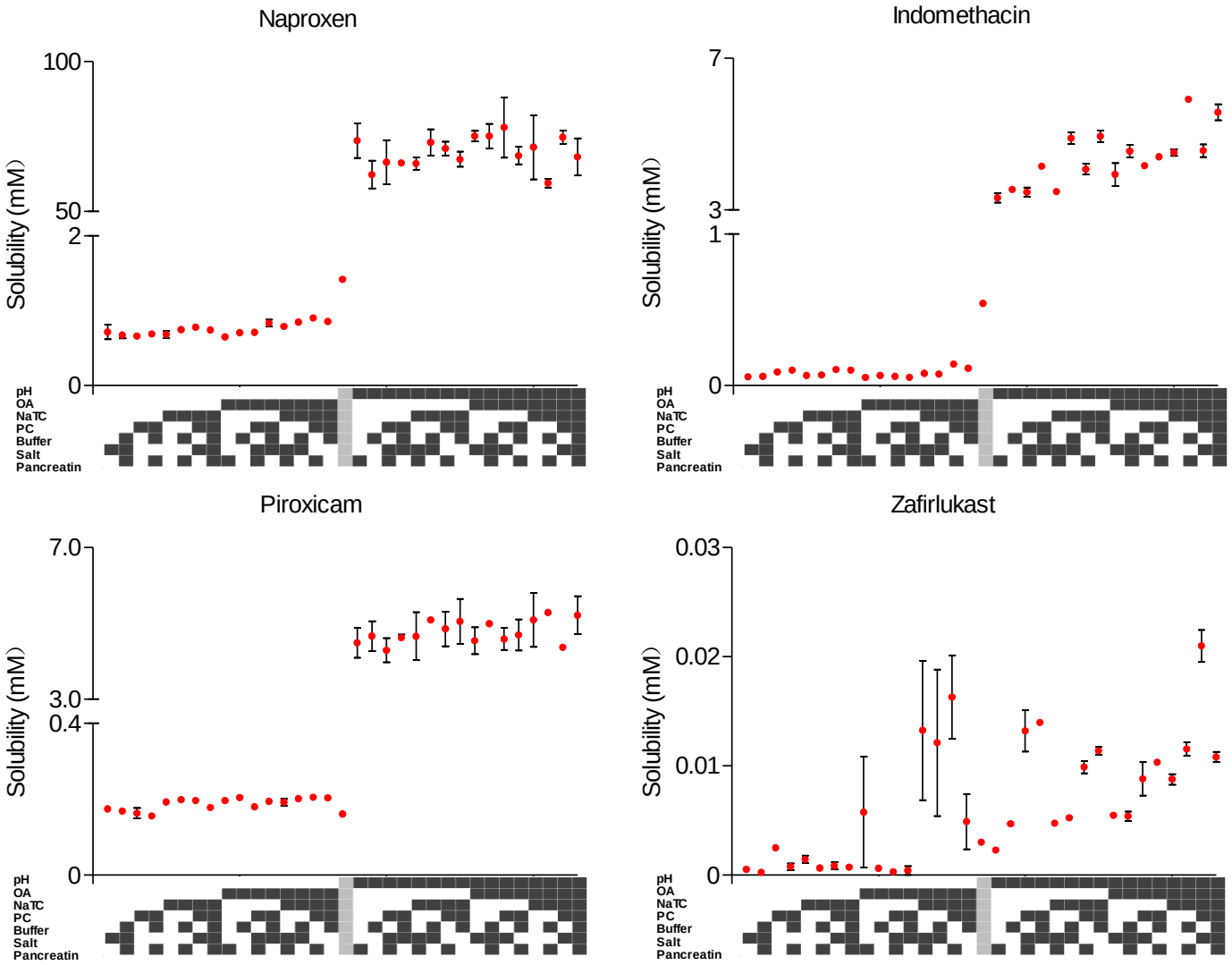


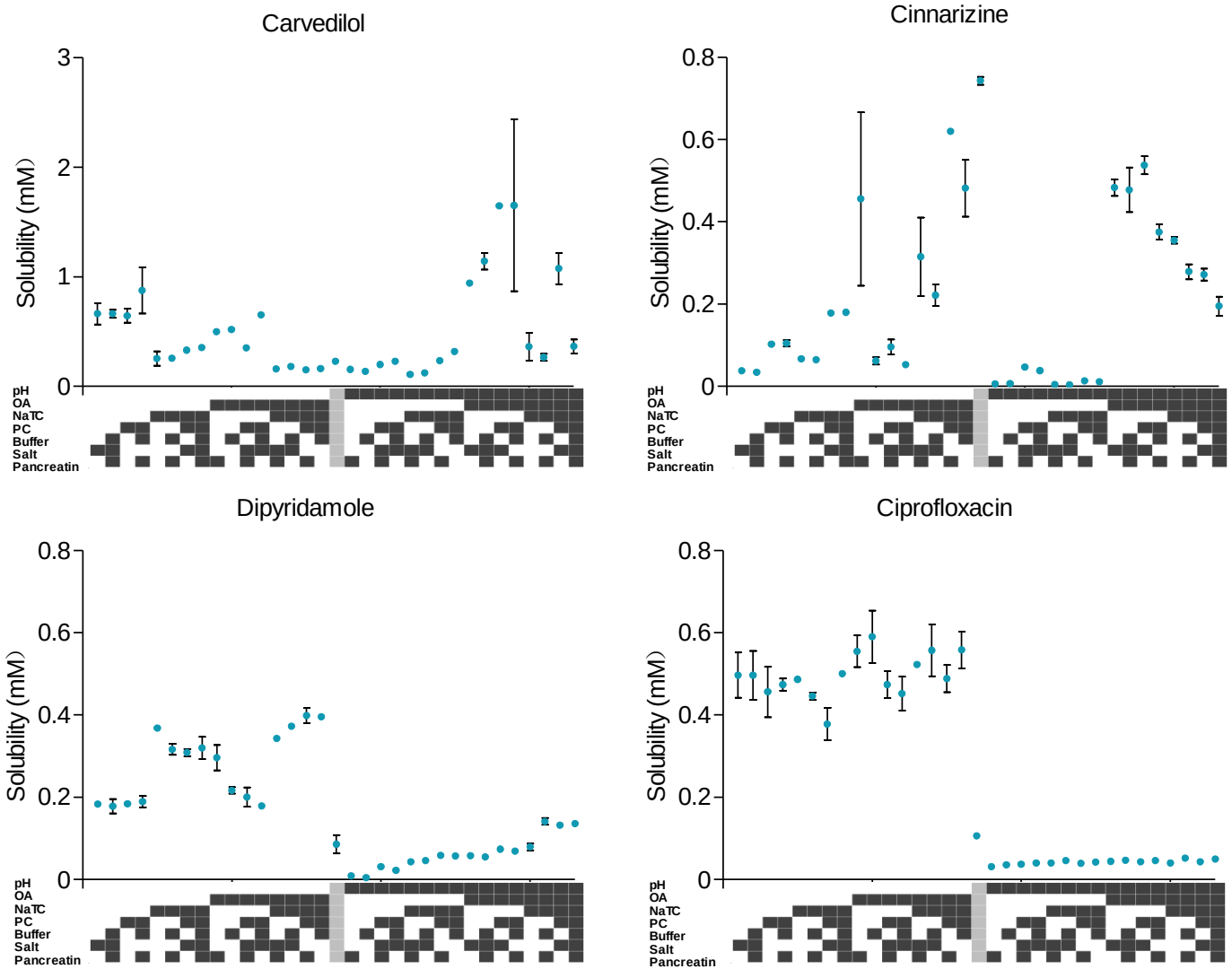
Figure 2. 5 Standardised effect values of 2-way interactions of each drug.

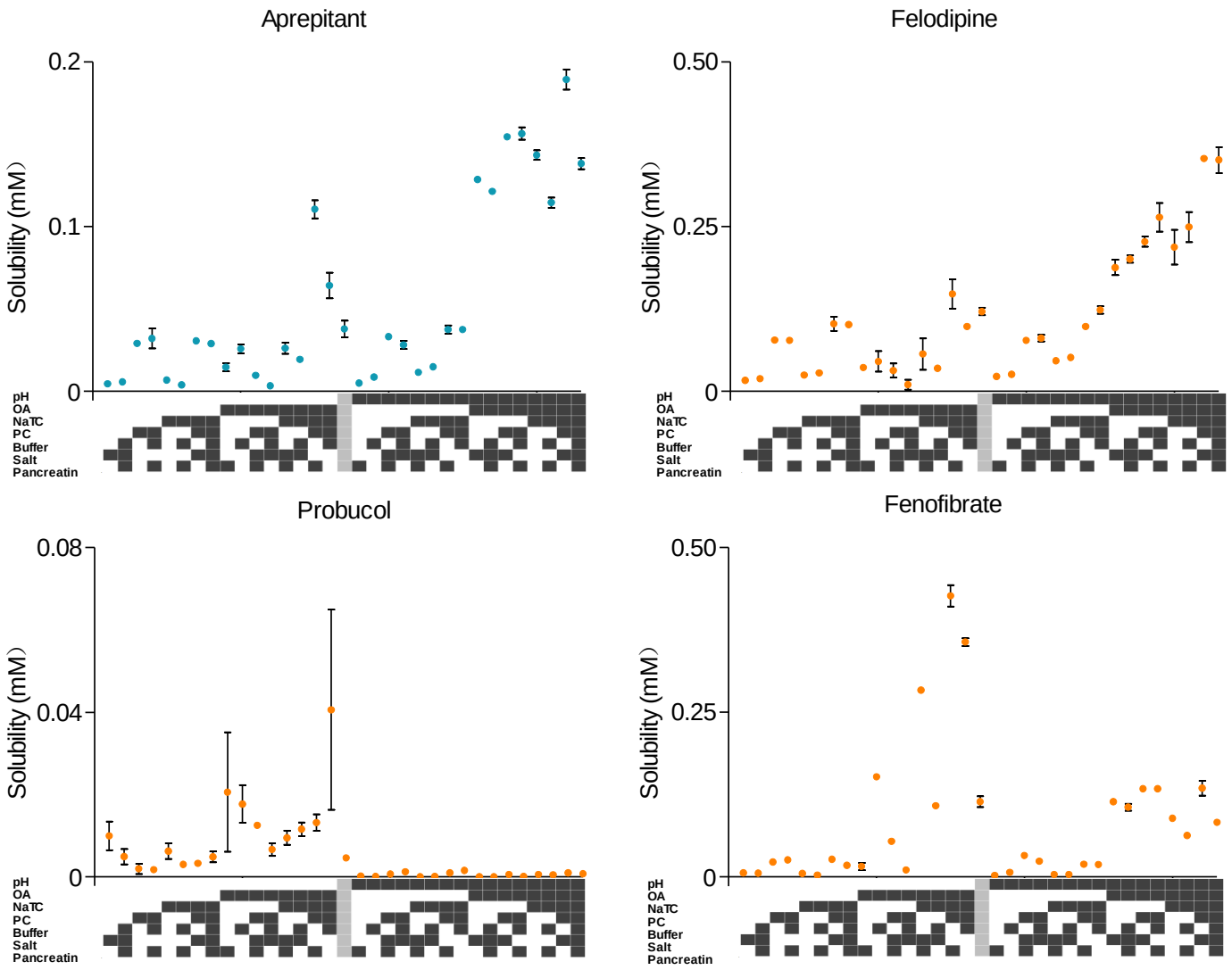
A bar over the dash line indicates the corresponding interaction is significant ($P < 0.05$). Figure 2.5s is the average value of the absolute standardised effect for each interaction grouped in three drug categories. For neutral drugs, the interactions are in descending order. Insignificant interactions NaTC*buffer, NaTC*pancreatin, PC*buffer, salt*pancreatin, buffer*OA, PC*pancreatin are not shown in Figure 2.5s.

2.2.5 Solubility patterns sorted by media factor

The DoE consists of 32 fasted SIF conditions and a centre point, all of which were measured in duplicate. Whilst the above figures display the summarised solubility and standardised effect, it does not provide visualisation of the solubility variation of individual drugs at different factor levels, except for pH effect to acidic compounds. Figure 2.6 presents the solubility of each drug in each medium; media were sorted from low to high level based on factors in the sequence of pH, OA, NaTC, PC, buffer, salt and pancreatin, according to their average magnitude ranking in Figure 2.3h. These solubility patterns visualise how each factor can influence the solubility of the drug.


Naproxen
Solubility (mM)
100
50
2
0
pH
OA
NaTC
PC
Buffer
Salt
Pancreatin
Indomethacin
Solubility (mM)
7
3
1
0
pH
OA
NaTC
PC
Buffer
Salt
Pancreatin
Piroxicam
Solubility (mM)
7.0
3.0
0.4
0
pH
OA
NaTC
PC
Buffer
Salt
Pancreatin
Zafirlukast
Solubility (mM)
0.03
0.02
0.01
0
pH
OA
NaTC
PC
Buffer
Salt
Pancreatin


Carvedilol
Cinnarizine
Dipyridamole
Ciprofloxacin
Solubility (mM)
pH
OA
NaTC
PC
Buffer
Salt
Pancreatin


Aprepitant
Solubility (mM)
0.2
0.1
0
Felodipine
0.50
0.25
Probucol
0.08
0.04
Fenofibrate
pH
OA
NaTC
PC
Buffer
Salt
Pancreatin

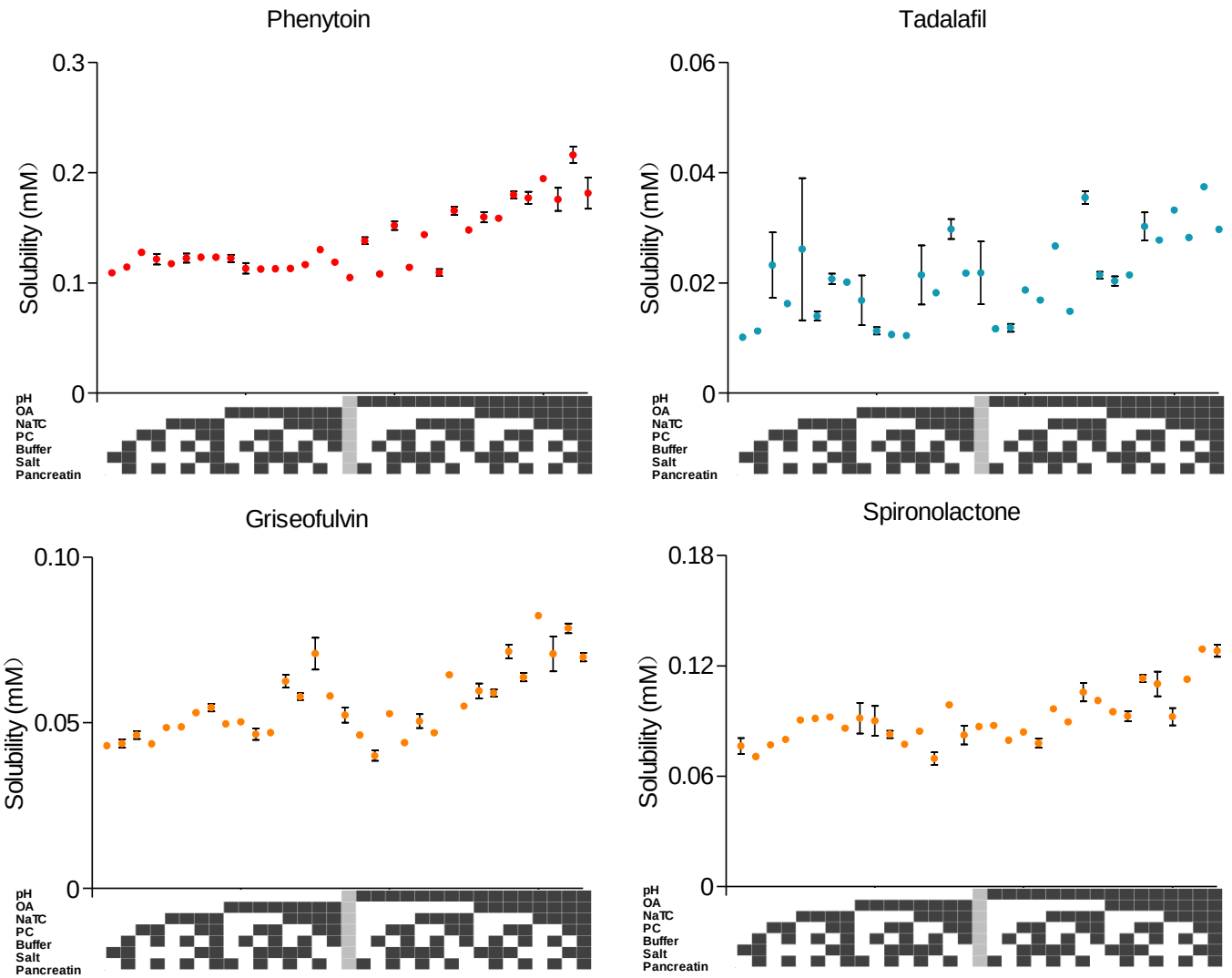

Phenytoin
Solubility (mM)
0.3
0.2
0.1
0
Tadalafil
0.06
0.04
0.02
Griseofulvin
0.10
0.05
Spironolactone
0.18
0.12
0.06
pH
OA
NaTC
PC
Buffer
Salt
Pancreatin

Figure 2. 6 Distribution of equilibrium solubility in relation to media components. Solubility value mean ± standard error of the mean (n = 2) for 33 media; Factors are grouped in the order of pH, OA, NaTC, PC, buffer, salt, pancreatin from low to high level; □ factor at low level in DoE; ■ factor at high level in DoE; ■ factor at central point in DoE. For values of levels see Table 2.1

As previously described, acidic drugs were distinctively discriminated by pH, with the exception of phenytoin and zafirlukast, where solubility distribution was in a range of 0.1 - 0.2 mM and 0 - 0.02 mM respectively. Phenytoin is located in a very tight range, with a slight increase across the distribution and the highest solubility appeared with high levels of pH, NaTC, OA and PC. The solubility of zafirlukast was scattered from 0 to 0.02 mM and had highest solubility with high level of PC and NaTC, but with no distinctive pattern. Zafirlukast with a pK_a 3.86, is fully ionised in the pH 5 to 7, however the in house Cheqsol data indicates the intrinsic solubility of zafirlukast is only 155 nM, and due to its lipophilicity, zafirlukast behaves like a weak base or neutral compound as its solubility is dominantly influenced by amphiphilic components. Notice the y-axis scale of the other three acids had to be divided into two parts to fit the data as the solubility has 10 to 100-fold magnitude variation induced by pH. The larger error bars at the high pH end also reflected the difficulty of pH adjustment with same precision during each experiment, as the solubility became very sensitive at small pH increments around pH 7, according to the Henderson-Hasselbalch equation (logarithmic relation between pH and solubility).

For basic compounds, except for ciprofloxacin, with a distribution distinctively divided by pH, the overall pH effect was less evident than acid and more interplay between the factors occurred as discussed in Section 2.2.4, leading to a unique distribution pattern for each drug. For example, under each pH subgroup of aprepitant, with OA changed from low to high level, there was a trend of increasing solubility, and it reached the highest solubility at the high level of both pH and OA, leading to an “N” shaped distribution, supporting the synergic interaction seen in

Figure 2.5a. Similarly, zafirlukast and cinnarizine had a similar tendency in each pH subgroup, complying with the result that OA was a significant factor for them, but also influenced by the involvement of other factors such as NaTC and PC, producing a more complicated pattern and requiring DoE to extract all the information. Multiple interactions affect the solubility of carvedilol in both directions (NaTC as negative effect, pH*OA as positive effect and NaTC *OA as negative effect), carvedilol gave a unique "U" shape solubility distribution.

For neutral compounds, fenofibrate and felodipine followed interesting patterns as explained in aprepitant, to indicate OA can solubilise the drugs and its solubilisation was affected by pH. For fenofibrate, highest solubility appeared at low pH and high OA (antagonistic interaction), while for felodipine, highest solubility appeared at high pH and high OA (synergic interaction), both visualise results from Figure 2.5a of positive (fenofibrate) and negative (felodipine) values of pH*OA. For griseofulvin and spironolactone, even with very tight solubility range, they also showed a subtle "N" shaped distribution, in accordance with Figure 2.5a. Probucol also had pH*OA interaction, but to the opposite direction, that is the highest solubility appeared at high level of OA but low level of pH, therefore an increase of solubility at the middle of the pattern. The above various results imply pH*OA was specifically related with drug properties. Probucol is a very lipophilic neutral drug with a log P around 10 and literature has shown that it was extremely insoluble even in biorelevant media (0.006 mM in FaSSIF, around 0.05 mM in fed HIF) (Persson *et al.*, 2005, Soderlind *et al.*, 2010), but has solubility of 0.155 mM in soybean oil (Zangenberg *et al.*, 2001). It is evident to observe its higher association with the neutral OA at pH 5 and similar studies support this phenomenon that probucol favoured the oil phase of triglycerides and solubility was affected by the hydrolysis of triglycerides (Zangenberg *et al.*, 2001). Christensen *et al.* also demonstrated its higher bioavailability when administered with food containing long chain

triglyceride than medium chain triglyceride (Christensen *et al.*, 2004). In summary, for neutral drugs or ionisable drugs with ionisation percentage unchanged at pH 5 - 7, the highest solubility generally appeared when at least three out of the four parameters, pH, OA, NaTC and PC, were in their high level, which not only demonstrates the importance of amphiphile solubilisation, but also the composition of the amphiphiles in the media and potentially the influence from pH.

Phenytoin, tadalafil, griseofulvin and spironolactone are four examples with tight solubility distributions in the DoE and they share some similarities. They are either neutral drugs or have a pK_a outside the DoE range of 5 - 7, thus ionisation percentage is not influenced by pH changes. In addition, they have comparatively low lipophilicity (log P phenytoin 2.29, tadalafil 1.64, griseofulvin 2, spironolactone 2.78) (Antunes *et al.*, 2008), MW (phenytoin 252, tadalafil 389, griseofulvin 353, spironolactone 416), and are rigid molecules compared with other BCS II drugs tested in DoE, therefore less sensitive to amphiphiles (Ottaviani *et al.*, 2010). Nevertheless, a subtle but evident solubility increase can still be identified from their solubility patterns with the increase of amphiphile concentration and pH (Figure 2.6). These indicate that they were still reactive to the amphiphiles solubilisation, but to a much lower extent than other drugs in the fasted SIF ranges and therefore solubility of those drugs are still not suggested to be analysed in a simple buffer, in addition their solubility variation still needs to be examined in fed state ranges. Comparison with literature for example, increased dissolution release was observed for phenytoin in both FaSSIF (36%) and FeSSIF (50%) compared with the corresponding aqueous buffer, due to the effect of bile components (Klein 2010). Solubility of griseofulvin was similar in buffer, fasted HIF, FaSSIF, and FaSSIF V2 (42 – 56 µM) (Soderlind *et al.*, 2010) but more than 3 time higher in fed HIF (170 µM) (Persson *et al.*, 2005). *In vivo* literature of those drugs reported food effect for phenytoin (C_{max} 18 µM to 25 µM, 40% increase for

postprandial) excluding the reasons of inhibited metabolism and reduced elimination half-life after food intake (Melander *et al.*, 1979, Sidhu *et al.*, 2004), another paper reported diverted variation of extraction rate among individuals (Sekikawa *et al.*, 1980). The individual variability and food effect were also reported for griseofulvin. For instance, the serum griseofulvin level, 4 h after a 1 g oral dose, was 0.9 μg/ml on average in fasted state and doubled to 2 μg/ml after food intake (n=10). The results proved that the increased plasma concentration was due to the fat in food which increased the GI absorption of griseofulvin rather than reduced serum clearance (Crounse, 1961a). The C_{max} for spironolactone was reported to be around 60 and 160 ng/ml with 200 mg oral dose before and after food intake (Overdiek and Merkus, 1986). Tadalafil is the only one among the four drugs to be reported with no food effects *in vivo* (Forgue *et al.*, 2006). However, when discussing about food effect, which is usually the ratio of blood concentration AUC at fasted and fed states, bioavailability is correlated with multiple factors such as food-induced decrease of liver first-pass metabolism (Pond and Tozer, 1984), dose (Singh, 2005), drug-food interactions (Lourenco, 2001), not just solubility. Additionally, the degree of "variability" can be subjective, as ratio and standard deviation can be artificially high if the denominator (i.e. original aqueous solubility) is small, while the actual relative increase is marginal. Meanwhile, more information is required in order to decide if the bioavailability change is of clinical importance, since if the drug has dose dependent metabolism or narrow therapeutic range, even a small bioavailability change can cause serious clinical consequences (Neuvonen, 1979). In summary, a tight solubility distribution in DoE does not negate high risk and food effect of the compounds, since it only indicates that the solubilisation capabilities of amphiphiles for these drugs are clearly limited. Those drugs probably have the "brick dust" characteristics with high crystal packing energy, which would hinder solubility (Stella and Nti-Addae, 2007, Wassvik *et al.*, 2008). From a formulation point of view, lipid formulation might be less

advantageous than particle size reduction (i.e. micronisation, solid dispersion) to achieve enhanced absorption (Kawabata *et al.*, 2011). A further study of the amphiphile effects was conducted and discussed with spironolactone and griseofulvin in Section 3.

2.2.6 Implication of design of experiment on SIF media selection

Phosphate buffer has long been used by pharmaceutical scientists for new drugs and dosage forms tests for routine quality control purposes (Vertzoni et al., 2004a). While carefully pH-adjusted buffer might be good enough to predict the behaviour of certain ionisable drugs (Soderlind *et al.*, 2010), this simple buffer does not sufficiently simulate the properties of GI fluids, and it is not suitable to predict the solubility of poorly soluble drugs and food effects (Dressman and Reppas, 2000). Using biorelevant media is not only mirroring the composition of GI fluids as close as possible, but also satisfying *in vitro* laboratory reproducibility and cost-efficiency (Wagner and Dressman, 2014), which leads to the concept of choosing the appropriate media according to the drug properties and testing requirements (absorption region, food effect etc.) based on a "decision tree" concept (Markopoulos *et al.*, 2015). Similarly, DoE of fasted SIF is a very useful tool to systematically illustrate that not all the GI components contribute the same proportion towards the solubility of different drugs and consequently the drug behaviour in GI tract. It is drug-dependent, and in certain scenarios, SIF can be simplified to a simple buffer, since the effects of other components are marginal, whilst for other drugs, care has to be taken to simulate the concentration of biological amphiphiles, food digestion products and ionic components such as buffer and salt.

For **acidic compounds**, pH is the vital factor and influences solubility in a range of 10 - 100 times, which can surpass the effects of other components. This substantial

variation has been reported before, for example Avdeef *et al.* presented pH-solubility profiles of 12 ionisable compounds, with solubility varying from 1 to 10^4 times difference in the whole pH range (1 - 12), in the pH 5 - 7, this range can still be as high as 10^3 times (Avdeef *et al.*, 2000). At pH 7, buffer capacity of the acidic drugs would shift the initial media pH and this is mainly contributed from more highly soluble ionised species dissolved in the media (Shoghi *et al.*, 2013). In fact, five acidic compounds (naproxen, indomethacin, piroxicam, ibuprofen, valsartan) with pK_a values between 4 - 5 were highly sensitive to the media pH and shifted the pH of the biorelevant media due to high solubility. For ibuprofen and valsartan, manually chasing the pH back to desired value and waiting for equilibrium were quite time-consuming and still cannot achieve accurate pH and reproducibility, therefore final data was not included in the DoE analysis (individual points shown in Figure 2.11 at the end of this section).

Similar to the above results, studies have shown that pH has much greater effect on the solubility and dissolution of acidic drugs glibenclamide (pK_a 4.32), glimepiride (pK_a 4.32), atorvastatin (pK_a 4.33) and furosemide (pK_a 4.25), while the concentration of NaTC and PC are not significantly relevant (Wagner and Dressman, 2014). A common characteristic of these compounds is their pK_a around 4 - 4.5, and here piroxicam, with a pK_a of 5.29 (Box *et al.*, 2006), also followed the same phenomenon (Yazdanian *et al.*, 2004). The development of SIF led to the addition of FFA and cholesterol into the fasted SIF (FaSSIF-V2plus, FaSSIF-V3), however both showed minimal contribution to better reflect the drug solubility in HIF for indomethacin, again due to the ionisation change dominated by pH (Koumandrakis *et al.*, 2014). In these examples, the biorelevant media did not show any advantages over a simple buffer. From a cost-efficient point of view, designing SIF for acidic drugs within this “pH-dominant zone” can be simplified, buffers covering proper pH range are sufficient to predict the drug behaviour in GI tract as a small

variation of pH can lead to an enormous drug ionisation and solubilisation. Due to the limited number of drugs tested, it is difficult to define the cut-off range of the "pH-dominant zone". Based on literature and results from DoE, compounds with pK_a between 4 - 5.5 and log P < 4 potentially fall inside the "pH-dominant zone". BCS was suggested to be too restrictive for a series of non-steroidal anti-inflammatory drugs (NSAID) (Yazdanian *et al.*, 2004, Rinaki *et al.*, 2004, Sheng *et al.*, 2006). Those weak acidic drugs contain carboxylic groups and low log P, thus potentially fall into this zone. Though classified as BCS II due to the limited solubility at gastric pH, they are highly permeable and can be regarded as BCS I drugs if considering solubility in the intestinal pH. In addition, the effect of BS and PL was marginal compared with pH and did not change the BCS category boundaries, which again emphasize the predominant role of pH for acidic drugs with pK_a close to intestinal pH. Figure 2.7 calculates the dose to solubility ratio, and shows that three pH-dominant drugs naproxen, indomethacin and piroxicam shifted from BCS II to BCS I based on solubility at pH 7. A shift of indomethacin from II to I was reported (Fagerberg *et al.*, 2010).

Importantly, phenytoin and zafirlukast, with pK_a value outside the 5 - 7 range, display much less pH effect and both remain in the BCS II region (Figure 2.7). However, the former has "brick dust" properties (rigid structure, low to moderate log P, high T_m) and showed tight solubility distribution (Wassvik *et al.*, 2008), while the latter is affected by amphiphile contents and has "grease ball" characteristics (rotatable structure, high log P, low T_m), which can be related to Lipinski's rule of five properties, such as lipophilicity and limited solubility even when ionised (Lipinski *et al.*, 1997). These acidic drugs can be considered as "neutral-like" drugs. However, the limited number of acidic drugs in this study leaves uncovered gaps for drugs with other property combinations. For example, drugs with pK_a values inside the DoE pH range but higher lipophilicity, or drugs with pK_a values outside DoE pH range

but generating moderate ionisation could be included for further study.

For **basic compounds**, a distinguishable boundary potentially exists for pK_a between 5 - 7 (±2). That is, when ionisation occurs in the fasted SIF pH (ciprofloxacin pK_a 6.2, cinnarizine pK_a 7.4, dipyridamole pK_a 6.4), pH effect is significant. Besides, lipophilicity can influence how dominant the pH factor is. Ciprofloxacin has a low lipophilicity and hence pH became the only significant factor that can negatively influence solubility through ionisation effect. While with more lipophilic drugs such as cinnarizine and dipyridamole, the pH effect is two-fold, one is the negative effect of ionisation and the other is directed by amphiphiles and their interactions with pH, which can be synergistic or antagonistic, making the overall results more complex. Figure 2.7 indicates only cinnarizine crosses the boundary of BCS II/I, while for dipyridamole and ciprofloxacin, even with 4 - 10 time solubility increase at low pH, they both stayed in BCS II due to their high oral dose (600 and 750 mg respectively). Similarly a few NSAID exceptions also raise the issue that in order to correlate BCS II with BCS I drug BA and potential biowaivers, dose effect has to be considered and solubility may not be sufficient for high dose drugs even when they are ionised substantially at pH 6.5 in intestine. One example is mefenamic acid with 25 times solubility enhancement from pH 5 to 6.5 (about 1 µg/ml at pH 5 and 25 µg/ml at pH 6.5), the recommended dose is 250 mg. In this case, the dose to solubility ratio is the major limitation for drug absorption (TenHoor *et al.*, 1991). On the other hand, when the compounds is fully unionised or ionised at intestinal pH (aprepitant, carvedilol, tadalafil), the weak basic drugs behave like neutral and pH alone would not dominate the solubility. Instead, the solubilisation of amphiphiles becomes dominate and lipophilicity determines how much the solubilisation effects could be. This can lead drugs such as carvedilol to cross the solubility boundary of BCS II/I. Nevertheless, similar as acidic drugs, gaps need to be covered in this category in order to gain more information.

Similar to these basic drugs that are lipophilic and ionisation unchanged, **neutral compounds** also show more pronounced solubilisation effects from biorelevant amphiphiles, with more convoluted interplay among amphiphiles and amphiphile(s) with pH, thus amphiphiles and pH have to be carefully adjusted in the media. Due to the negative net charge mainly contributed by OA and NaTC, micelles seem to have larger capacity to solubilise neutral or positively charged basic compounds at GI pH, whilst for acidic drugs, solubilisation is directed by pH rather than mainly benefit from micelle formation because of the charge repellent with negatively charged micelles (Zaki *et al.*, 2010b, Ottaviani *et al.*, 2010). Additionally, drugs with "brick dust" properties (griseofulvin, spironolactone) may have solid-state limited solubility profile and not sensitive to the changes in GI contents (Zaki *et al.*, 2010a). Not only neutral drugs but also ionisable drugs with ionisation state not affected by pH variation in fasted SIF can have "brick dust" properties (phenytoin, tadalafil).

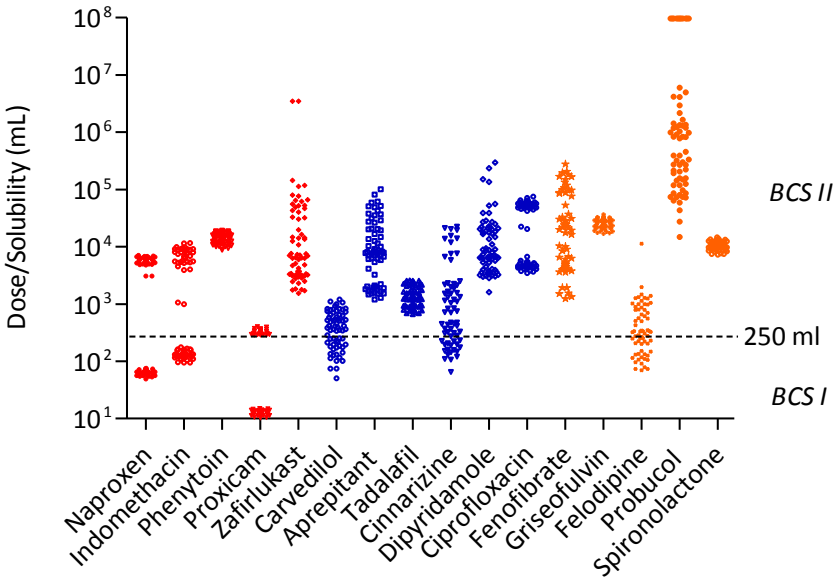


Figure 2. 7 The dose to solubility ratio (ml) of each drug.
Points include all solubility data from DoE (N=66). Ratio equals to volume required to dissolve the highest dose. Therefore the boundary between BCS I and II is when ratio equals to 250 ml.

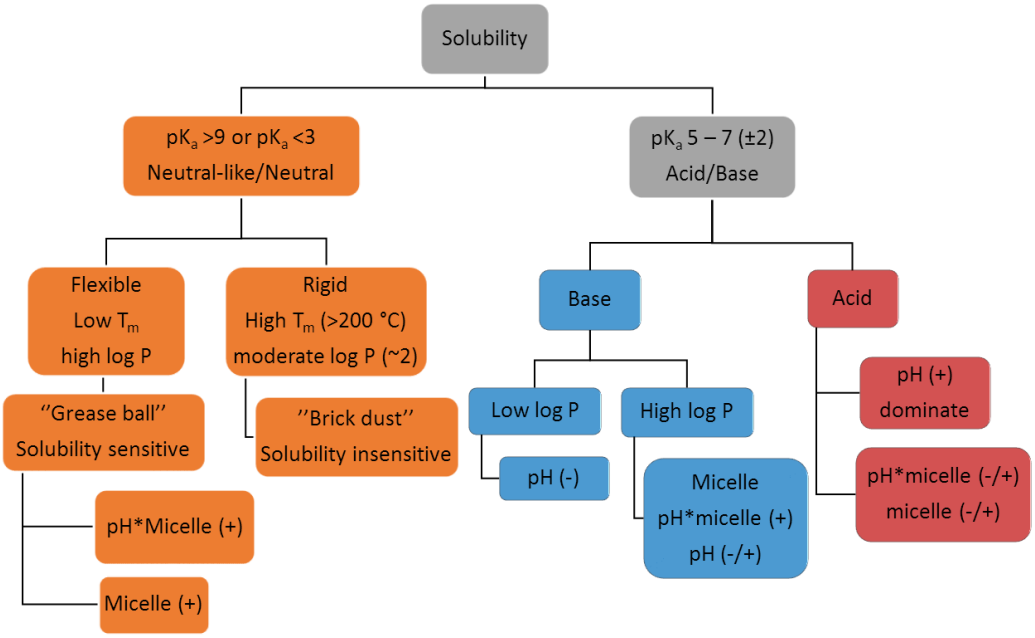


Figure 2. 8 A decision tree of the significant factors affecting solubility of insoluble drugs. The pK_a range of 3 - 9 are based on the equation 2 and pH 5 - 7 (±2) range in DoE, hence it is suggestive and need further validation. "Micelle" represents amphiphile contents NaTC, PC, OA in the media but not specified, similar as their potential interactions (*) with pH. (+) and (-) represent the factor as positive or negative effects.

In summary, a decision tree of SIF factors that could significantly affect solubility of insoluble drugs is presented in Figure 2.8. It is not necessary to have clear boundaries among acidic, basic and neutral drugs, but rather to identify if they are "ionisable" or "ionisation unchanged" under the fasted SIF pH range. Ionisable compounds include all acidic or basic drugs pK_a around 5 - 7 (±2). The suggested value of 4 - 5.5 and low to moderate log P (<4) give the "pH-dominant zone", where pH takes the lead to determine solubility. Solubility variation may arise with ionisable compounds or neutral-like compounds with "grease ball" properties (Wassvik *et al.*, 2008). When biorelevant media are utilised, ionisable drugs with low log P (naproxen, indomethacin, piroxicam) tend to shift from BCS II to BCS I, whilst dose has to be considered (dipyridamole and ciproflexacin). Lipophilic drugs are mainly affected by amphiphiles and pH, although the extent of amphiphile is

lower than pH but they can still induce solubility variation, in this case low dose drugs cinnarizine, carvedilol and felodipine can cross the BCS I/II boundaries (Zaki *et al.*, 2010a) (Figure 2.7). Due to the limited number of drugs tested, the map is suggestive only and requires further studies to establish general cut-off values.

Several papers have demonstrated that mixed micelles composed of lipids and NaTC can significantly increase the intestinal solubility of drugs with log P > 3 - 4 (Dressman and Reppas, 2000, Bergstrom *et al.*, 2007, Gamsiz *et al.*, 2010). These drugs are usually BCS II and IV drugs. This simple rule of thumb can be used to decide drug assessment using level II biorelevant media such as FaSSIF and FeSSIF (Markopoulos *et al.*, 2015) which simultaneously capture the pH and amphiphiles variation. However, influence of amphiphiles and their mixtures are generally positive, the interaction with pH can be more divergent, as observed in DoE, depending on the ionisation and charge change of both API and amphiphiles, therefore both pK_a and lipophilicity are critical knowledge to consider before media selection.

The variation of the composition and solubilising capabilities of HIF is substantial (Clarysse *et al.*, 2009b, Clarysse *et al.*, 2009a), thus it is important to target the solubilisation capability of HIF, rather than the exact composition of HIF when designing SIF (Augustijns *et al.*, 2014). However, replacing all components with simple/pure ingredients at a single level is still difficult. More importantly, based on current knowledge, most drug display large solubility variation and utilising ingredients at their single levels only lead to one solubility value that potentially can locate anywhere in the solubility distribution, without gaining information of the value range of the drug. A more practical strategy is to mirror the critical components in the media in a variation window and looking at the sensitivity of API in a series of media capturing the variation of critical media factors.

2.2.7 Principal component analysis

To find out the correlation of the solubility distribution and the physiochemical properties of the drugs, a principal component analysis (PCA) was attempted for the current data. PCA is a factor extraction technique to handle data with high dimensionality and inner correlation. It transforms the original variables and extracts principle components, which are not linearly correlated to represent the original variables (loading fractional information of variables). The number of principle components is less or equal to the number of original variables, and the principle components are in ranking so that the first few components represent most of the variables and the information extracted is displayed as percentage loading (Jolliffe, 2002).

The physiochemical variables considered were MW, log P, pK_a, polar surface area (PSA), hydrogen bond donor and acceptor, rotatable bond (RoB), T_m and intrinsic solubility (S_o). DoE variables considered were solubility average (AveSol), solubility standard deviation (SD) and relative standard deviation (RSD) in DoE. Since pK_a has different meaning for acidic and basic compounds, the data were transformed to the percentage ionisation at pH 7 and 5 (denoted as “pH5ion” and “pH7ion”), “+” and “-” indicated the charge of basic and acidic drugs respectively. Ionisation percentage difference (IonDiff) is the ionisation percentage difference at pH 7 and pH 5, and calculated according to pK_a in Table 2.5 and Henderson-Hasselbalch equation (Equation 3).

Table 2. 5 Physiochemical properties and solubility data of DoE for each drug

Drug	MW	Log P	pH 5 ion	pH 7 ion	IonDiff	PSA (Å2)	HB Donor	HB Acceptor	RoB	T_m	So(μM)	SolAve (mM)	SD	RSD	pKa
Ionisation unchanged															
Spironolactone	416.57	2.78	0.00	0.00	0.00	60.44	0	3	2	208	44.00	0.09	0.01	0.16	
Griseofulvin	352.766	2	0.00	0.00	0.00	71.06	0	6	3	220	14.79	0.06	0.01	0.20	
Tadalafil	389.404	1.64	0.00	0.00	0.00	74.87	1	4	1	301	19.83	0.02	0.01	0.39	15.17
Felodipine	384.259	3.86	0.00	0.00	0.00	64.63	1	5	6	145	0.28	0.11	0.10	0.88	
Aprepitant	534.427	4.8	1.00	1.00	0.00	75.19	2	11	6	254	1.50	0.05	0.06	1.04	9.7
Fenofibrate	360.831	5.24	0.00	0.00	0.00	52.6	0	4	7	80.5	0.81	0.08	0.10	1.29	
Probucol	516.844	10	0.00	0.00	0.00	40.46	2	4	8	126	0.01	0.01	0.01	1.77	
Ionisable															
Zafirlukast	575.676	6.4	-0.95	-1.00	-0.05	115.73	2	6	9	139	0.15	0.01	0.01	0.92	3.68
Cinnarizine	368.51	5.77	1.00	0.72	-0.28	6.48	0	2	6	119	3.20	0.22	0.24	1.11	7.4
Dipyridamole	504.6	3.95	0.96	0.20	-0.76	145.44	4	12	12	163	7.90	0.17	0.14	0.82	6.4
Carvedilol	406.474	3.91	1.00	0.86	-0.13	75.74	3	5	10	114.5	24.60	0.48	0.44	0.90	7.8
Indomethacin	357.787	3.8	-0.76	-1.00	-0.24	68.53	1	4	4	158	16.60	2.16	2.20	1.02	4.5
Naproxen	230.2	3.24	-0.83	-1.00	-0.17	46.53	1	3	3	153	72.44	34.26	35.13	1.03	4.3
Piroxicam	331.4	3.06	-0.24	-0.97	-0.73	99.6	2	6	2	199	122.21	2.41	2.34	0.97	5.5
Phenytoin	252.268	2.29	0.00	-0.07	-0.07	58.2	2	2	2	286	58.90	0.14	0.03	0.22	8.1
Ciprofloxacin	331.3	-1.08	0.94	0.14	-0.80	72.88	2	6	3	256	253.00	0.26	0.23	0.88	6.2

Log P, intrinsic solubility and pK_a are experimental data from literature where available (Cordero *et al.*, 1997, Wassvik *et al.*, 2006, Völgyi *et al.*, 2007, Llinas *et al.*, 2008, Mehanna *et al.*, 2010, Planinsek *et al.*, 2011), other information from DrugBank. Acidic, basic and neutral drugs are coloured in red, amber and blue respectively.

The correlation matrix in Table 2.6 indicated the linear correlation of every two parameters. High linearity has been highlighted such as MW and RoB, log P and S_o, PSA and hydrogen donor/acceptor, and pH5ion and pH7ion. Interestingly, pH5ion has a correlation of 0.901 with pH7ion and AveSol has a correlation of 1 with SD. SD is an indication of variability of the data and affected by the magnitude of the original data especially when different set of data have varying absolute magnitude. In figure 2.2, the solubility of the 16 drugs has a magnitude range of 10^{-4} – 10^{2} mM, therefore it is reasonable to see the linearity between AveSol and SD. Thus inclusion of RSD, which divides SD with the mean, makes it less biased to compare different drugs.

Initial setting of two principle components explained a total of 53.8% of the whole information, with the first component extracted 30.4% and the second component 23.5%. A third component added 17.4% information, making the total of 71.3% information extracted. Numbers of aromatic rings and total rings were used initially but only made 65% information extracted; therefore they were not included in the database.

Figure 2.9a displays the position of each factor in the space of component 1 and 2. This figure indicates how each factor is correlated with component 1 and 2, for example MW has 0.842 loading for component 1 and -0.138 loading for component 2, therefore component 1 reflects major information of MW. Points far from original points (or close to circle) are better extracted. In summary component 1 mainly extracts information of MW, RoB, hydrogen bond donor and acceptor, pH5ion, pH7ion and DoE solubility information (AveSol and Std, but not RSD). Component 2 mainly describes S_o, T_m, IonDiff and log P. Additionally, factors on the diagonal lines across the zero point have a close negative correlation, and factors close to each other are positively correlated, with a prerequisite that both points are

far from the centre. Therefore, S_o and T_m are both negatively correlated with log P, MW is positively related with RoB, supporting the information from Table 2.6. High lipophilicity is correlated with poor aqueous solubility (Lipinski *et al.*, 2001). Solid-state limited solubility (crystal packing and high T_m) is more likely to occur when compounds have lower to moderate lipophilicity (log P <3) (Zaki *et al.*, 2010a), which supports the negative correlation of log P and T_m. T_m negatively correlated with RSD and log P positively correlated with RSD, though both r values are small (~0.65). Veber *et al.* reported RoB, PSA and hydrogen bonds tended to increase together with MW (Veber *et al.*, 2002). Unfortunately only around 70% information is accumulatively extracted from DoE attributes (AveSol, SD), it is difficult to draw conclusion what factors they are correlated to. Information such as hydrogen bond acceptor and donor, PSA, even though they may be correlated with each other, it is very hard to find their practical correlation with other drug physiochemical properties.

In Figure 2.9b, component 3 complimentary extracts information from DoE attributes (AveSol, SD), but not as much information from other factors as component 1 and 2, as most of the factors are close to y = 0 axis. Solubility average and SD are negatively correlated with pH7ion. Notice that negativity is caused by the "-" sign given to the acidic drugs, rather than the literal "ionisation percentage" negatively correlated with solubility. Conversely, it indicates the more negatively ionised drugs in the media, the more average solubility and SD, again implying the dominating influence of acidic drugs in the database. Log P and T_m were mainly extracted from component 2 and were negatively correlated according to Figure 2.9a and Figure 2.9c.

Table 2. 6 Correlation matrix.

Correlation	MW	logP	pH5ion	pH7ion	IonDiff	PSA	HBdonor	HBacp	RoB	Tm	So	SolAve	SD	RSD
MW	1													
logP	0.572	1												
pH5ion	0.184	-0.136	1											
pH7ion	0.253	0.03	0.901	1										
IonDiff	0.133	0.376	-0.311	0.131	1									
PSA	0.424	-0.18	-0.027	-0.226	-0.432	1								
HBdonor	0.363	0.057	0.319	0.107	-0.493	0.674	1							
HBacp	0.58	-0.016	0.406	0.252	-0.378	0.735	0.611	1						
RoB	0.653	0.581	0.323	0.302	-0.074	0.355	0.552	0.487	1					
Tm	-0.194	-0.642	0.107	0.076	-0.08	0.154	0.063	0.118	-0.65	1				
So	-0.447	-0.661	0.129	-0.167	-0.664	0.061	0.146	-0.043	-0.438	0.399	1			
SolAve	-0.477	-0.078	-0.408	-0.431	-0.011	-0.19	-0.094	-0.211	-0.204	-0.125	0.152	1		
SD	-0.475	-0.076	-0.408	-0.43	-0.009	-0.191	-0.095	-0.21	-0.202	-0.127	0.15	1	1	
RSD	0.3	0.69	0.029	-0.018	-0.106	-0.194	0.183	0.087	0.505	-0.649	-0.107	0.124	0.127	1

SolAve, solubility average in DoE; HBdonor and HBacp, hydrogen bond donor and acceptor; S_o, intrinsic solubility; T_m, melting point (°C). Highlighted numbers mean the correlation is significant at 0.01 level

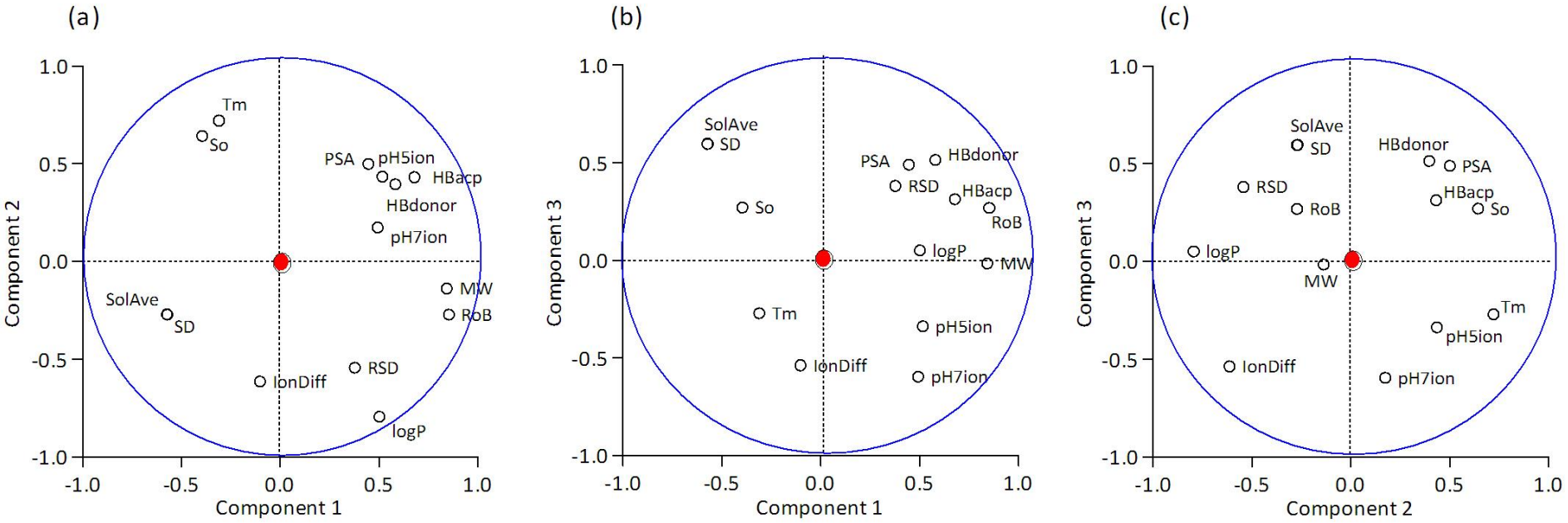


Figure 2. 9 Loading information of factors.

Figures in the space of component 1 and 2 (a), component 1 and 3 (b), component 2 and 3 (c). SolAve, solubility average in DoE; HBdonor and HBacp, hydrogen bond donor and acceptor; S_o, intrinsic solubility

PCA gives some useful information, but not to a predictable level due to the limited numbers of drugs (n=16) in the input covering the drug space (Lindenberg *et al.*, 2004) for statistical analysis and modelling. Even though high MW, high lipophilicity, number of hydrogen bond donor/acceptor can have high probability of poor solubility, but this simply cannot cover all possibilities as variables are inter-connected but not linearly correlated. Each parameter described one aspect of the drug but did not reflect the overall features holistically. Therefore it is very difficult to gain simple correlation of drug variability and drug properties, as the relationship may be divergent and non-linear.

However, if analysing the drug sensitivity represented by RSD strategically, interesting information can be found based on the decision tree concept in Section 2.2.6. Compounds are divided into two groups according to their pK_a and IonDiff values (Figure 2.10). In the "ionisable" subgroup, though pH is a critical factor, the magnitude of IonDiff is not the main driver for solubility variability. For example acid drugs can still have high RSD even though IonDiff is small (i.e. Naproxen -0.17). Instead, in the ranking of log P, low lipophilic drugs (mainly acid) either display segmental solubility by pH or insensitivity due to solid-state limited solubility (phenytoin), while high lipophilic drugs can also display high RSD due to a combination of pH and amphiphile variation in different media (i.e. zafirlukast). For "ionisation unchanged" group, log P has a clearer correlation with RSD (r^2=0.86), indicating the interaction between drug and amphiphile is mainly directed by lipophilicity and causes solubility variation.

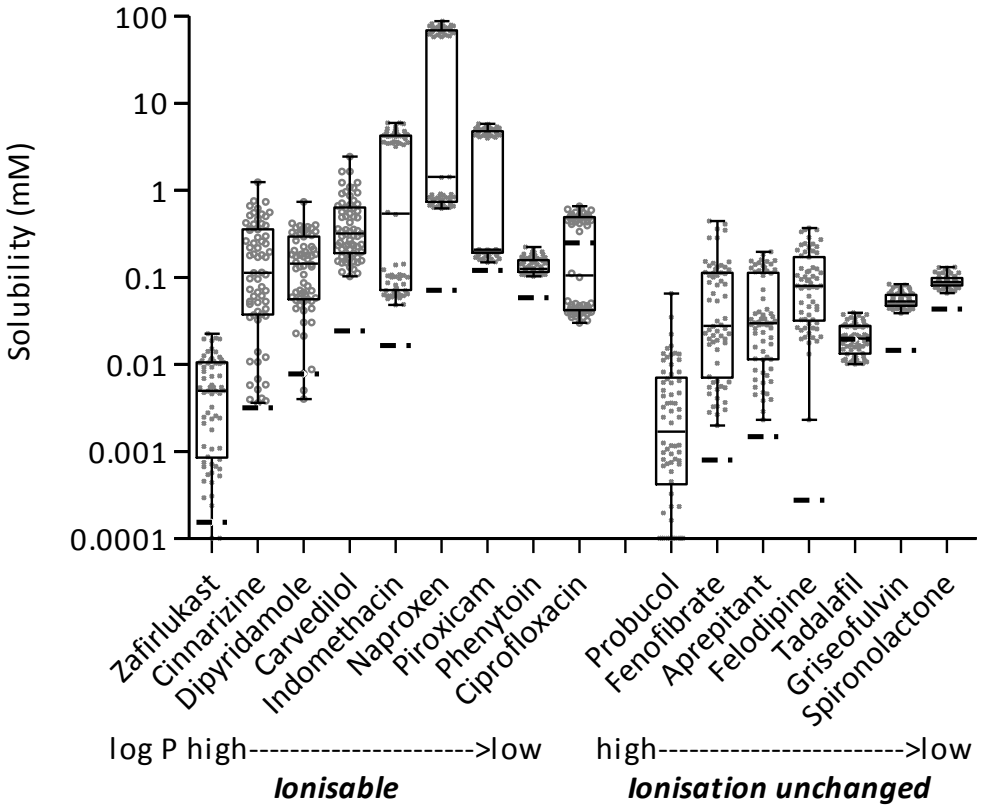


Figure 2. 10 Solubility distribution with the decision tree concept.

Left side displays "ionisable" compounds in descending order of log P. Right side displays "ionisation unchanged" compounds also in descending order of log P. Box whiskers cover min to max value, indicate the magnitude of RSD, and dash line (- -) is the S_o.

2.3 Conclusion

This section investigated the equilibrium solubility of 16 BCS II drugs in 66 different fasted SIF recipes. This series of fasted SIF were designed in fractional factorial design to systematically and simultaneously investigate the influence of seven commonly considered factors in fasted SIF on drug solubility, which were pH, NaTC, PC, buffer, salt, OA and pancreatin. The range of each factor covered the physiological data in GI tract and also potential individual variability. The technique is applicable to drugs or candidate compounds. The solubility data

obtained was comparable to previous literature measured in both fasted biorelevant media and HIF.

DoE illustrates the statistical significance of the factors and also to what extent and direction it may potentially influence drugs' solubility. Except pancreatin, all of the remaining six factors (pH, OA, NaTC, PC, buffer and salt) display statistically significant effects on drug solubility. Some of the solubilisation effect and drug specific interactions have been previously reported, such as NaTC, PC and pH (Persson *et al.*, 2005, Kleberg *et al.*, 2010, Soderlind *et al.*, 2010), while some interactions have not been extensively investigated or reported, such as pH*OA. This indicates that DoE methodology is capable of capturing drug performance in fasted intestinal fluid *in vivo*, and also providing interesting insight and quantification measurements of the effects of different media components. Ionisable drugs with a pK_a close to physiological pH and low lipophilicity were predominately affected by pH, in accordance with Henderson-Hasselbalch equation. Solubility of acidic drugs increased with pH and difference can reach a magnitude of 100 times, while solubility of basic drugs decreased with increased pH. Lipophilic drugs were mainly affected by amphiphiles OA, NaTC and PC. These lipophilic drugs were mainly neutral drugs or ionisable drugs, for which ionisation less affected between pH 5 - 7. The mean analysis of the effects for all 16 drugs indicates pH was the predominant single factor, followed by OA, NaTC, PC, buffer and salt. OA and pH had the highest magnitude among all the interactions. With only five acidic, six basic drugs and five neutral drugs, it only provides a gross overview and the mean analysis of the effects and detailed information has to be interpreted from individual drugs.

The current DoE is still a time-consuming and labour intensive experiment. A partially automated solubility screening (PASS) approach was developed which

involves robotic liquid handling and sample separation for HPLC analysis on a 96-well plate, and can provide high throughput results that complied well with literature data (Alsenz *et al.*, 2007). This would be possible equipment but require adaptation such as pH adjustment and centrifugation.

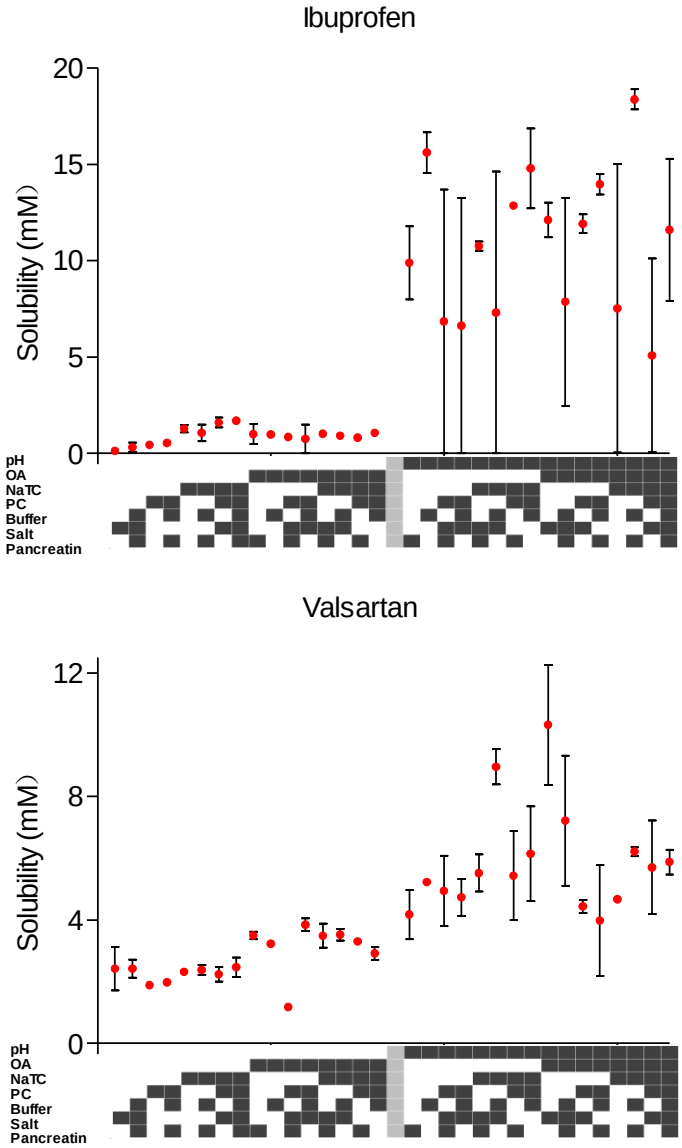


Figure 2. 11 Solubility data of ibuprofen and valsartan in relation to media components. Solubility value mean ± standard error of the mean (n = 2); Factors are grouped in the order of pH, OA, NaTC, PL, buffer, salt, pancreatin; □ Factor at low level in DoE; ■ factor at high level in DoE; ■ factor at central point in DoE. For values of levels see Table 2.1.

3. Mixture Design

In Section 2, the results indicate that the solubility of several basic, neutral drugs and one acid drug zafirlukast is predominately influenced by OA, NaTC and PC. In order to fully visualise the amphiphile interactions and solubilisation capacity of NaTC, PC and OA, a solubility study was conducted in a series of mixed amphiphile/water media in which the amphiphiles were maintained at physiological concentrations. The fourth amphiphile used was monoglyceride (MG), representing one of the main digestion products in the fed state. The system consists of NaTC, PC, OA and MG at various molar ratios but a constant total molar concentration, constructed by a 4-component Mixture Design (4MD) (Eriksson *et al.*, 1998). There were one acidic, two basic and four neutral BCS II drugs selected from Section 2 due to their various interactions with amphiphiles. In order to eliminate the effect of pH and OA interaction, the 4MD was only conducted at pH 7 where OA were better ionised and solubilised. The buffer and salt concentrations were also kept at biorelevant levels identical to the previous DoE.

3.1 Materials and methods

3.1.1 Total concentration of 4-component Mixture Design

Based on DoE media in Table 2.2, there are eight possible combinations of NaTC, PC and OA at high or low levels, which are displayed in Table 3.1. A medium total amphiphile concentration (11.7 mM) was chosen for the 4MD. 4MD contained 39 different combinations in the tetrahedron contour plot, including 4 combinations inside the tetrahedron, and 35 combinations on the four surfaces of the tetrahedron. Each surface represented a phase with the absence of one of the four amphiphiles and therefore the four faces were NaTC/PC/OA, NaTC/PC/MG, NaTC/OA/MG and PC/OA/MG surfaces. Each face had 15 combinations, with 5 points on the side shared by two surfaces (Figure 3.1). Concentrations were provided in mol% of the total amount. The water content was more than 99 wt% for all the composition,

indicating this was a dilute system.

Table 3. 1 Concentration (mM) of three amphiphiles used in the previous DoE based on Table 2.2. The possible combinations for 3 factors in 2 levels are 2^3=8.

	NaTC	PC	OA	Total concentration
1	1.5	0.2	0.5	2.2
2	1.5	1	0.5	3
3	5.9	0.2	0.5	6.6
4	5.9	1	0.5	7.4
5	1.5	0.2	10	**11.7**
6	1.5	1	10	12.5
7	5.9	0.2	10	16.1
8	5.9	1	10	16.9

4MD was constructed using Minitab® 16.0, simplex lattice with four component input. Degree of lattice of 4 and the design augmented with axial points, which means there were also points inside the tetrahedron rather than just on the surface. This 4MD has 39 different media with detailed concentrations and composition in Table 3.2. The solubility was determined in these media and data were analysed in Minitab® 16.0 to fit into quadratic and special cubic models. Solubility of each surface of the tetrahedron was also fitted into ternary contour plots with smoothing in OriginPro® 2015.

Table 3. 2 4MD detailed media composition, with component ratio of the total 11.7 mM and the concentration of NaTC, MG, PC and OA

	Molar ratio				**Concentration (mM)**			
	NaTC	MG	PC	OA	NaTC	MG	PC	OA
1	0	0	0.25	0.75	0.0	0.0	2.9	8.8
2	0.25	0	0	0.75	2.9	0.0	0.0	8.8
3	0	0	0	1	0.0	0.0	0.0	11.7
4	0.25	0	0.25	0.5	2.9	0.0	2.9	5.9
5	0	0.25	0.25	0.5	0.0	2.9	2.9	5.9
6	0.25	0.25	0.5	0	2.9	2.9	5.9	0.0

7	0	0.5	0.5	0	0.0	5.9	5.9	0.0
8	0.125	0.125	0.625	0.125	1.5	1.5	7.3	1.5
9	0.25	0.5	0.25	0	2.9	5.9	2.9	0.0
10	0.25	0	0.5	0.25	2.9	0.0	5.9	2.9
11	0.5	0	0.25	0.25	5.9	0.0	2.9	2.9
12	0.5	0	0.5	0	5.9	0.0	5.9	0.0
13	0.125	0.625	0.125	0.125	1.5	7.3	1.5	1.5
14	0.25	0.25	0.25	0.25	2.9	2.9	2.9	2.9
15	0.625	0.125	0.125	0.125	7.3	1.5	1.5	1.5
16	0	0.25	0	0.75	0.0	2.9	0.0	8.8
17	0.75	0	0.25	0	8.8	0.0	2.9	0.0
18	0	0.25	0.75	0	0.0	2.9	8.8	0.0
19	0.125	0.125	0.125	0.625	1.5	1.5	1.5	7.3
20	0	0	1	0	0.0	0.0	11.7	0.0
21	0.25	0	0.75	0	2.9	0.0	8.8	0.0
22	0	0	0.5	0.5	0.0	0.0	5.9	5.9
23	0.5	0.25	0	0.25	5.9	2.9	0.0	2.9
24	0.5	0.25	0.25	0	5.9	2.9	2.9	0.0
25	0.25	0.25	0	0.5	2.9	2.9	0.0	5.9
26	0.5	0.5	0	0	5.9	5.9	0.0	0.0
27	0.5	0	0	0.5	5.9	0.0	0.0	5.9
28	0.25	0.75	0	0	2.9	8.8	0.0	0.0
29	0.75	0.25	0	0	8.8	2.9	0.0	0.0
30	0	0.75	0.25	0	0.0	8.8	2.9	0.0
31	0.25	0.5	0	0.25	2.9	5.9	0.0	2.9
32	1	0	0	0	11.7	0.0	0.0	0.0
33	0.75	0	0	0.25	8.8	0.0	0.0	2.9
34	0	0	0.75	0.25	0.0	0.0	8.8	2.9
35	0	0.25	0.5	0.25	0.0	2.9	5.9	2.9
36	0	0.5	0.25	0.25	0.0	5.9	2.9	2.9
37	0	0.5	0	0.5	0.0	5.9	0.0	5.9
38	0	0.75	0	0.25	0.0	8.8	0.0	2.9
39	0	1	0	0	0.0	11.7	0.0	0.0

3.1.2 Stock solution preparation

Phosphate buffer containing 68 mM NaCl and 45 mM NaH_2PO_4 was prepared with

deionised water and pH adjusted to 7. The NaTC /PC/OA surface of the 4MD was prepared as follows: stock solutions (according to the concentration in Table 2.1) of NaTC, OA and PC were freshly prepared from solids dissolved in the phosphate buffer. The PC stock solution was prepared by dissolving lipid in chloroform, removing the chloroform by evaporation under nitrogen and dissolving the dried PC film into phosphate buffer. Stock solutions of NaTC, PC and OA were added into each tube and the required volume of phosphate buffer added for a final volume of 4 ml. The rest of the 4MD were prepared as follows: stock solutions of NaTC, PC, OA and MG were prepared with phosphate all in the concentration of 11.7 mM. MG cannot dissolve in buffer, so the stock MG was prepared by mixing NaTC (1 mM) and MG (10.7 mM), making it to a total concentration of 11.7 mM, and for practical experimental reasons this solution was employed as 100 mol% MG. All mixtures were prepared in duplicate and solubility determined.

3.1.3 Equilibrium solubility measurement and HPLC

The equilibrium solubility measurement and HPLC methods used were the same as in Section 2.1.

3.1.4 Zeta potential

For the surface containing NaTC/PL/OA mixture, the Zeta potential of each mixed solutions was measured in a Malvern™ clear disposable zeta cell (DTS 1060C) by using a Malvern™ Zetasizer Nano instrument and each medium measured in triplicate. Average zeta potential data were fitted into ternary contour plot with smoothing in OriginPro® 2015.

3.2 Results and discussion

3.2.1 Structure of the contour plot

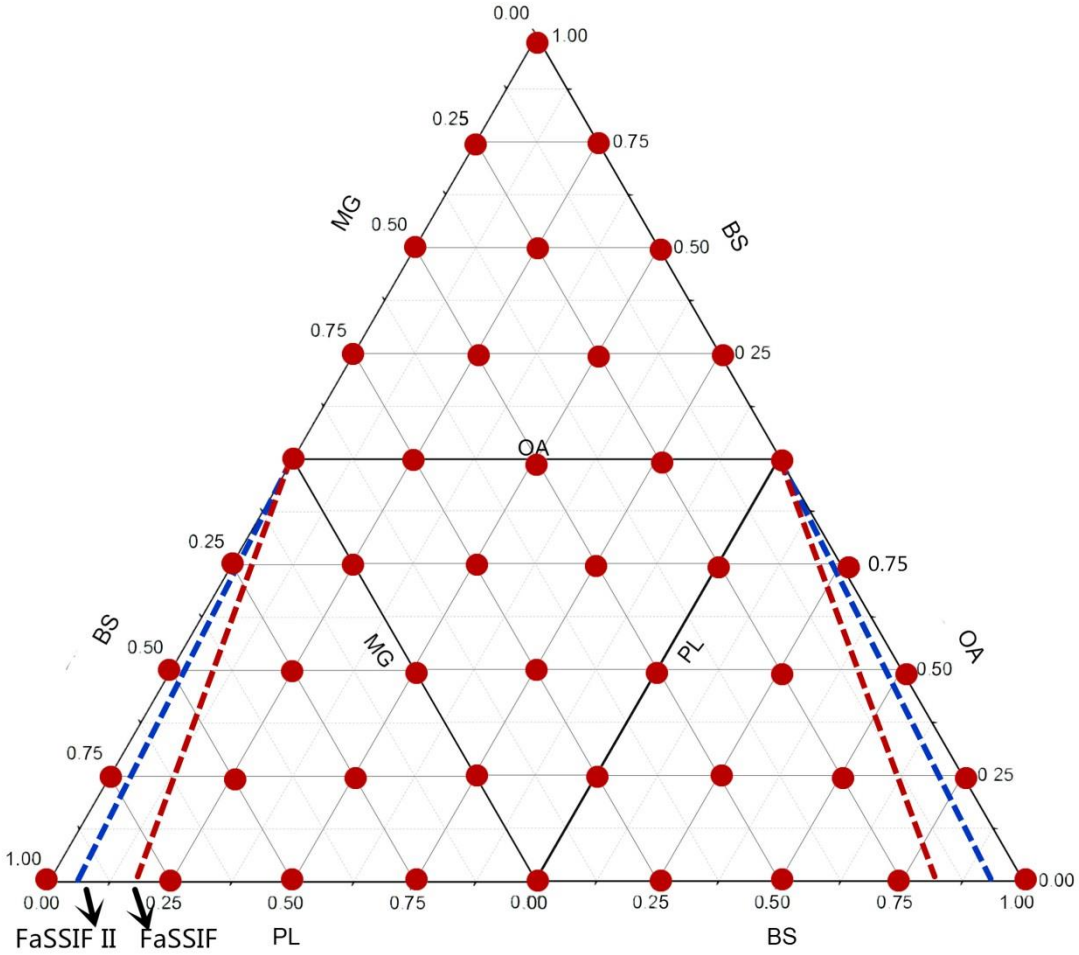
A tetrahedron included all the media combinations in the 4MD. The tetrahedron

was composed of four surfaces; each surface was a triangle and represented a medium composed of 100 mol% of three amphiphiles and 0% of the forth amphiphile. Each side of the triangle represented media composed of 100 mol% of two of the three amphiphiles. For practical visualisation, the tetrahedron was peeled from the vertex representing the medium containing 100 mol% NaTC. Therefore the four pieces of triangles can be placed flat in two dimensions as a large main triangle, and the three vertices of the big triangle all represent the same medium of 100% NaTC. The first graph in Figure 3.1 indicates the four pieces. Each crossing points represent a medium combination in the 4MD and solubility measured for each drug, and notice that there are 4 points inside the tetrahedron that cannot be shown in the graph.

3.2.2 Equilibrium solubility contour plots

Figure 3.1 presents solubility contour plot drawn according to the measured solubility in each point in 4MD, excluding points inside the tetrahedron. The colour shades indicate solubility magnitudes, with darker the colour the higher the solubility. None of the seven drugs had identical high solubility zones and also the solubility variability was different for each drug. In addition, each drug displayed distinctive interactions with the four amphiphiles, for example, the solubility of zafirlukast only increased when the proportion of PC increased, while the carvedilol solubility increased with OA ratio. In the case of fenofibrate and felodipine, an appropriate mixture of amphiphiles provided better solubility (also see Table 3.2). Individual solubility data points on each facet of the tetrahedron for each drug are present in Figure 3.2. The solubility of spironolactone and griseofulvin only varied in a narrow range, while others (i.e. carvedilol) can have a 250-fold variation. The fifth column displays the additional four points inside the tetrahedron with mixture of all amphiphiles. For each drug, Figure 3.2 shows that the four facets almost have the same range of drug solubility, with a few exceptions that did not cover the

lower solubility points. For example, the NaTC/PC/OA combination for carvedilol did not cover solubility below 0.1 mM, while the other three faces have. In addition, the media representing the four points inside the tetrahedron did not increase solubility variability.



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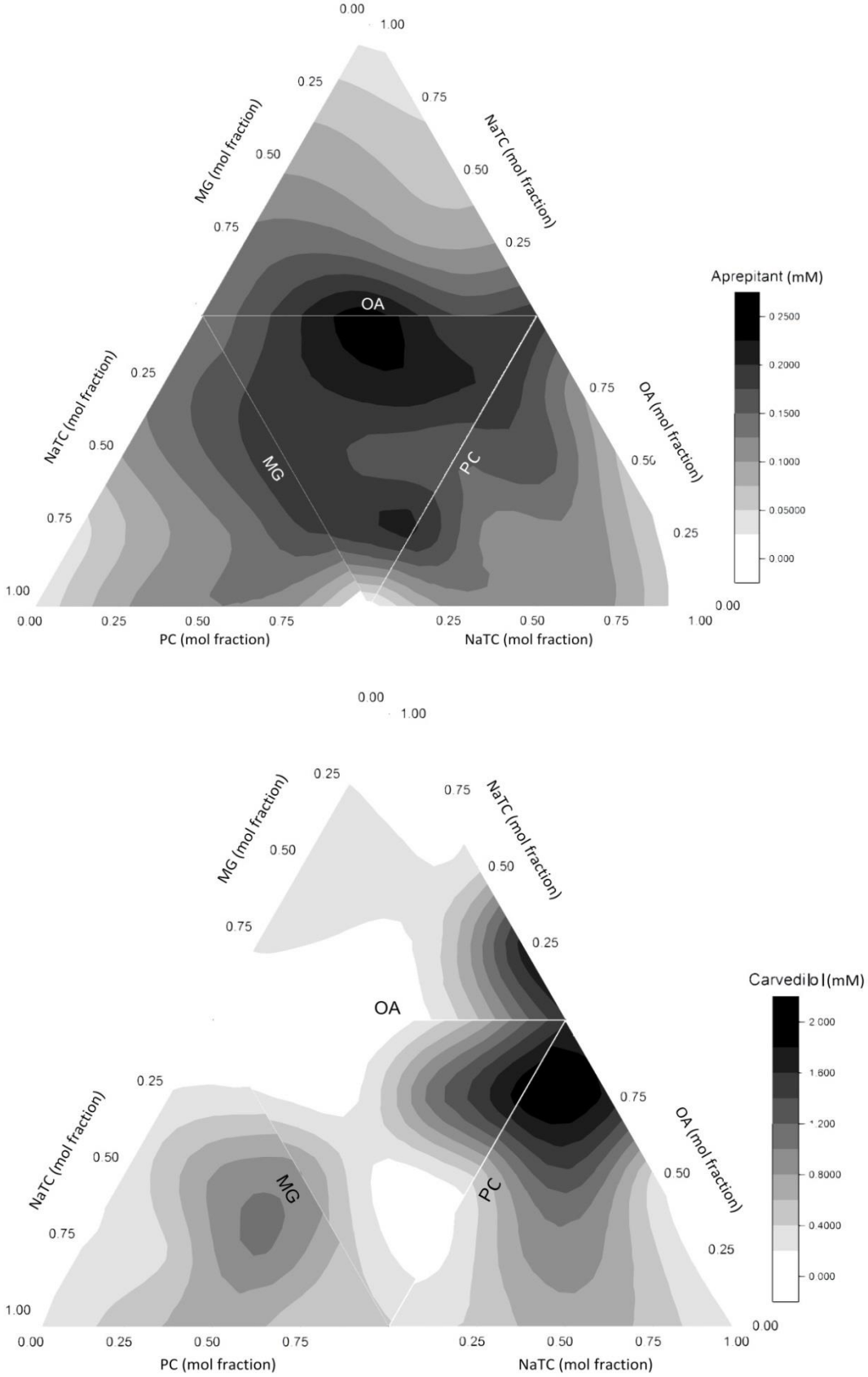

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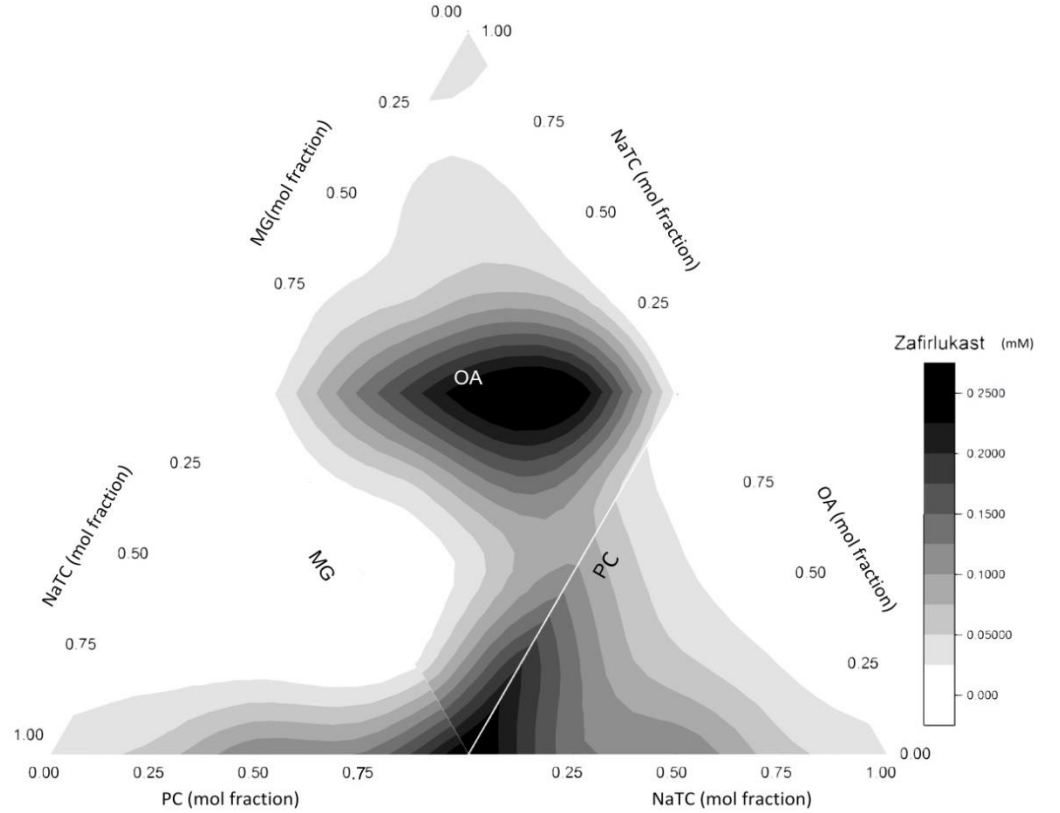

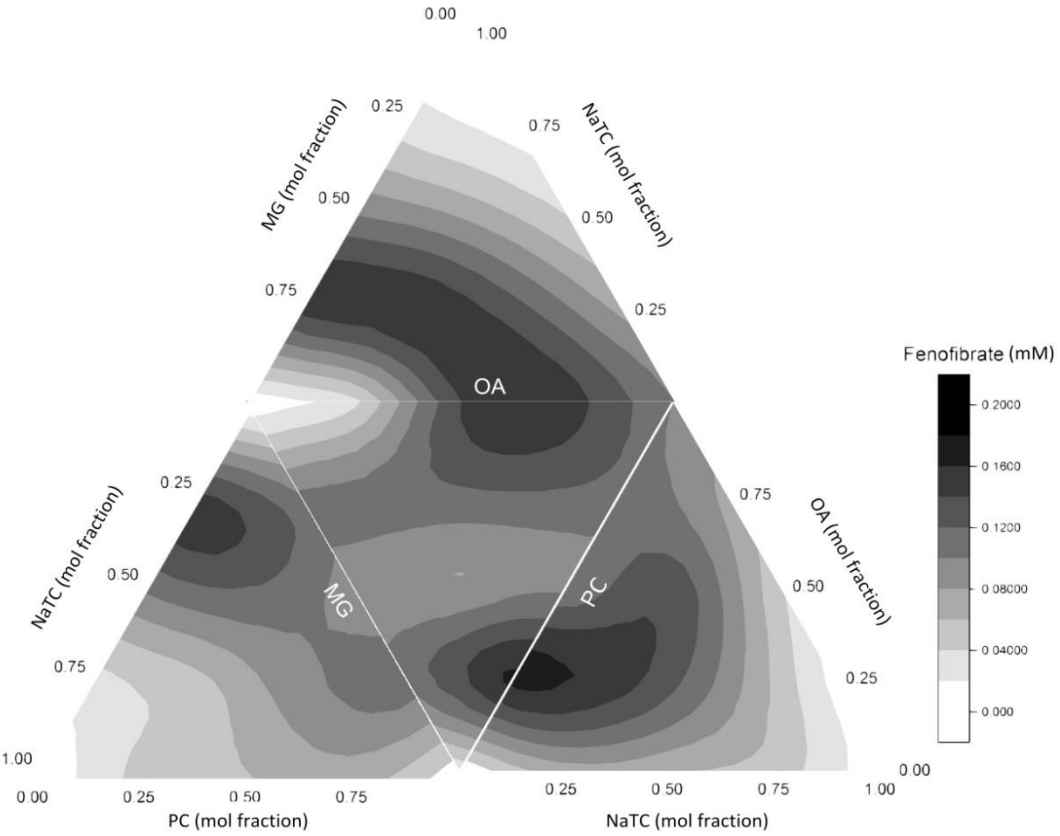

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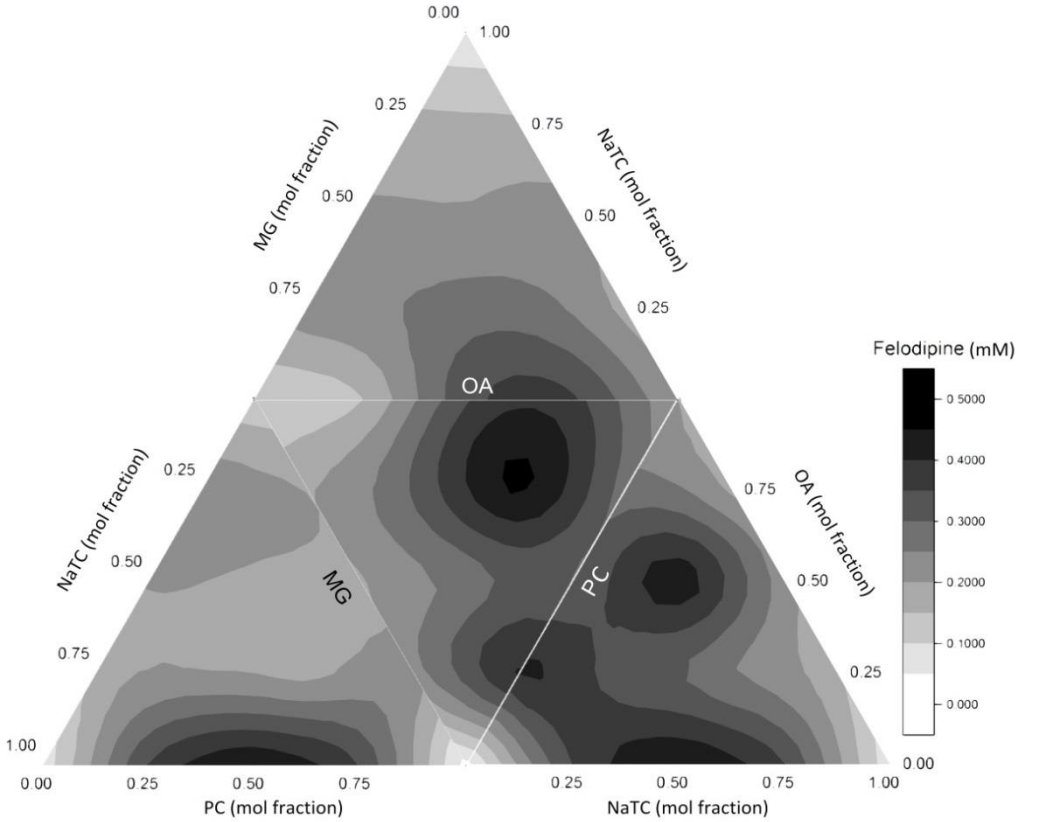

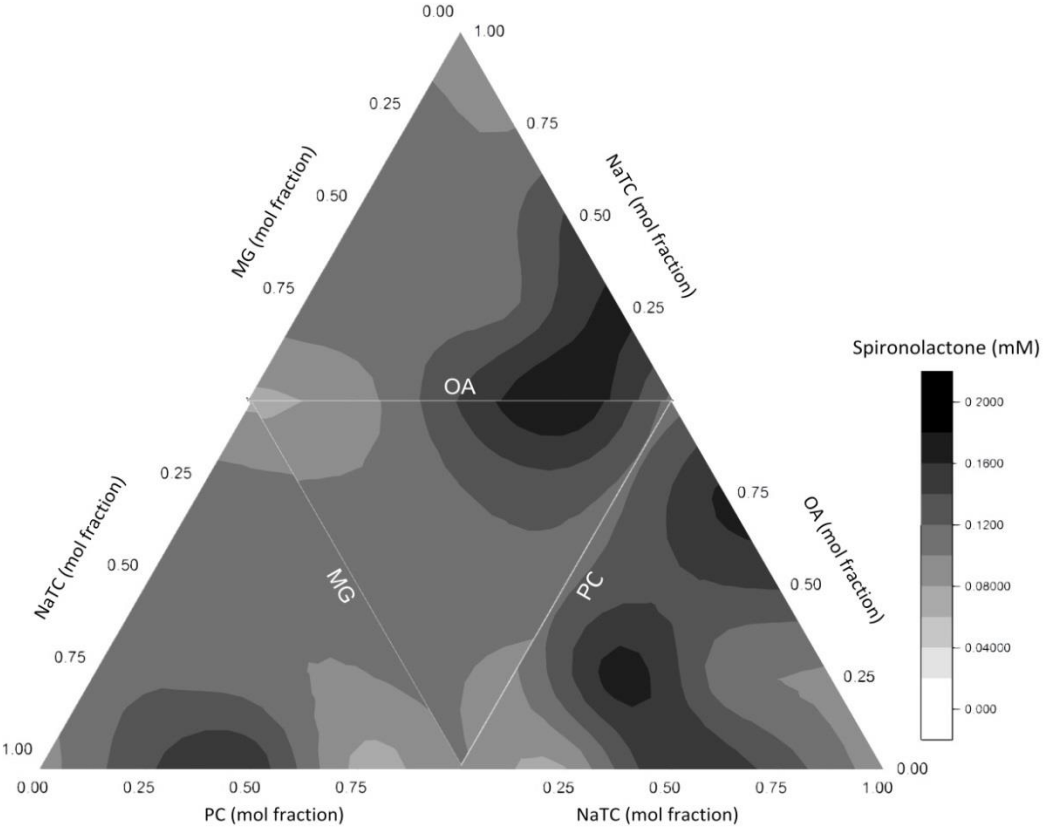

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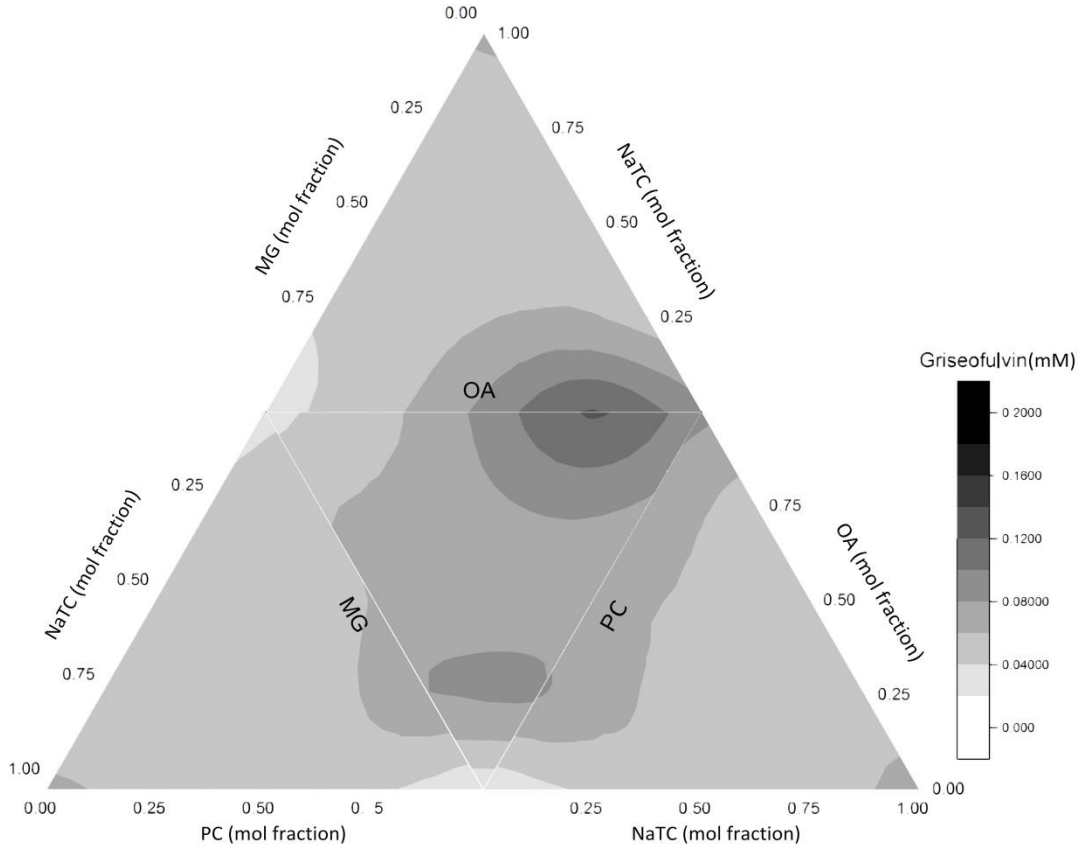


Figure 3. 1 Solubility contour plots.

Solubility contour plots determined by the 4MD, main triangle consists of four smaller triangles representing the four surfaces of the tetrahedron when open from the top vertex of 100 mol% NaTC. The colour shades attached to individual figures represent the solubility (mM) for each drug, note solubility scales vary. In the first graph, points are where solubility measured in 4MD. The dash lines cover possible media containing NaTC/PC in a ratio of 4 (typical ratio in FaSSIF media) or 15 (typical ratio in FaSSIF II). NaTC: sodium taurocholate; OA: sodium oleate; MG: monoglyceride; PC: soya phosphatidyl choline.

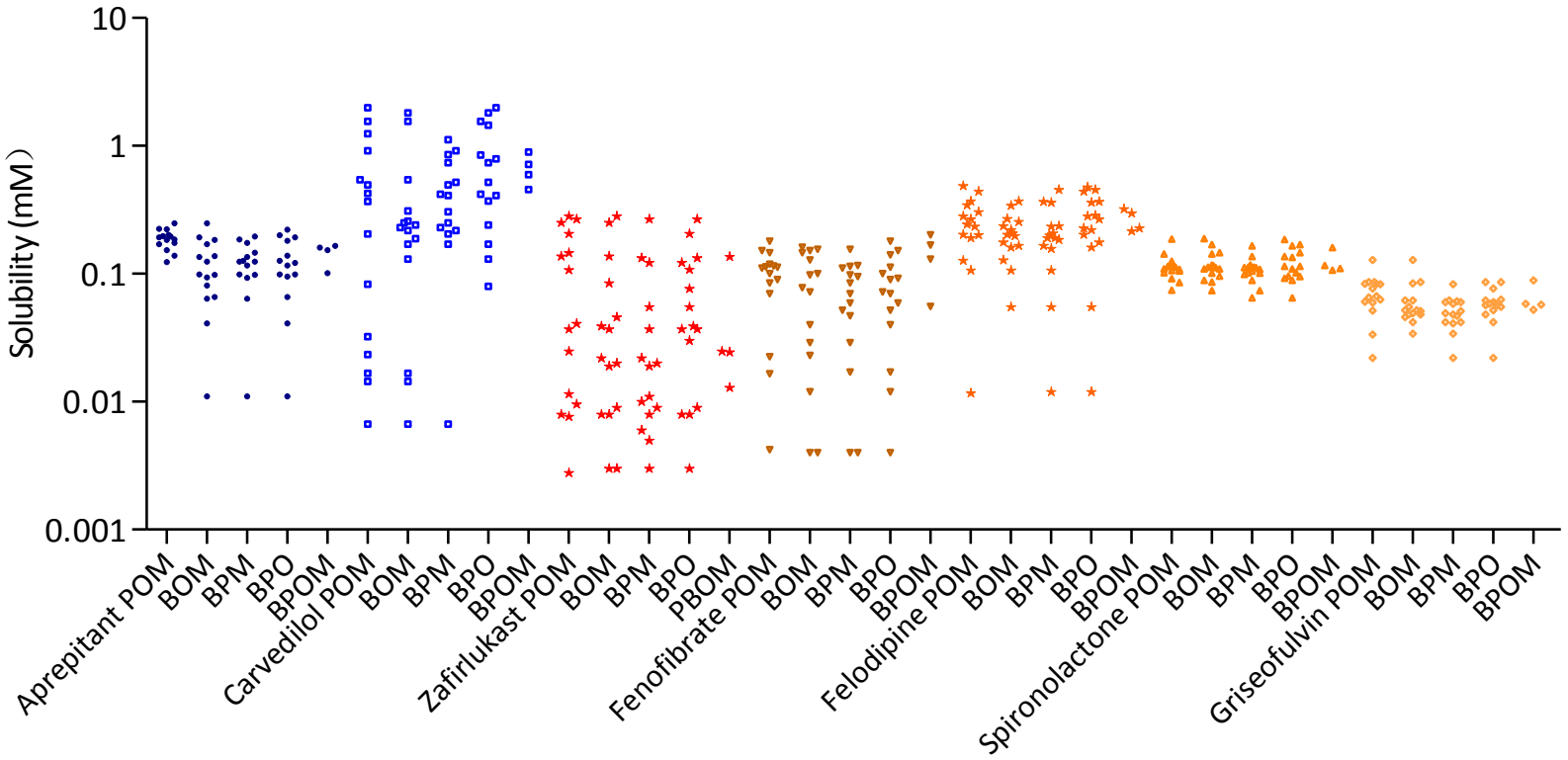


Figure 3. 2 Equilibrium solubility measurements in 4MD

Equilibrium solubility of each drug is present in five columns (each surface 15 points, inside 4 points, n=2). Each column represents solubility measured on one surface of the tetrahedron (B for NaTC; P for PC; O for OA; M for MG; for example BPO represents media containing NaTC, PC and OA, with no addition of MG), and "BPOM" represents media containing 4 amphiphiles, which therefore is inside the tetrahedron.

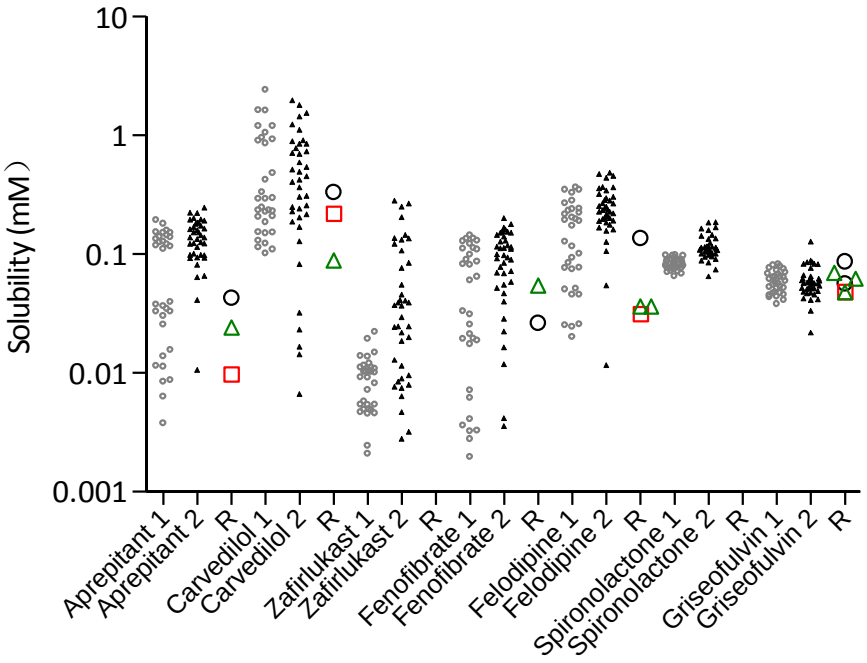


Figure 3. 3 Equilibrium solubility of DoE and 4MD of seven drugs

Equilibrium solubility for each drug based on various media combinations presented in previous DoE (data at pH 7) and 4MD. "1" represents DoE values, "2" represents 4MD values, "R" solubility values from literature (Soderlind *et al.*, 2010, Augustijns *et al.*, 2014). Δ reported drug solubility in fasted HIF, o reported drug solubility in FaSSIF, □ reported drugs solubility in FaSSIF V2.

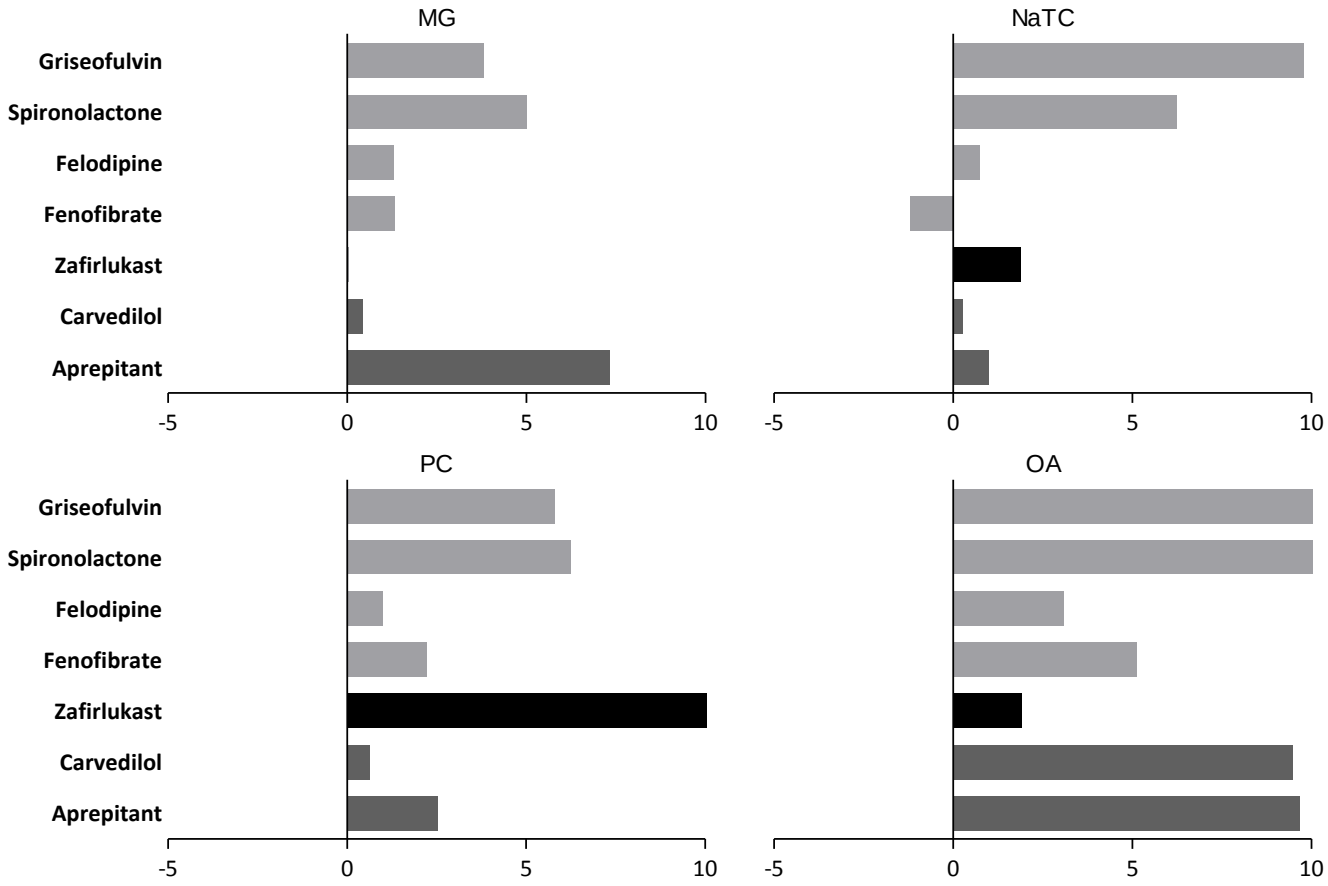
Figure 3.3 presents solubility of the seven drugs in DoE, 4MD and literature fasted HIF, FaSSIF, FaSSIF V2. The equilibrium solubility from each drug's individual 4MD matches with the literature solubility values indicating that the experiment is exploring a relevant solubility zone. In addition, the 4MD approach made it possible to explore the solubility profiles in a different dimension, focussing on the proportion of each amphiphile, while maintaining the same total molar concentration. Although a medium total concentration was chosen based on DoE, the 4MD extended the concentration range of NaTC, PC and OA (0 - 11.7 mM), especially PC (0.2 - 1 mM in DoE) and NaTC (1.5 - 5.9 mM in DoE). Therefore, the comparison between 4MD and DoE indicates that solubility ranges of most drugs

increased in the 4MD to different extents; for example, zafirlukast exhibited much higher solubility in 4MD, while the lower solubility range of carvedilol was extended. Importantly, the 4MD only covered solubility at pH 7 and some drugs have already displayed large pH variation in DoE, therefore they have the potential to expand their solubility range when another dimension was added. However, drugs like spironolactone and griseofulvin, with consistent solubility in the DoE, they were less likely to have dramatic change in the 4MD scope.

3.2.3 Solubility influence of amphiphiles

The standardised effect values, followed with a P-value, determine which factors in the model are statistically significant (Figure 3.4). There is no P-value for each amphiphile in their single term, but only standardised effect values to show the magnitude. The standardised effect value of each single amphiphile is closely related with the solubility at the media that has 100 mol% of that amphiphile, thus the higher the effect value, the higher the solubility in this single amphiphile medium. OA exhibited the highest solubilisation capabilities for six out of seven drugs (except zafirlukst), while NaTC and MG have the least solubilisation on their own. The low solubility value of carvedilol in NaTC agrees with reported paper that NaTC has a negative impact on carvedilol solubility (Chakraborty *et al.*, 2009, Khadra *et al.*, 2015). According to Figure 3.1, the three vertices in the main triangle representing 100 mol% NaTC showed lower solubility, implying that a high ratio of NaTC alone did not show any solubilisation advantages for these seven drugs.

(a)



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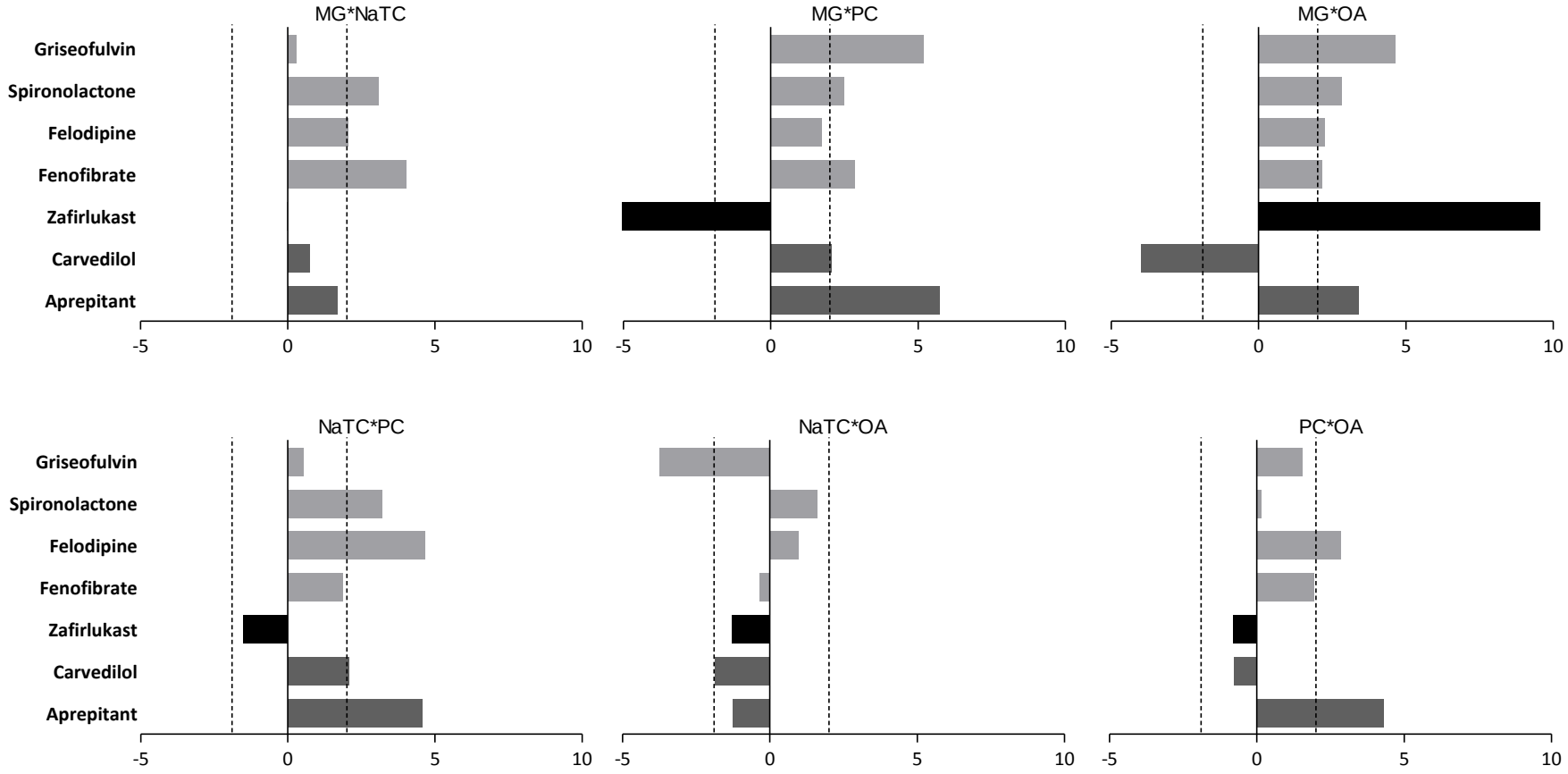

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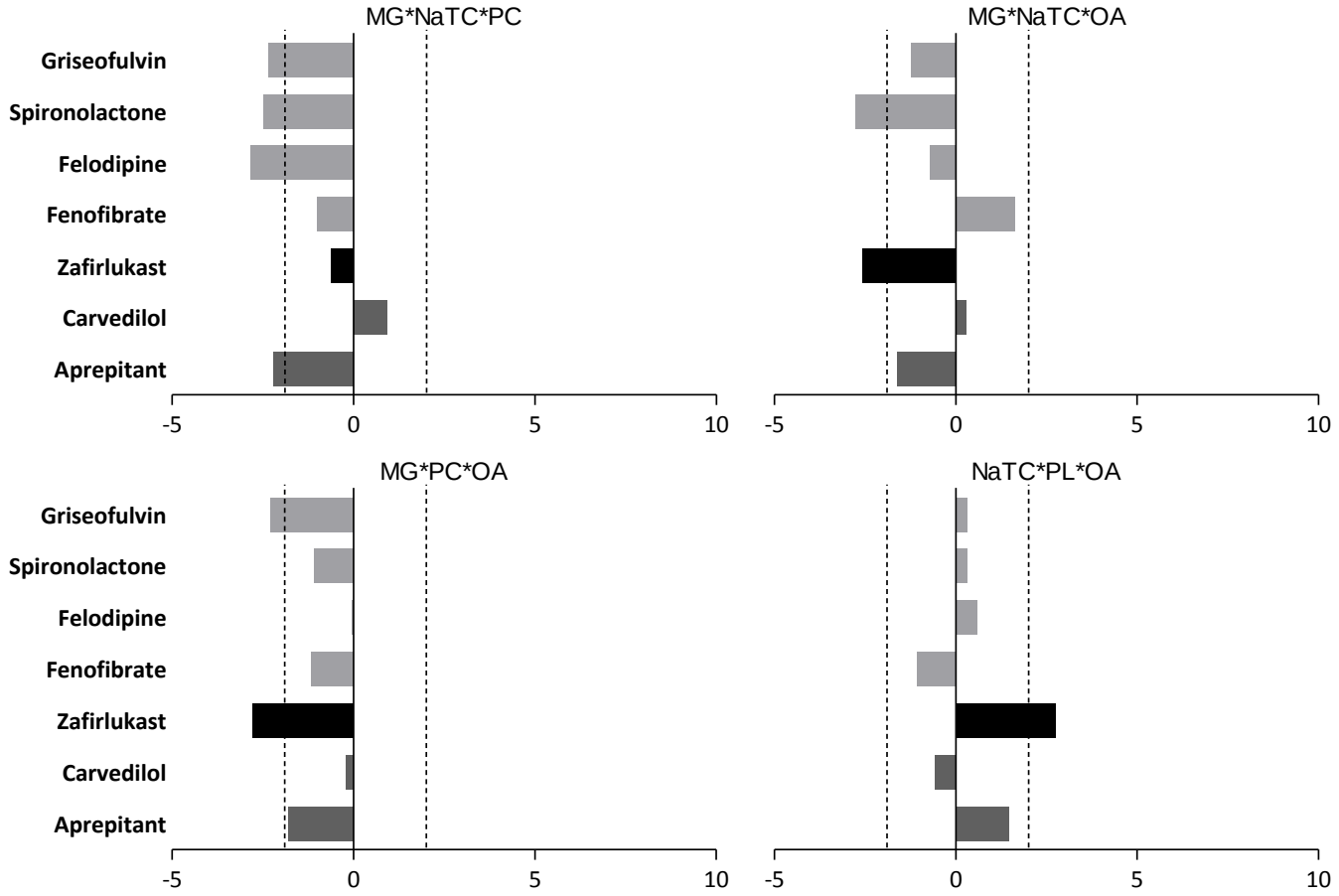

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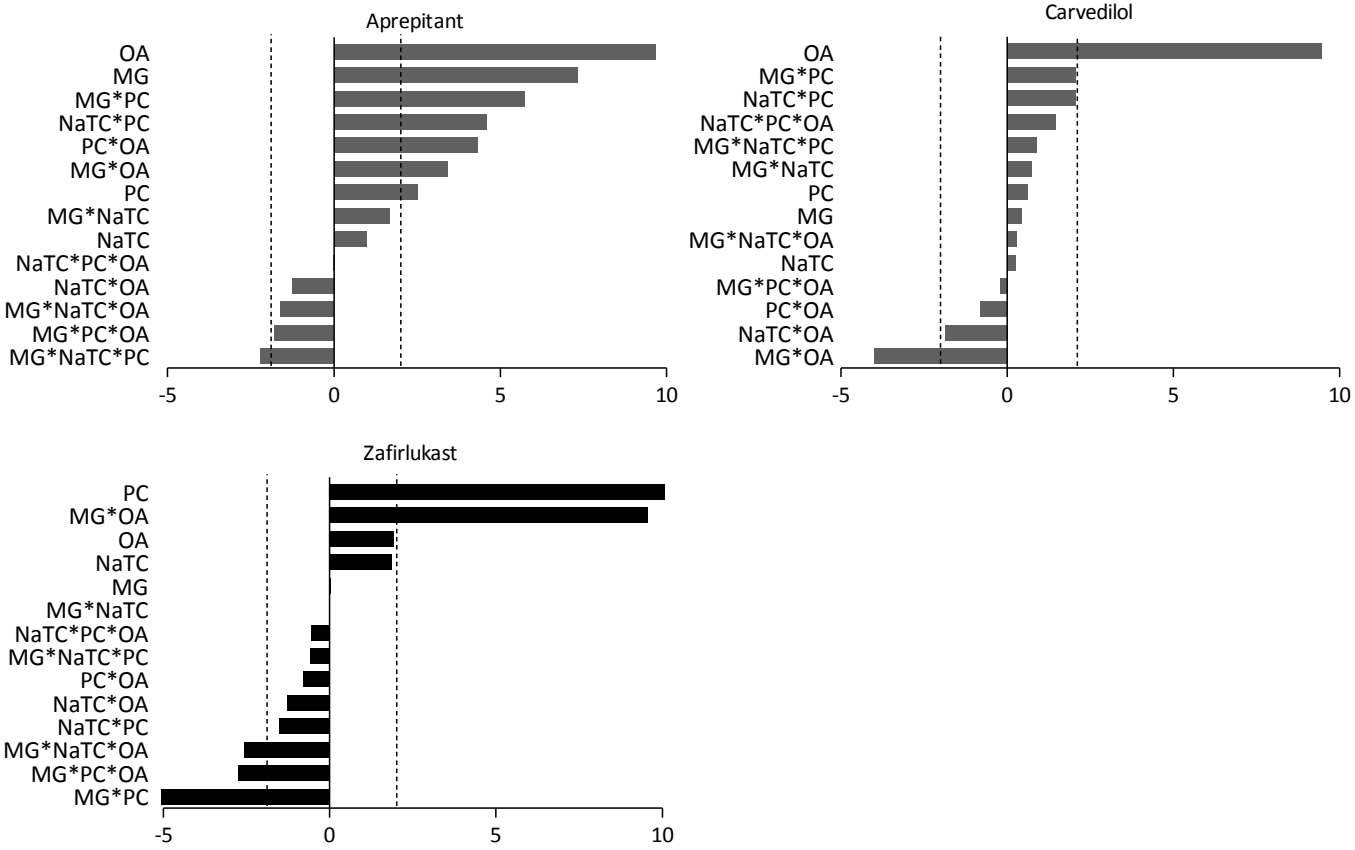
(d)



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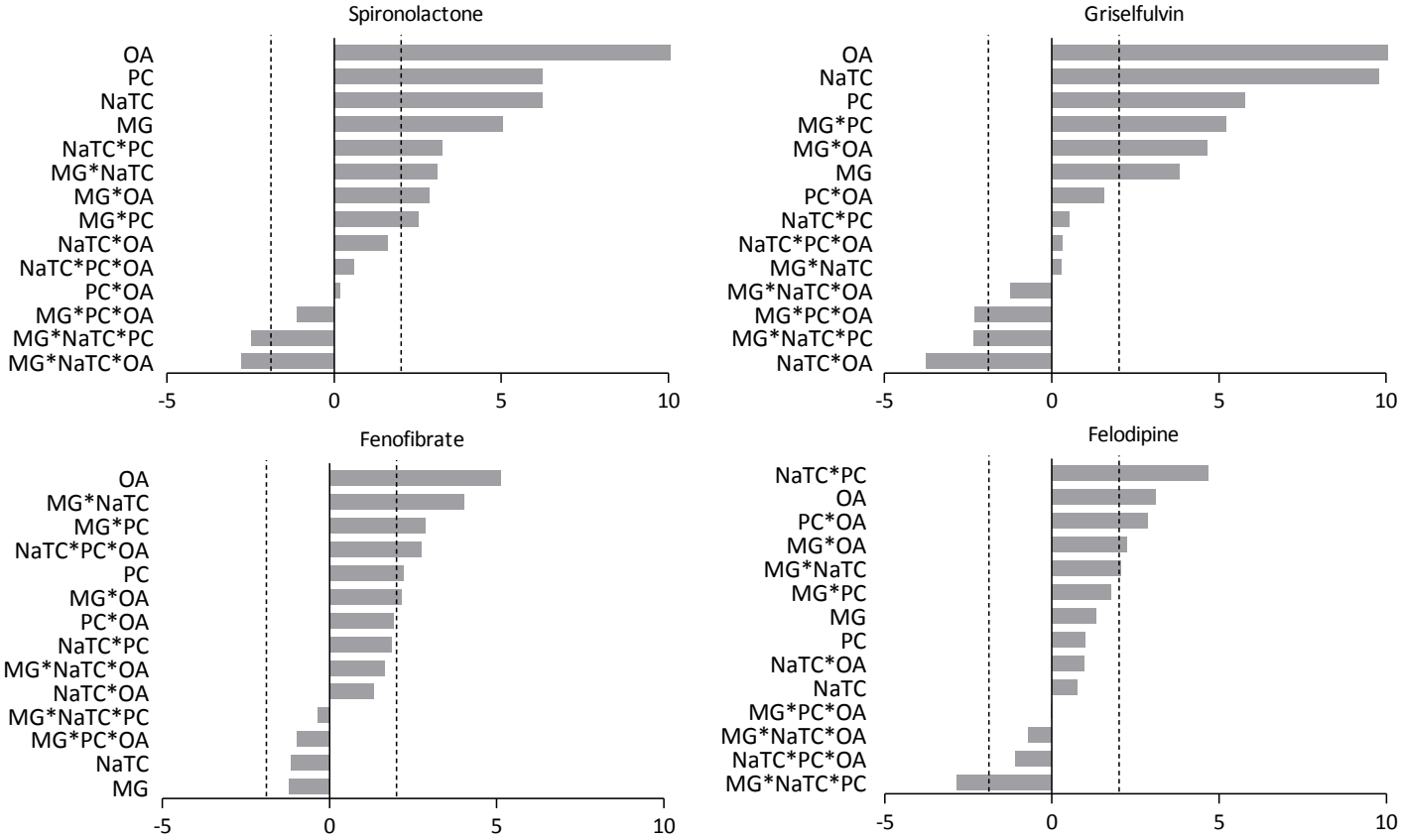


Figure 3. 4 Standardised effect values (x-axis) of (a) individual amphiphiles for each drug; (b) two amphiphile interactions (c) three amphiphile interactions; (d) each drug; factors/interactions in decreasing order of magnitude. Bars over the dashed line show statistical significance ($p<0.05$).

For two-way amphiphile interactions (Figure 3.3b), seventeen out of a possible forty two interactions had a statistically significant positive standardised effect on solubility and three had a statistically significant negative standardised effect on solubility, with more than half of the possible amphiphile interactions not influencing solubility. For example, MG*OA negatively affected carvedilol solubility, MG*PC negatively impacted zafirlukast solubility and NaTC*OA negatively impacts griseofulvin solubility. The three amphiphile interactions were only statistically significant in nine out of twenty eight possible occurrences and eight of these have a negative effect on solubility with the combination of MG*NaTC*PL negatively impacting four out of the seven drugs. There was also evidence of drug specific behaviour for example, except for zafirlukast, OA exhibited a dominantly significant positive effect for all drugs (Figure 3.4d). Aprepitant was also positively affected by MG whilst zafirlukast and carvedilol were not, and the MG*PC negatively impacted zafirlukast in contrast to carvedilol and aprepitant. OA had a remarkable effect to increase solubility of carvedilol, whereas a combination with MG (MG*OA) in the media decreased its solubility.

3.2.4 Model fitting according to amphiphile solubilisation capabilities

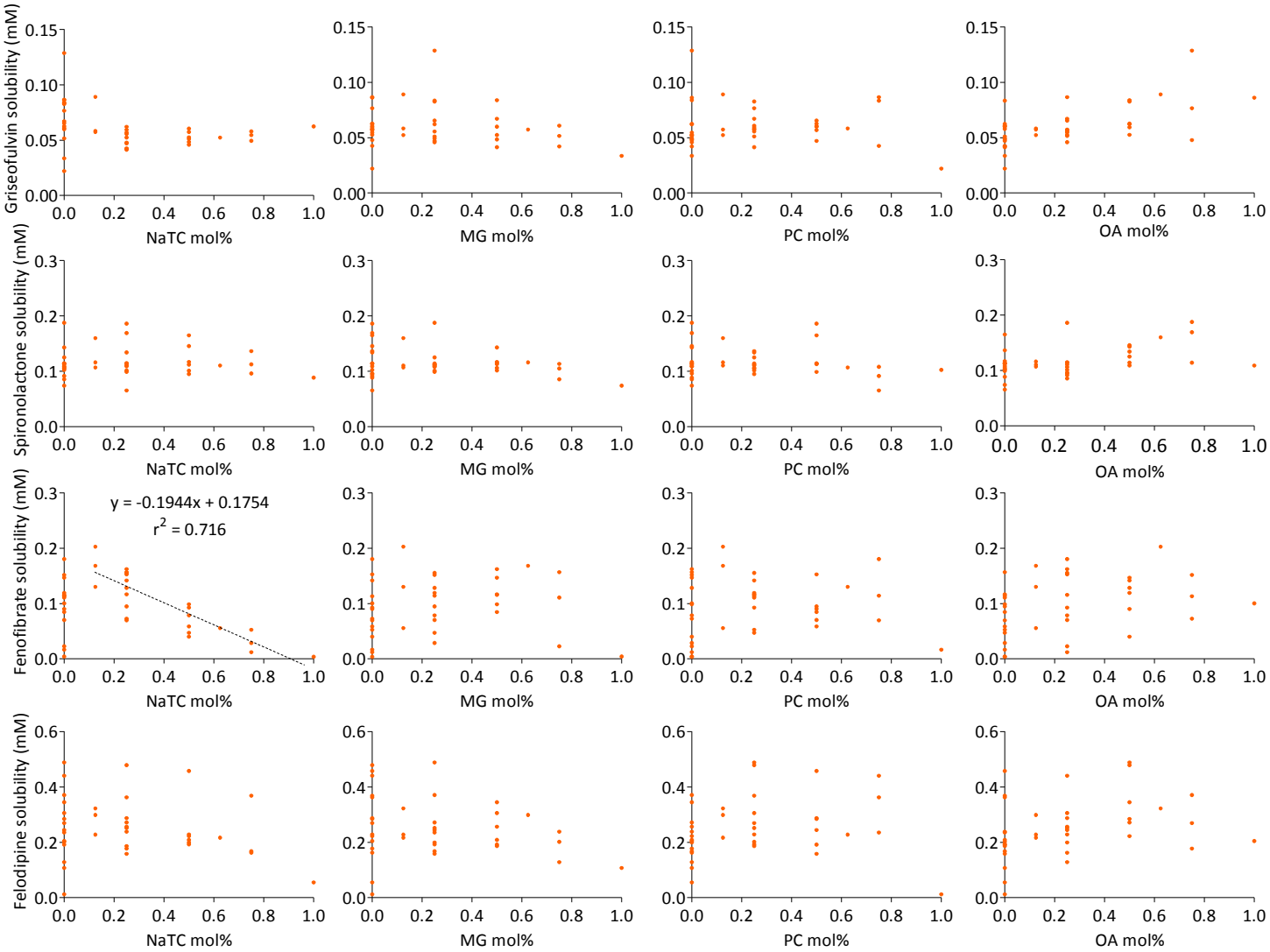
Minitab® fits the mixture design data into quadratic and special cubic models, however the coefficient of determination r^2 (<0.8) is not significant for any of the drugs. This indicates that the predictors (amphiphile ratio/concentration) in the model are not sufficient to explain the solubility variation of the drugs. Drug property induced drug-micelle interaction is also a predominant factor (Persson *et al.*, 2005), which was not included in the model due to the limited drug property diversities. A simple linear correlation to fit the total ratio (x, expressed as mol proportion of the total 11.7 mol, mol%) of one of the amphiphiles and the drug solubility (y, mM) was attempted. However, only two out of twenty-eight possibilities showed linearity (Figure 3.4). NaTC ratio has a negative linear

relationship with aprepitant (r^2 = 0.7164) and fenofibrate (r^2 = 0.716, without the points at 0 mol% NaTC). This suggests the relationship of drug solubility and amphiphile concentration is not linear and that the amphiphiles undergo a more complicated interaction with each other and with drugs rather than simply a sum solubilisation capacity of individuals.

Several papers have reported the equivalent solubilising capacity of different biorelevant amphiphiles on a molar basis in biorelevant media. One example is danazol, the highest coefficient of determination (r^2 = 0.99) was evident when plotting its solubility and the total concentration of BS, OA and MG in the media, while for probucol, r^2 was comparatively lower (0.79) (Zangenberg *et al.*, 2001). Similarly, Sunesen *et al.* reported the solubility of danazol has positive linearity with the presence of four amphiphiles (BS, PL, OA, MG) on a molar basis (r^2 = 0.98) (Sunesen *et al.*, 2005). Both studies used porcine BS extract. The solubility of estradiol was proportional to the total mass and mass concentration of either two or four amphiphiles systems (NaGC/PC or NaGC/PC/OA/MG) regardless of composition and aggregation structures of the mixed micelles (Ilardia-Arana *et al.*, 2006). They used media with several different amphiphile levels, while the ratios were constant (i.e. NaGC/PC ratio of 4 - 5). In contrast, this study systematically utilised a series of media containing the same total concentration of amphiphiles but various ratios of four components. Only two out of seven insoluble drugs tested have comparatively constant solubility across the tetrahedron. Moreover, this is not due to the constant total amphiphile concentration employed in 4MD but because these two drugs (spironolactone and griseofulvin) were proved to be relatively insensitive to any changes in different fasted SIF from the previous DoE. For the other five drugs, with the same total amphiphile concentration, the proportion of each component can have a remarkable influence on the system and in some cases, the highest and lowest solubility points are close to each other (Figure 3.1 and Table 3.3). Therefore, not only the amphiphile concentration, but

also the ratios of the amphiphiles can influence drug solubility, which could be related to the drug's properties such as log P (Kleberg *et al.*, 2010). In systems designed to determine solubility in biorelevant media with a fixed ratio of components (Kossena *et al.*, 2003, Ilardia-Arana *et al.*, 2006), some variability may be easily overlooked.

The difficulty of fitting 4MD data into linear models based on amphiphile concentrations again suggests the complicated interaction among the amphiphiles, which is in agreement with DoE studies, in which the amphiphile component interactions were significant for lipophilic compounds. For example, significant interactions of NaTC and OA affected felodipine, griseofulvin, fenofibrate, zafirlukast, aprepitant and carvedilol (Figure 2.6b and 2.6c), while interactions with PC had a lower magnitude of effect on drugs which might be due to the narrow range of PC concentration utilised (0.2 - 1 mM) in the DoE. However, the roles and interactions of PC became more evident in 4MD, as the range was expanded evenly for all amphiphiles (0 - 11.7 mM). Although 4MD covers all possibilities of the selected concentration and some of the combination ratios are not physiologically relevant in fasted or fed state, they can provide a larger experimental space permitting examination of how the variations happen and indicate potential risks of sensitivity issues. The dashed line in Figure 3.1 indicated a possible space by providing BS/PL in a 4:1 and 15:1 ratio, with multiple levels of either MG or OA, which best resemble the possible scenarios in FaSSIF and FeSSIF media (Galia *et al.*, 1998, Jantratid *et al.*, 2008). This clearly covers a set of very limited solubility possibilities that could happen in the intestine.


Griseofulvin solubility (mM)
Spironolactone solubility (mM)
Fenofibrate solubility (mM)
Felodipine solubility (mM)
NaTC mol%
MG mol%
PC mol%
OA mol%
y = -0.1944x + 0.1754
r2 = 0.716

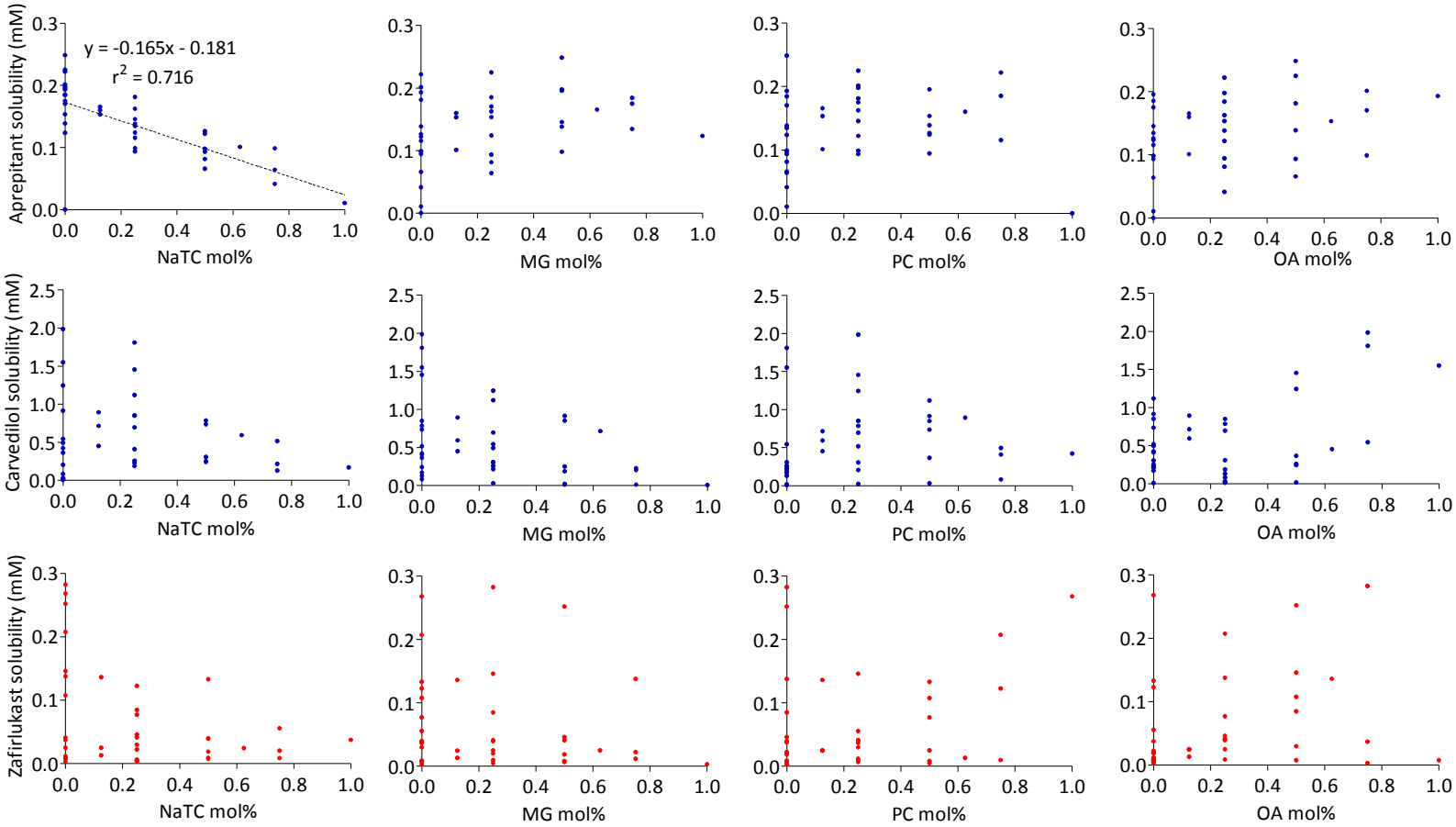


Figure 3. 5 Correlation between drug solubility and molar fraction of amphiphile (NaTC, MG, PC and OA mol%) in the media (n=2).

Table 3. 3 Composition (mol% of surfactants) of media providing the highest and the lowest three solubility values of each drug

	Aprepitant	Griseofulvin	Felodipine	Fenofibrate	Spironolactone	Zafirlukast	Carvedilol
High solubility zone	75% PC 25% OA	25% MG 75% OA	25% MG PC 50% OA	12.5% NaTC MG PC 62.5% OA	25% MG 75% OA	25% MG 75% OA	25% PC 75% OA
	25% MG PC 50% OA	12.5% NaTC MG PC 62.5% OA	25% NaTC PC 50% OA	75% PC 25% OA	25% NaTC OA 50% PC	100% PC	25% NaTC 75% OA
	50% MG 50% OA	75% PC 25% OA	50% NaTC 50% PC	12.5% NaTC PC OA 62.5% MG	25% NaTC 75% OA	50% MG 50% OA	100% OA
Low solubility zone	100% PC	100% PC	100% PC	100% NaTC	25% NaTC 75% PC	100% MG	100% MG
	100% NaTC	100% MG	100% NaTC	100% MG	100% MG	25% NaTC 75% OA	75% MG 25% OA
	75% NaTC 25% OA	25% NaTC PC 50% MG	100% MG	75% NaTC 25% OA	75% MG 25% OA	25% NaTC MG 50% PC	50% MG 50% OA

3.2.5 Individual drug-amphiphile interactions

Vertices, which represent single amphiphile dominant media, tend to show that single material has poor solubilisation capabilities. For example, in Table 3.3, 100 mol% PC is the low solubility zone of fenofibrate, aprepitant and felodipine, 100 mol% MG is the low solubility zone of fenofibrate, zafirlukast and carvedilol and 100 mol% NaTC is the low solubility zone of fenofibrate, aprepitant and felidipine. The only exception is that 100 mol% PC is the high solubility zone of zafirlukast. Interestingly, apart from high solubility zones, carvedilol and zafirlukast illustrate extensive low solubility zones where the system apparently dislikes the drug.

100 mol% NaTC is usually a low solubility zone which suggests that NaTC alone or at a high ratio tends to have less solubilisation capability compared with the same concentration of a mixture of different amphiphiles for the tested drugs. Mixed micelles of different BS and PC are believed to have larger core size to incorporate more hydrophobic compounds (de Castro *et al.*, 2001). This is especially advantageous in a system only containing NaTC, as the low aggregation number (Hofmann and Small, 1967) and steric hindrance may limit the solubilising capacity of NaTC and mixing with PL under physiological condition (based on FaSSIF 4:1 proportion) can reduce the CMC of NaTC (Gómez *et al.*, 2013). Anionic compounds (acidic drugs) may also be better accommodated into the mixed micelles due to the lower net surface charge upon addition of PL (Schwarz *et al.*, 1997).

PC and MG are both poorly dispersible/soluble amphiphiles in aqueous buffer without the assistance of other solubilisation agents such as NaTC (Hofmann, 1963) and Table 3.3 shows that generally 100 mol% PC and 100 mol% MG media provide very poor solubilisation. However, PC exhibited excellent solubilisation for aprepitant, felodipine and fenofibrate, provided that an appropriate ratio of NaTC or

OA (see Table 3.3) was present. Salt forms of OA are more soluble and ionised in physiological pH and the zeta potential contour plot in Figure 3.5 indicates that the addition of OA increases surface charge of PC that can stabilise the mixed micelles, emphasising the importance of adding charged surfactants to solubilise and stabilise the neutral PC in aqueous buffer in order to maximise its solubilisation ability (Xu *et al.*, 2005, Sezgin *et al.*, 2006).

Combinations of amphiphiles exhibit better solubilisation and this study incorporated four different amphiphiles. However, the recipe providing highest solubility in 4MD varies from drug to drug. NaTC and OA are two ionisable amphiphiles, the carboxylate group of OA has a pK_a about 5, however pK_a could increase considerably to above 7 in PL vesicles or other mixed aggregates (Small *et al.*, 1984, Edwards *et al.*, 1995). Temperature and ionic strength can also affect the apparent pK_a. These ionisation changes may affect solubilisation capacities of the amphiphiles and the charge interaction with ionisable drugs. Carvedilol (pK_a 7.8, log P 3.91) (Mannhold, 2005, Loftsson *et al.*, 2008) is a moderately hydrophobic and ionic compound, which is protonated at pH 7. These data show that solubilisation is aided by a more negatively charged system with higher OA (75% - 100% OA). The solubility distribution within the NaTC/PC/OA surface of carvedilol resembles the zeta potential contour plot (Figure 3.6), which implies that electrostatic attraction becomes a predominant factor for carvedilol-amphiphile interaction. Formation of ion-pair complexes have been reported between FFA and organic cationic drugs including a serious of beta-blockers and improve drug permeation (Green *et al.*, 1989).

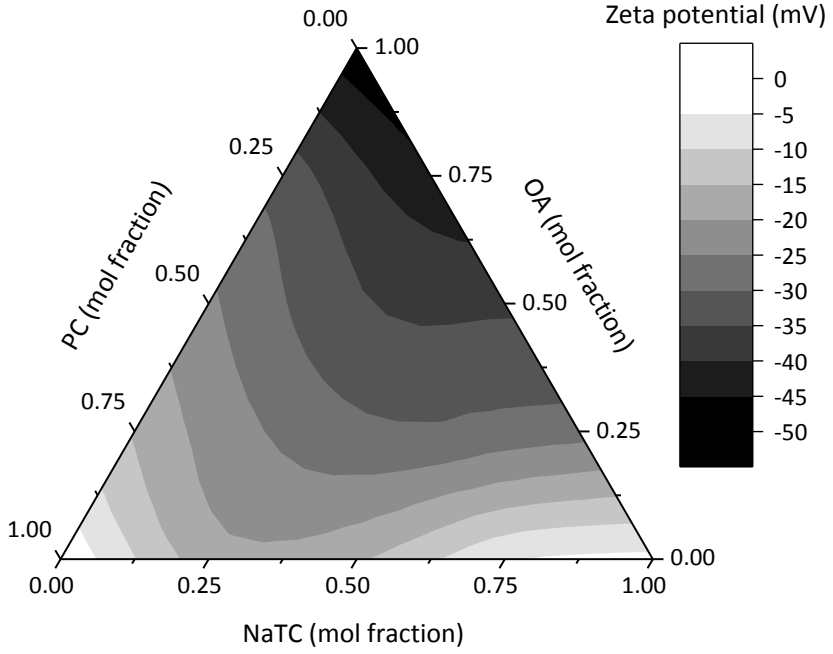


Figure 3. 6 Zeta potential (mV)) distribution in NaTC/PL/OA mixture surface (0% MG). Data based on 15 different combinations. Each points are average number from 3 measurements, and processed in OriginPro® 9.0.0 SR1.

The lipophilicity of a drug can affect how much it engages with the lipid-rich micelles (Zangenberg *et al.*, 2001, Kossena *et al.*, 2003). Therefore, it is not surprising to find that the solubility of spironolactone (log P 2.78) (Sora *et al.*, 2010) and griseofulvin (log P 2.18) (Mithani *et al.*, 1996) was not significantly affected by amphiphiles at this concentration, since both of them have a comparatively lower log P than the other drugs tested. Previous DoE also indicated that their solubility was not heavily affected with the presence of NaTC, PC and OA at different levels. For the steroidal drug spironolactone, Hammad and Muller (Hammad and Muller, 1998) reported similar phenomenon with three other steroidal drugs, prednisolone, progesterone and estradiol. Their low degree of interaction with BS/PC mixed micelles might be conformationally related and the lack of a nonpolar moiety may reduce the hydrophobic interaction with micelles. Similar results were also

observed in the study of Kossena and colleagues(Kossena *et al.*, 2004), who tested hydrocortisone and four hydrocortisone esters, providing drugs with a range of log P values (log P 1.4 - 5) and the solubility was similar in blank buffer and biorelevant media containing 4 mM BS mixtures and 1 mM lyso-PC, only increasing after the addition of fairly high concentration of FFA and digested glycerides (calculated as > 10 mM). This is more representative of the fed state or when a lipid formulation is administered. The data obtained from griseofulvin in this study is in accordance with the Persson's study (Persson *et al.*, 2005), where the solubility only has a slight increase from fasted to fed state with a 4-fold increase of BS and 14-fold increase of PL. The increase of dissolution rate was even more marginal, while the solubility increase of felodipine was much higher under the same conditions.

Zafirlukast has a comparatively high log P (log P 6.4 ChemAxon) and flexibility (RoB of 9) and on the NaTC/PL/OA surface, its solubility was dominantly affected by the concentration of PC, which might be attributable to its flexible planar and rotatable structure which could fit between the hydrophobic chains of PC. Additional components in the 4MD reveal another high solubility zone for zafirlukast with equal blend of MG and OA, both of which have long alkyl chain tails. Warren *et al.* used molecular dynamics and found that more lipophilic molecules tend to interact with the lipid alkane chain region of the system, which again indicates the interaction of the drug with the lipid system is highly dependent on the polarity of both the drug and lipid molecules (Warren *et al.*, 2013). Nevertheless, apart from the multiple phase forms and micelle sizes observed across different ratios, no standardised rules and clear correlation have been identified between the composition of the media and different drug solubility (Kleberg *et al.*, 2010).

Mithani *et al.* developed a model to predict the solubility in the presence of NaTC by using the partition coefficient (log P) and aqueous solubility of the drugs. The

model was predictive for a series of drugs, however only considering BS in GIT (Mithani *et al.*, 1996). Studies have shown that both the concentration (Zughaid *et al.*, 2012) and conjugation (de Castro *et al.*, 2001) of BS may influence the solubility of certain drugs, considering only one BS (NaTC) was included in this study, it would be more complex for the *in vivo* situation with a variety of BS and food digests under dynamic changes of concentration and pH. Incorporating four amphiphiles in the 4MD increases the difficulties for model fitting and reveals the complex interplay between drug-amphiphile and amphiphile-amphiphile interactions. Therefore, not only lipophilicity of the drugs and amphiphiles, but also the molecular size, shape, charge and structure can show specific effects on drug solubilisation (Schwarz *et al.*, 1997).

3.3 Conclusion

4MD provide complimentary information for the previous fractional factorial DoE and mainly focuses on the influence of the biorelevant amphiphile ratios on the equilibrium solubility of seven BCS II drugs. Solubility in 4MD is comparable with literature from both fasted HIF and biorelevant media. Two out of seven drugs (griseofulvin, spironolactone) have small variation on solubility when changing the amphiphile ratios in the media, which adds additional information to previous DoE that both drugs are not only insensitive to the amphiphile concentration but also amphiphile ratios. For the other five drugs, results imply that the media solubilisation capacities are not a simple accumulation of the four amphiphiles since drug solubility does not have a linear relationship with the total concentration of amphiphiles or concentration of a single amphiphile. This is different from a few literature studies reporting the linear relationship of solubility and amphiphile concentration regardless of the type of amphiphiles used (Zangenberg *et al.*, 2001, Sunesen *et al.*, 2005, Ilardia-Arana *et al.*, 2006) and maybe drug related. Complicated drug-amphiphile and amphiphile-amphiphile interactions can affect drug solubility, due to drug lipophilicity and amphiphile types (Hammad and Muller,

1998, Kossena *et al.*, 2004, Persson *et al.*, 2005).

4MD again confirms the importance of considering the mixture of digestion-related lipids in biorelevant media and the issue of mixture ratios. Biorelevant media for evaluation of API and formulated drug on the GI absorption were introduced and developed to multiple media with various combinations of BS and PL, commonly in the ratio of either 4 or 15, with addition of MG and OA in fed states (Table 1.1). Utilising those media with limited ratios can potentially miss other possibilities and affect the interpretation of drug solubility. Therefore, biorelevant media should target a physiological related ratio range of BS, PL and digested lipids, while covering the concentration of fasted and fed states in order to provide information on the variation and sensitivity of a drug to specific combinations of biorelevant components. This would be useful to predict the individual variability, food-induced or disease-related absorption changes that can affect BA of the drug.

One important merit of the 4MD is that the levels of amphiphiles in the previous DoE were selected based on the physiological level, which can overweight one amphiphile relative to another if the selected concentration levels intrinsically vary in magnitude (levels of OA, NaTC much higher than PC, MG). However, in the 4MD, all components are positioned in an equal molar range, which can give more straightforward insight into their individual solubilising capacity on a molar basis. Only one total concentration was investigated in this study, which did not reach the fed state level where more diverse drug behaviour is expected. For example, griseofulvin absorption from GIT has been proved to increase with the administration of high fat meal (Crounse, 1961b). This is not shown in this study, which might be due to the limited scope of the 4MD and suggest further study.

4. Dissolution in DoE media

The objective of this study is to compare drug dissolution rates in two different media reflecting the fasted SIF variation based on DoE in Section 2 and investigate how it correlates with equilibrium solubility in the same media and conditions.

4.1 Materials and methods

4.1.1 Preparation of fasted simulated intestinal fluids

According to the results of DoE, two dissolution media were chosen where the highest and median equilibrium solubility of each drug was determined, among all the DoE media recipes. The corresponding recipes can be different for each drug and are generally referred as "high point (HP) media" and "median point (MP) media". Off-line sampling was utilised to avoid UV interference of the turbid media to drug concentration measurement. Media were prepared from corresponding powder ingredients and pH adjusted according to concentrations in Table 2.2.

4.1.2 Powder dissolution test and sample collection

Dissolution test was performed in Sirius InForm (Sirius Analytical, Forest Row, UK) with built-in pH monitoring under the "GI dissolution" assay model. At least 2-3 fold excess of powder needed to reach the equilibrium solubility was added to the vials unless elsewhere stated, which confirmed excess materials present during the whole experiment. Same drug batch, drug weight, media volume (40 or 50 ml) and stirring rate (300 rpm) were used in two media for each drug. Off-line sampling of 250 µl was taken every 10 min up to 2 h through a 96-well plate filter (Millipore MultiScreen® 0.22 µm pore size) and 200 µl filtrate analysed in HPLC. HPLC methods used were the same as in Section 2.1. After 2 h, 1 ml of each sample was also withdrawn from the vial and centrifuged for 5 min at 13,000 rpm and supernatant analysed in HPLC, data were compared with the last filtered

sample to estimate a filter absorption (%). For each medium, dissolution was run in triplicate. Additional protocols: the dissolution of naproxen was also conducted in corresponding aqueous buffer equivalent to its HP and MP media but without amphiphile contents. The dissolution of carvedilol was repeated with three different carvedilol powder weights: 20, 30 and 35 mg.

4.1.3 Shake flask method for carvedilol

Shake flask method was used for carvedilol to monitor the concentration in 15 ml Corning® centrifuge tubes in HP and MP media, rotating for 24 h. 20 mg carvedilol was added into 10 ml of HP or MP media in centrifuge tubes and rotated at 12 rpm. Samples of 500 µl were withdrawn at 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h and 24 h, centrifuged at 13,000 rpm for 5 min and 100 µl supernatant analysed in HPLC. Experiments were conducted in triplicate for each medium. This part was used to compare with the dissolution data obtained from Sirius inform in Section 4.1.2.

4.1.4 Measurement of particle surface area

Dara of particle surface area (m^2/g) of each drug were in house data measured with Malvern Mastersizer Hydro 2000 (Malvern, UK). The particles were placed in a water suspension and dispersed by ultrasound.

4.1.5 Calculation of intrinsic dissolution rate

The intrinsic dissolution rate (IDR) is expressed as equation 7, where *V* is the dissolution media volume, *C* is the concentration, *t* is the time and *A* is the surface area of the disk or powder (Yu *et al.*, 2004).

$$IDR = \frac{V}{A} * \frac{dC}{dt} \qquad \text{(Equation 7)}$$

4.2 Results and discussion

4.2.1 Relationship of intrinsic dissolution rate and equilibrium solubility

Due to the instant burst at the beginning of the dissolution, phenytoin, piroxicam, naproxen, tadalafil and griseofulvin achieved their maximum concentration before the first off-line samples were taken, the IDR were difficult to calculate by curve fitting. Therefore, the rate of concentration change of these drugs was calculated as an average rate in the first 10 min (dC/dt = maximum concentration/10 min), the actual IDR may therefore be underestimated.

Comparisons of IDR and equilibrium solubility in HP and MP media are shown in Figure 4.1. Table 4.1 lists the calculated intrinsic dissolution rates (IDR) of all the drugs in HP and MP media and literature powder or disk IDR. These were detailed in the following Figure 4.2 - Figure 4.5, which display the powder dissolution profiles of 11 compounds. Dissolution of naproxen and carvedilol has additional protocols. Except for zafirlukast and carvedilol, the HP media representing high solubility exhibited higher IDR than MP media representing median solubility. It is in agreement with Noyes-Whitney equation (Equation 4) and IDR equation (Equation 7) that IDR is positively related with drug solubility under the same dissolution condition (media volume, particle surface area, stirring rate etc.); solubility is highly affected by the nature of the dissolution media, as demonstrated in DoE and 4MD.

IDR of zafirlukast was quite similar in two media, provided with different equilibrium solubility (0.012 and 0.003 mg/ml respectively) (Figure 4.2). In addition, the concentration after 2 h dissolution was 0.347 and 0.319 mg/ml in MP and HP media, more than 25 times of the equilibrium solubility, implying that zafirlukast underwent a dissolution applying kinetic solubility rather than equilibrium solubility. Zafirlukast is poorly soluble in its crystal of both anhydrous and monohydrate forms. The amorphous are comparatively stable but can

transform to the monohydrate in water (Llinas et al., 2015). Commercial amorphous products include zafirlukast in Accolate® (Zhang et al., 2014). The kinetic dissolution and supersaturation rate of amorphous zafirlukast have not been reported or captured *in vitro* before (Kataoka et al., 2013). In this study, the original powder used has been reported as amorphous form by OrBiTo colleagues and zafirlukast clearly showed supersaturation from amorphous form for the initial two hours. The IDR might not correlate with the corresponding equilibrium solubility, but determined by the maximum solubility of the drug, in this case kinetic solubility. The media contents can influence the time of supersaturation and rate of precipitation (Kostewicz *et al.*, 2002). This "spring and parachute" dissolution was described in Section 1.7.

Initially, carvedilol dissolution was carried out with 20 mg powder in both MP and HP media. However, results indicate that the IDR in MP medium (Recipe 12) was higher than HP medium (Recipe 23) (Figure 4.1), contradictory to the ranking of their equilibrium solubility. For every single measuring point, the concentration was lower in HP medium than MP medium with a median difference of 0.035 mg/ml. A Wilcoxon signed-rank test determined that there was statistically significant difference between samples of 20 mg in HP and MP media ($P<0.05$). Questions about the accuracy of data obtained from DoE and the filter absorption were the initial reasons to explain this result. The first question was solved by conducting the shake flask method for carvedilol again. Moreover samples were withdrawn at different time points to monitor the concentration and all samples were centrifuged rather than filtered before HPLC analysis. The equilibrium solubility matched with previous data in Section 2 and sensibly explained the relationship between equilibrium solubility and IDR. However, comparison with current experiment, Figure 4.5a and Figure 4.5d indicate that for MP medium, the concentration matched quite well, but in HP medium, concentration was 5 times

higher in shake flask method than dissolution from off-line sampling data after 2h. For the second question, the filter absorption was less than 40%, which explained only part but not all of the lower concentration. Another possibility is the available powder was not enough to reach the required concentration, so an attempt was to increase the powder weight and check if the concentration increases. However, when using 30 and 35 mg of carvedilol, the concentration did not increase accordingly. A Wilcoxon signed-rank test determined that there was no statistically significant difference between samples of 20 mg and 30 mg, 30 mg and 35 mg in HP media ($p>0.05$). There was a significant difference between samples of 20 mg and 35 mg, with difference median of 0.01 mg/ml ($p<0.05$). Therefore, this is not the main reason to explain the results. Further study should analyse whether there are any media composition change throughout the experiment in the Sirius inForm sample holding vial, and compare it with media in shake flask method to identify if the IDR difference is attributed to the changed media and solubility.

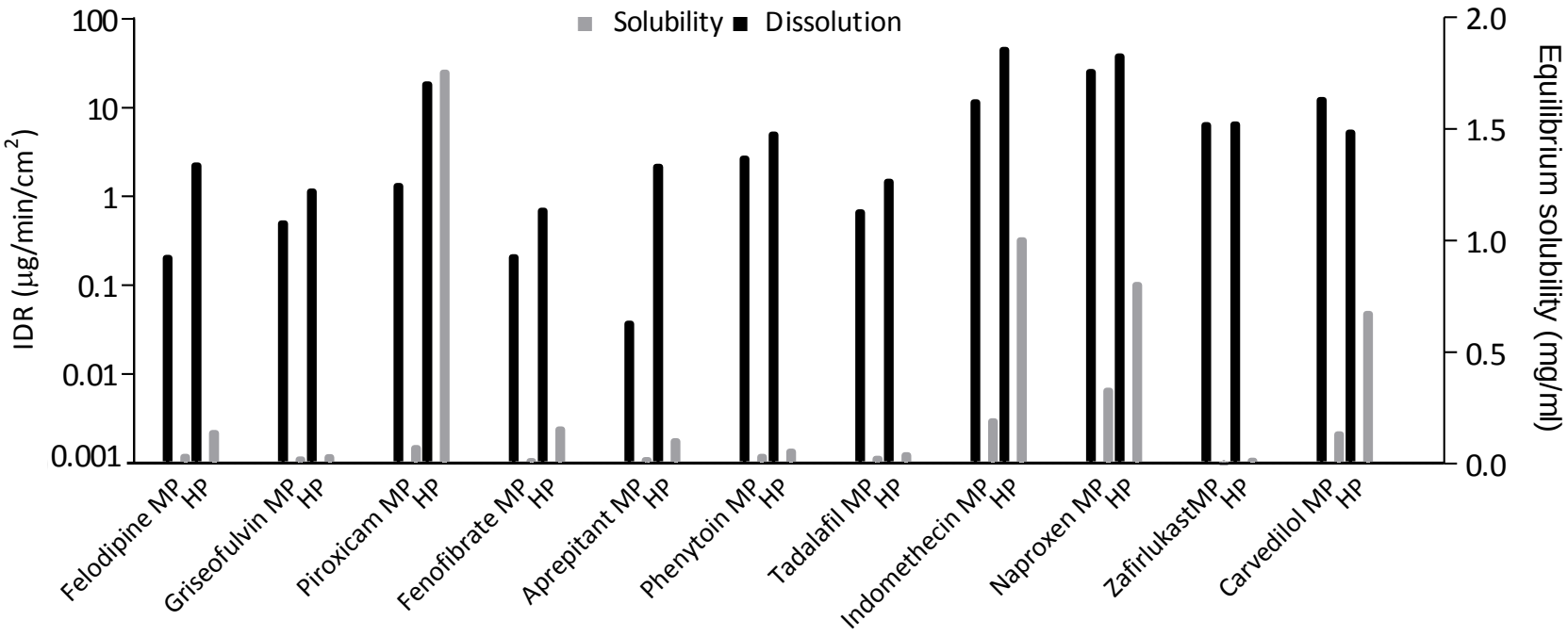


Figure 4. 1 IDR and equilibrium solubility in corresponding MP and HP media.

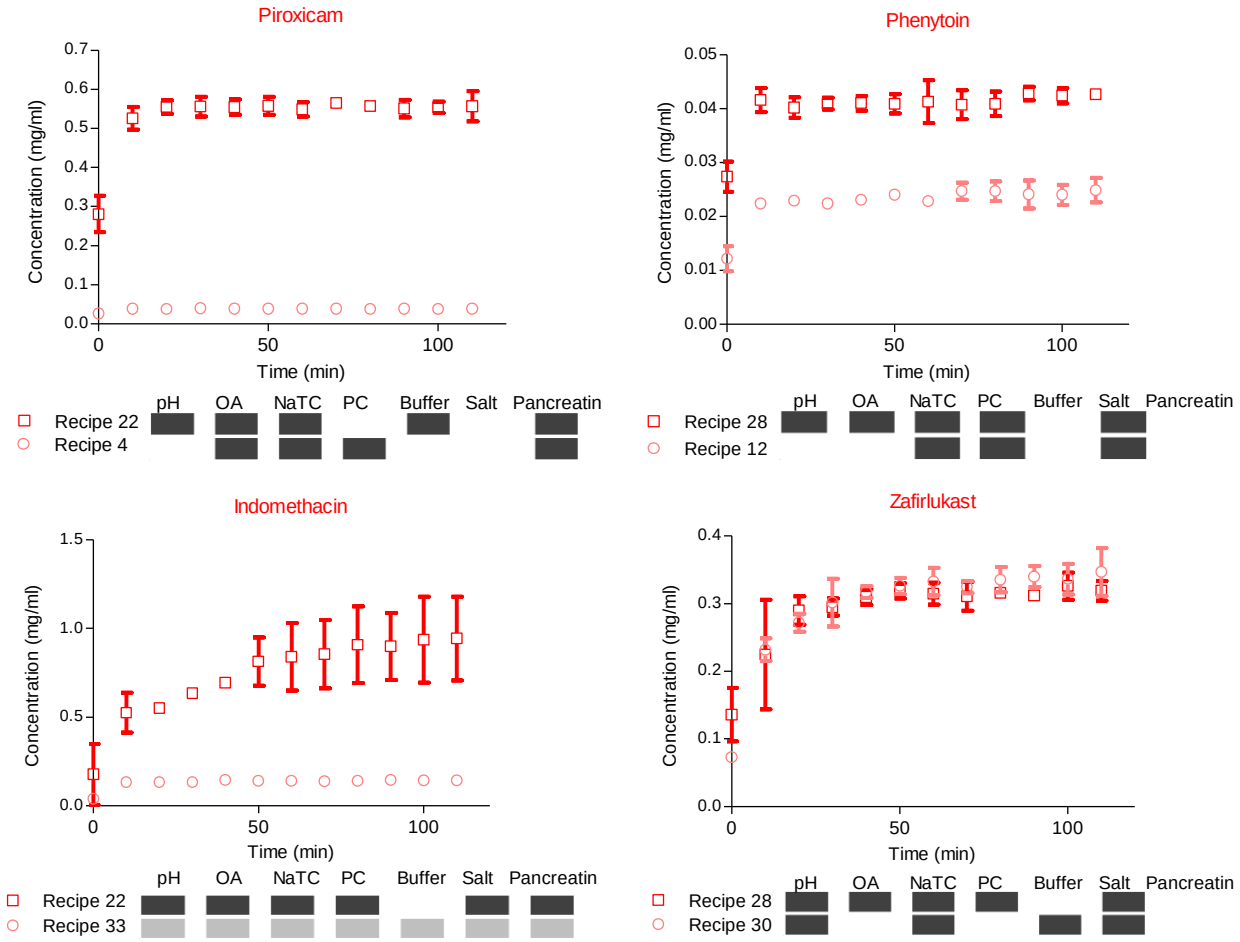


Figure continues.

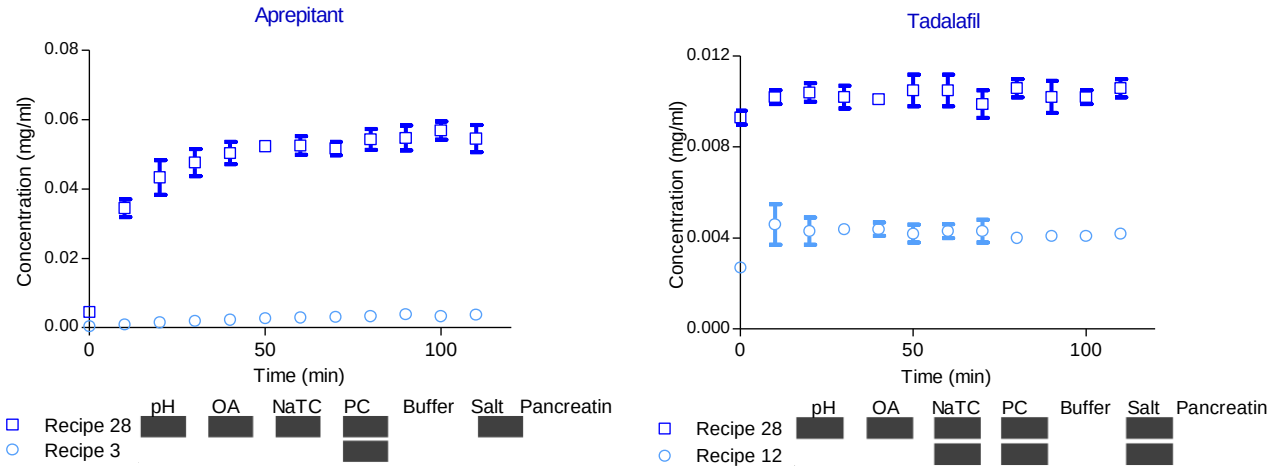


Figure 4. 2 Powder dissolution profiles of four acidic drugs piroxicam, phenytoin, indomethacin, zafirlukast and two basic drugs aprepitant, tadalafil Notes apply to all graphs in Figure 4.2 - 4.5 in the following pages: Error bars are SD of 3 measurements of dissolution, covered inside points if too small. □ represents data from HP media and o from MP media. The composition of each media recipe was shown below each graph, where blank represents component in low level, filled grey square for median level and dark for high level. For exact levels of each component, refer to table 2.2.

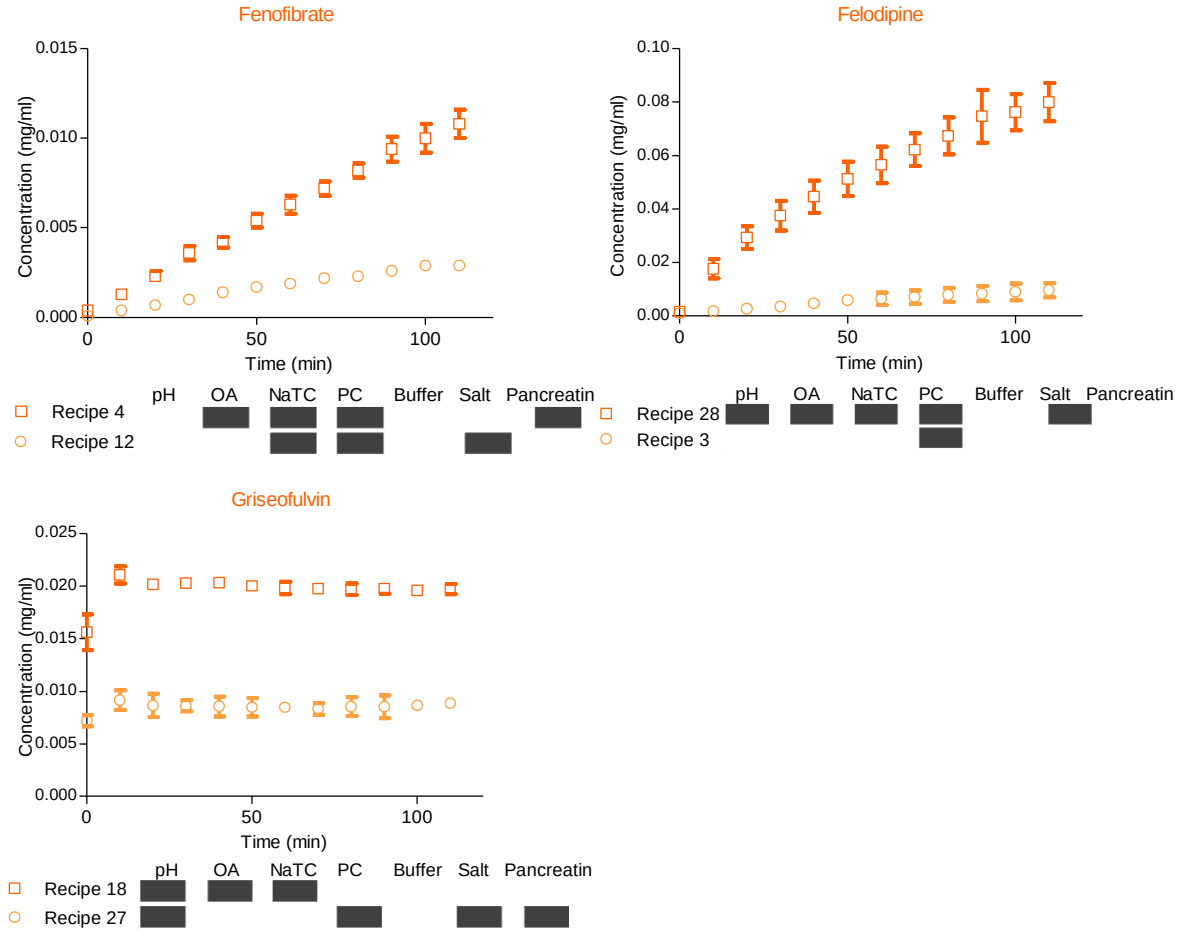


Figure 4. 3 Powder dissolution profiles of three neutral drugs fenofibrate, felodipine and griseofulvin

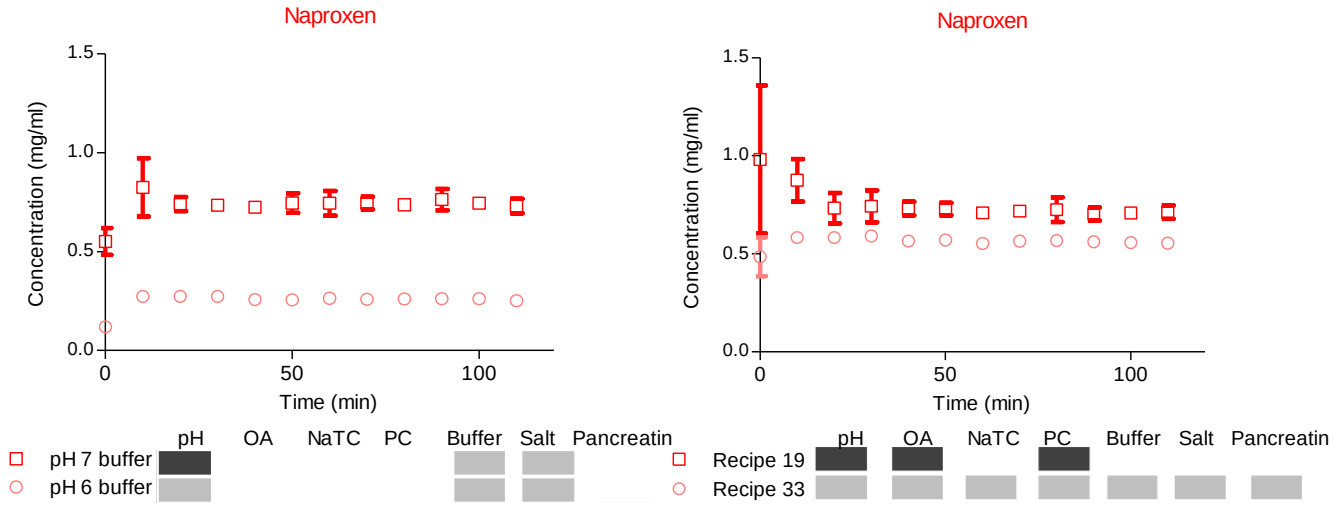


Figure 4. 4 Powder dissolution profiles of naproxen in aqueous buffer and biorelevant media
Right graph was conducted in HP (recipe 19 pH 7) and MP (recipe 33 pH 6) media, left graph was conducted in corresponding buffer without presence of NaTC, PC and OA

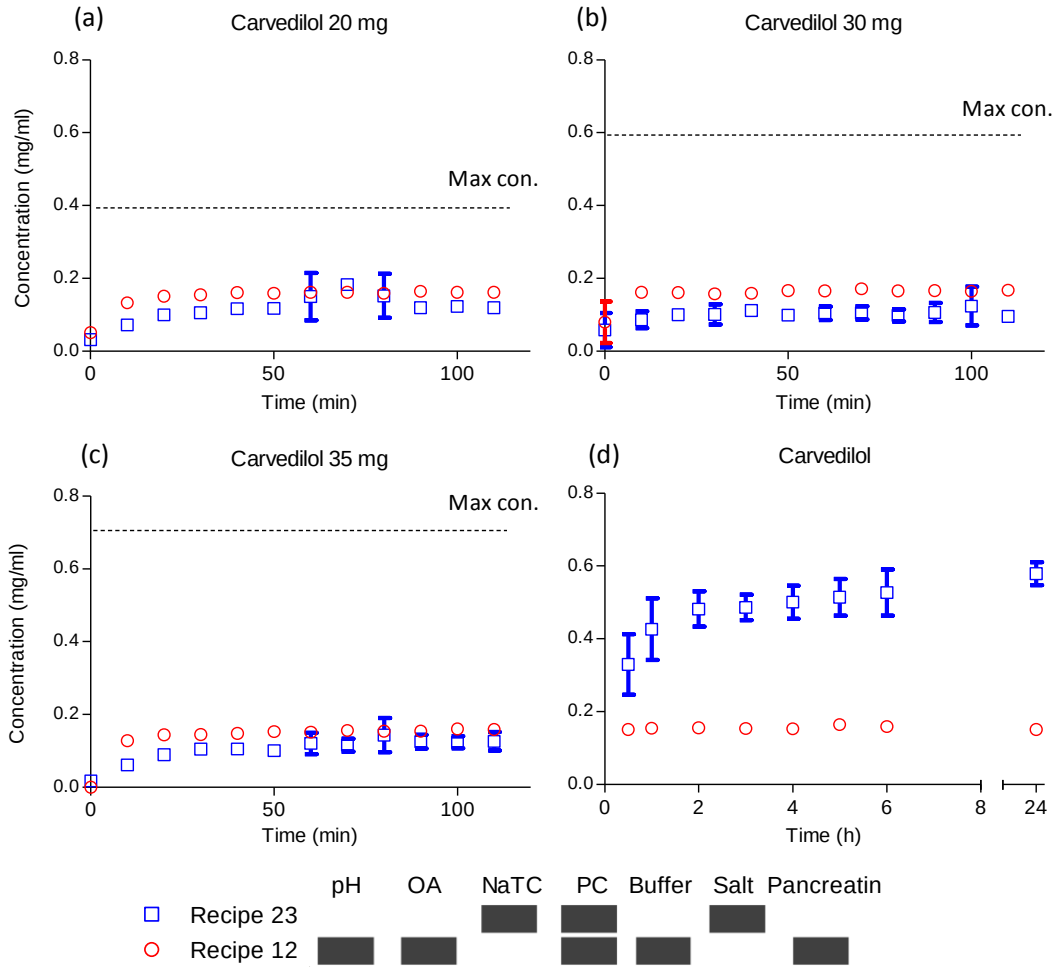


Figure 4. 5 Powder dissolution profiles of carvedilol

(a)(b)(c) were conducted with initial carvedilol powder of 20, 30, 35 mg in Sirius inForm, with sample filtered through 96-well plate before HPLC analysis. The dashed line is the theoretical maximum media concentration achievable with the added drug quantity. Figure 4 (d) was conducted by shake flask for 24 h in centrifuge tubes, with sample centrifuged and supernatant extracted for HPLC analysis. Theoretical maximum concentration was 2 mg/ml (20 mg in 10 ml media).

Table 4. 1 IDR, solubility and literature IDR of BCS II drugs in different media

	Powder (mg)	Volume (ml)	PS (m^2/g)	Solubility* (mg/ml)	Filter absorption	IDR ($\mu g/min/cm^2$)	IDR in literature ($\mu g/min/cm^2$)
Indomethacin MP	40	40	0.12	0.190		11	0.83 (pH 4.5) [t]
HP				1.000		44	33.5 (pH6.5 FaSSIF) [f]
Piroxicam MP	30	40	0.39	0.068	9%	1.3	2.0 (pH 4.5) [t]
HP				1.750	9%	18	DIDR 4.1 (pH 4.5) 91 (pH 6.8) [a]
							DIDR 43 (pH 4.5) 88 (pH 6.8) [y]
Naproxen MP	40	50	0.29	0.326	-4%	25	17 (pH 4.5) [t]
HP				0.800	1%	37	DIDR 13 (pH 4.5) 334 (pH 6.8) [a]
buffer pH6						11	DIDR 12 (pH 4.5) 264 (pH 6.8) [y]
buffer pH7						36	
Phenytoin MP	15	50	0.28	0.031	8%	2.6	
HP				0.054	-4%	5.0	
Zafirlukast MP	30	50	0.96	0.003	24%	6.4	
HP				0.012	21%	6.4	
Carvedilol MP	35	50	0.26	0.130	13%	12	5.4 (pH6.5 FaSSIF) [f]
HP				0.670	38%	5.2	
Tadalafil MP	15	50	0.23	0.008	20%	0.66	
HP				0.015	0%	1.5	
Aprepitant MP	10	50	1.09	0.016	61%	0.05	
HP				0.101	15%	2.8	

Felodipine MP	40	40	0.05	0.030	36%	0.40	0.64 (pH6.5 FaSSIF) [f]
HP				0.136	21%	4.2	
Griseofulvin MP	20	40	0.37	0.019	27%	0.50	2.5 (pH1.2, 4.5, 6.8) [t]
HP				0.029	-9%	1.14	DIDR 2.21 (pH 4.5) 1.61 (pH 6.8) [a]
							DIDR 1.9 (pH 4.5) 2.2 (pH 6.8) [y]
Fenofibrate MP	25	40	0.02	0.010	48%	0.28	
HP				0.154	28%	0.69	

HP, media provided high solubility in DoE; MP, media provided median solubility in DoE; PS, particle surface area per gram; IDR, intrinsic powder dissolution in this study; DIDR, disc IDR; Solubility*, equilibrium solubility in the corresponding HP or MP media (from Section 2); References are all from buffer unless specifying using "FaSSIF"; Filter absorption, for 96-well plate, filter absorption = 1 – (concentration at 2h from filtrate)/(concentration at 2h from supernatant after centrifugation). References: [a] (Avdeef and Tsinman, 2008) [f] (Fagerberg *et al.*, 2010) [t](Tsinman *et al.*, 2009) [y] (Yu *et al.*, 2004)

4.2.2 Comparison with literature

Generally, IDR in the MP media (pH 5) of acidic drug indomethacin, naproxen and piroxicam correlates with the literature values measured in pH 4.5 buffer. IDR of HP media (pH 7) correlates with the literature measured in pH 6.8 buffer, though for naproxen and piroxicam, the experimental data were lower than literature. MP and HP media represented low and high pH respectively, due to the predominant effect of pH on equilibrium solubility of acidic compounds; hence it is sensible to have pH dominated IDR for these compounds due to their equilibrium solubility, with higher pH gave higher IDR and vice versa. The data lower than literature from HP media were mainly attributed to the calculation for rapid dissolution underestimated IDR, but also can be related with presence of NaTC, PC and OA in biorelevant media. A comparison of buffer and biorelevant media were conducted for naproxen and showed dissolution profiles were similar at in HP media and aqueous buffer (pH 7), although with amphiphiles present in HP medium, indicating again the dominating effect of pH on drug solubility and therefore IDR. The dissolution in HP medium showed supersaturation at the beginning followed with precipitation, which was not present in the aqueous buffer (Figure 4.4). Additionally, dissolution was slightly lower in aqueous buffer (pH 6) than MP medium, which may be related with OA and PC added in HP media. The solubility of naproxen in recipe 33 (pH 6) was 0.5 mg/ml (1.5 mM), tied up with dissolution profile, while reported solubility of naproxen in buffer (pH 6) was around 0.9 mg/ml (4 mM) (Avdeef *et al.*, 2000, Avdeef and Tsinman, 2008). Thus solubility in fasted SIF recipe 33 and pH 6 buffer in current study were lower than literature. This decrease may be related with salting out effect from phosphate buffer or sodium salt, or determination difference. Therefore, though IDR was mainly driven by pH due to pH-dependent solubility, the addition of amphiphiles and electrolytes may affect IDR to some extent. Literature reported piroxicam underwent a transformation from anhydrous form to monohydrate and had supersaturation in

the dissolution (Jinno *et al.*, 2000, Tsinman *et al.*, 2009), while the supersaturation was not observed in this study.

The literature IDR of felodipine was obtained in pH 6.5 FaSSIF media and correlates well with the MP medium, while the IDR in HP medium was approximately ten times higher than that in MP medium. Accordingly, the solubility of felodipine was 14 μg/ml in pH 6.5 FaSSIF (Persson *et al.*, 2005), linked well with the MP medium solubility 3 μg/ml, while the solubility in HP medium was 136 μg/ml (data from Section 2), which was around ten times of the FaSSIF solubility, explaining also the ten time variation of IDR between MP and HP media. Griseofulvin had a slightly higher IDR in HP than MP media, even though both were in pH 7, this difference was introduced by alteration of OA, NaTC and PC concentration, while literature reported only one IDR for dissolution at pH 1.2, 4.5 and 6.8 for this neutral compound, which potentially overlooked the effect from amphiphiles and interactions with pH (Tsinman *et al.*, 2009).

All of the drugs tested were BCS II drugs and their IDR were less than 50 μg/min/cm^2 in selected DoE fasted SIF. Yu *et al.* suggested a disk IDR cut-off value of 100 μg/min/cm^2 as the boundary to distinguish high and low IDR in aqueous buffer media using a USP Wood Apparatus after comparing dissolution of 6 poorly soluble and 9 highly soluble drugs in line with BCS solubility classification criteria (Yu, Carlin *et al.* 2004). Based on this value, with the introduction of biorelevant media, all of the compounds tested were still regarded as low IDR. DoE distinguished indomethacin, piroxicam and naproxen as high solubility when utilising pH 7 fasted SIF while in dissolution they still perform as low IDR. This indicated that the biorelevant media may improve drug solubility and shift from BCS II to BCS I, it is still necessary to determine dissolution behaviour, as it may not be related with the increased solubility only. Additionally, this cut-off value should also be adjusted in

consideration of dose similar to solubility classification, which can bring justification with drugs in different dose.

DoE pointed out the significant positive relationship between the equilibrium solubility of lipophilic basic/neutral drugs and the amphiphile concentration in the media. HP media recipe 28, with high level of NaTC, PC and OA, provided highest solubility for zafirlukast, tadalafil, aprepitant, felodipine and phenytoin, and accordingly provided a higher IDR. Literature reported the different dissolution profiles in buffer and biorelevant media such as FaSSIF, FeSSIF and milk, especially for poorly soluble drugs (Galia *et al.*, 1998). Differences not only occurred between buffer and biorelevant media, but also between fasted and fed states (Dressman and Reppas, 2000), hence using biorelevant media to predict drug dissolution *in vivo* should account for the levels of the components, as they can significantly affect dissolution through changing drug solubility.

4.2.3 Ratio of intrinsic dissolution rate and ratio of solubility in two media

Figure 4.6 is the IDR ratio and solubility ratio at HP media and MP media. Solubility ratio is always above 1 according to the definition of HP and MP media. IDR ratio is above 1 except carvedilol. The IDR ratio in HP and MP media is lower than the solubility ratio for piroxicam, fenofibrate, indomethacin and zafirlukast. On the other hand, IDR ratio is higher than solubility ratio for felodipine and aprepitant. The solubility of aprepitant has about 6-fold increase, while the IDR had a more than 60-fold increase. For naproxen, phenytoin, griseofulvin and tadalafil, the ratios of IDR and solubility were pretty close, also for those drugs, the ratios were all in the lower range of 1 - 5, while in higher range, the difference of IDR ratio and solubility ratio was larger.

The IDR of aprepitant has a much greater ratio than that of solubility in HP and MP

media. This may be attributed to the poor wetting at MP media with low level of NaTC and OA compared with both at a high level in HP media, thus altering the efficient surface area. Dissolution can be further enhanced by efficient powder wetting with increasing biorelevant amphiphiles (Weintraub and Gibaldi 1969). Aprepitant is well known for its particle size-dependent BA and food effects, and nanoparticle suspension can significantly increase *in vivo* exposure and absorption (Wu, Loper *et al.* 2004).

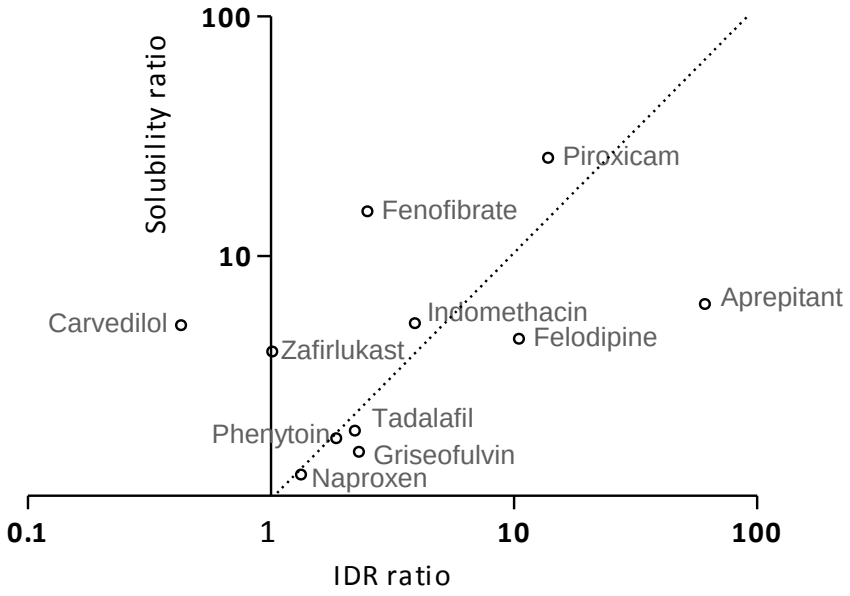


Figure 4. 6 Plot of IDR ratio and solubility ratio at HP media and MP media. Dash line has a slope of 1, indicating when IDR ratio equals to solubility ratio. Carvedilol is an outlier that has an IDR ratio < 1.

The solubility ratio in HP and MP media did not exactly match the IDR ratio in the corresponding media because IDR is a combination result from solubility (C_S), diffusion coefficient (D) and diffusion layer (h). According to Noyes-Whitney equation, both drug solubility and diffusion coefficient in amphiphile mixtures (NaTC/PC/OA) can positively affect IDR. The increase of solubility is usually associated with increased amount of drug ionisation and/or amphiphile solubilisation. This can affect the equilibrium of free drug and drug partitioned in

amphiphile aggregates and lead to changes of proportion of drug at different states with different diffusion coefficients (Jinno *et al.*, 2000). Okazaki *et al.* tested the solubility and dissolution of griseofulvin and danazol in FaSSIF and FeSSIF and compared with data in buffer (ratios of FaSSIF/buffer and FeSSIF/buffer). The solubility ratios ranged from 1.5 to 5.6 for griseofulvin and from 86 to 450 for danazol, while the IDR ratios only increased from 1.5 to 2.2 for griseofulvin and from 2.1 to 5.6 for danazol (Okazaki *et al.*, 2008). This is because the effective diffusion coefficient was a combination of diffusion coefficient of free drug and drug partitioned into amphiphile aggregates, the latter can be ten times lower due to the increased aggregate size than free drug (Okazaki *et al.*, 2008, Gamsiz *et al.*, 2010). Therefore IDR ratio is a result of both solubility ratio and effective diffusion coefficient ratio in free drug and aggregates. In Okazaki's example, griseofulvin and danazol are both neutral, while for ionisable drugs, there are another two forms: the free ionised drug and ionised drug in aggregates, both may introduce different diffusion coefficients. In the current study, though the knowledge of solubility was available, there was no systematic control of amphiphile levels used in the media. Studies analysing solubility in blank buffer and biorelevant media with known amount of amphiphiles can be used to calculate the fraction of drug partitioned in amphiphile aggregates; diffusion coefficient can be obtained experimentally using particle size from dynamic light scattering and Stokes-Einstein equation; pK_a indicates the drug percentage ionised at certain pH; all of these parameters can be used to build the dissolution model in different media (Okazaki *et al.*, 2008). Lipophilic drugs were more likely to show different dissolution in buffer, FaSSIF and FeSSIF, due to more interaction with mixed amphiphile aggregates compared with drugs with log $P < 0$ (Gamsiz *et al.*, 2010). Moreover, type of amphiphiles used introduces coexistence of multiple aggregate species, which can introduce different interactions with drug. This has been supported by the 4MD experiment. Models considering drug partition significantly improved the simple Noyes-Whitney

model and predict amphiphile effects on dissolution, however assuming one partition coefficient and one total amphiphile concentration can still generate errors compared with experimental data (Gamsiz *et al.*, 2010).

4.3 Conclusion

Both solubility and dissolution are important parameters for PBPK modelling and understanding drug absorption *in vivo* (Dressman and Reppas, 2000). Powder dissolution in biorelevant media containing different composition of fasted SIF display different dissolution profiles, indicating the positive relationship between IDR and equilibrium solubility, though IDR tends to increase to a smaller extent. Due to this correlation, the significant effects from pH, amphiphile contents, buffer and salt to drug solubility are potentially transferable to affect IDR, for poorly soluble drugs. Significantly different IDR were reported between simple aqueous buffer and lipid-rich biorelevant media (Klein 2010). For BCS II, since permeability is not rate limiting step of drug absorption, using biorelevant media for dissolution is essential to establish IVIVC.

The endogenous GI contents and impact of food on solubility and dissolution may vary, thus dissolution should not be interpreted solely from equilibrium solubility based on linear relationship according to Noyes-Whitney equation, at least for poorly soluble drugs. More sophisticated dissolution theories considering media influence should be added (Persson *et al.*, 2005), which include the drug-amphiphile interactions based on drug lipophilicity and amphiphile species. Alternatively, experimental dissolution tests utilising physiologically relevant media in multiple levels with automatic systems are still recommended. Additionally, due to the interference of UV signal in turbid media, off-line sampling and sample filtering have to be used for biorelevant media with high levels of lipids (Okazaki *et al.*, 2008, Gamsiz *et al.*, 2010), even though the commercial FaSSIF and FeSSIF (Biorelevant,

2016) can be used on-line (Gamsiz *et al.*, 2010). These procedures can generate experimental errors, and the lack of points in the initial time (<1 min) can be a disadvantage for rapid powder dissolution (Gamsiz *et al.*, 2010). The development of an on-line concentration analysis technique is highly required to deal with turbid media for real time dissolution.

5 Conclusion & future work

5.1 Conclusion

GI tract is a dynamic system with highly variable fluid composition among different absorption locations and individuals (Kleberg *et al.*, 2010), consequently a single medium will only reveal the drug behaviour under one set of condition and neglect potential possibilities in others. Moreover, changing one factor at a time, depending on the drug tested, may disguise interactions with other media components. DoE allows for a comprehensive understanding of the solubility profile, which is inside the variation window of the physiological conditions; it also simultaneously provides information on the critical media factors and interactions that cause the variation, therefore indicating how the drug absorption may vary and where the limitation could arise *in vivo*.

Except pancreatin, all other factors can significantly affect drug equilibrium solubility. The average effect magnitudes are in the descending order of pH, OA, NaTC, PC, buffer and salt. OA and pH have the highest magnitude among all significant interactions. However, the significant factors, interactions, the magnitude and the ranking are different for individual drugs, rather than generalised rules. A decision tree concept has been developed to understand the critical factors, with pH dominantly affects ionisable compounds, while amphiphiles affect compounds that have no ionisation change at the biorelevant media pH range (5 - 7). The results proved the importance to use biorelevant media, and further emphasize that drug reactions are indeed case by case, thus a series of systematically designed media have to be used to capture the whole solubility variation space, rather than a single or a few media (FaSSIF/FeSSIF) measuring drug solubility in only certain conditions. In addition, using these media with multiple factor changes at once, one cannot diagnose the reason of the solubility changes (Kostewicz *et al.*, 2004). Fasted biorelevant media can be prepared without pancreatin; however the remaining

factors have to be considered according to physiological data.

Following interesting information about the influence of amphiphiles to BCS II drugs in DoE, 4MD focuses on designing biorelevant media with various ratios of amphiphiles and on their influence on the equilibrium solubility. It sheds light on the drug-amphiphile interactions, some effects were in good agreement with reported studies (Hammad and Muller, 1998, Kossena *et al.*, 2004, Persson *et al.*, 2005), indicating the interactions were related with the drugs. Changing amphiphile ratios has marginal effect to two out of seven drugs (griseofulvin, spironolactone), the other five drugs solubility varies and implies non-linear relationship between amphiphile solubilisation capacities and amphiphile concentrations. There were drug and amphiphile specific interactions that are related with the lipophilicity of the drug, molecular structure, ionisation of both the drug and amphiphiles.

DoE and 4MD both indicated the solubility sensitivity to media composition levels and ratios. Biorelevant media should explore a physiologically related and compacted level and ratio range of pH, amphiphiles, buffer and salt to reveal information on solubility variation and the reasons for it. Both experiments covered the literature values, indicating they are possible to be applied practically and transferred to new design covering the conditions in both fasted and fed states.

Besides solubility, simulating dissolution *in vitro* is also a key assessment to predict *in vivo* absorption. The dissolution study using selected fasted SIF from DoE supported the simple concept from Noyes-Whitney equation that IDR is positively correlated with solubility. Solubility change is usually induced by the change of media composition, however this correlation is usually not linear because IDR is influenced by both solubility and diffusion coefficient, both of which can be

divergently altered by the media composition. The influence on solubility has been highlighted by DoE; the specific drug-amphiphile interactions, which leads to varying diffusion coefficient, has been demonstrated in 4MD, indicating the need for developing a more complicated dissolution model or using experimental data. Small scale dissolution on inForm platform and HPLC off-line analysis allow for the use of turbid biorelevant media with diverse composition even at the early stage of drug development. However, dissolution instrument handling turbid media for on-line concentration monitoring and even detecting solid form transformation could give many advantages compared with current methods.

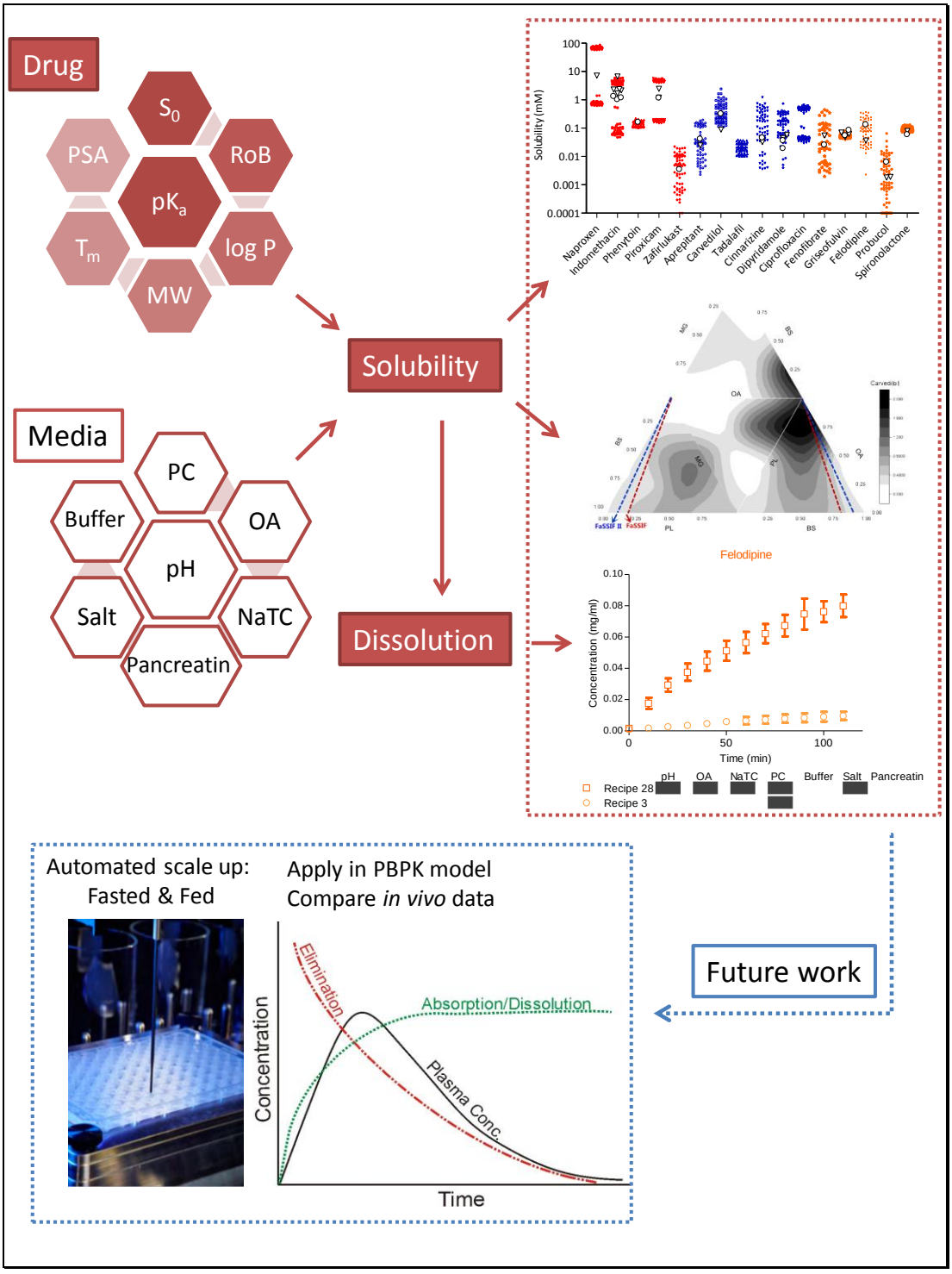
5.2 Future work

Current study only investigated the significant factors and interactions in the fasted intestinal state. Further study can employ adjusted levels of each factor correlated with fed state. Griseofulvin was reported to double the solubility in FeSSIF (FaSSIF 15 µg/ml; FeSSIF 34 µg/ml; BS/PL=30/7.5 mM solubility 58 µg/ml) (Okazaki *et al.*, 2008), while current study only reports solubility in a tight range 19 ± 3 µg/ml. Therefore, a DoE covering fed state levels can clarify if an enlarged range of parameters can alter the solubility distribution and significant factors. Digestion products such as MG should be considered which potentially increase the number of experiments in the design. Development of automated system can reduce experimental errors, and reduce DoE measurement from duplicates to single point, significantly minimising the number of experiment and amount of drug used. Alternatively, a fractional factorial design can be used to reduce the scale, with knowledge of confounded parameters and avoid parameter of interests confounded with each other. Similarly, multiple amphiphile total concentrations can be employed for 4MD, since drug-amphiphile interactions are expected to change due to the formation of different lipid phases (micelles, vesicles) (Staggers *et al.*, 1990). This can be accomplished in a full range mixture design or an optimised design (such

as D-optimal) only considering physiologically relevant space as preparation of solubilised single lipid media may be not possible.

Another limitation is the lack of application in PBPK modelling and correlation establishment with *in vivo* plasma concentration data. DoE captures the variation of GI media that can induce the solubility fluctuation, which would be useful information for PBPK models such as GastroPlus™ Advanced Compartmental And Transit (ACAT) model and Simcyp® advanced dissolution absorption and metabolism (ADAM) model (Sjogren *et al.*, 2016), in order to maximise the prediction powder of the models and identify food effects (Jones *et al.*, 2006). The results of DoE imply the possibility to choose the complexity of biorelevant media based on the test purposes and the drug categories. The proposed decision tree needs to be further developed to identify more inter-correlation of critical physicochemical properties and cut-off values. Markopoulos *et al.* have started to define the level of biorelevant media based on its content complexity and degree of biorelevance, though more studies have to be done to refine the model and justify the prediction power (Markopoulos *et al.*, 2015). Number of drugs has to increase to a statistical level; however this number is related with drugs under each sub categories (i.e. high/low log P, MW). Practical strategies are required for studying drugs with diversity of different properties and property combinations, alternatively using a series of structurally similar drugs can help dissect the relationship of one drug property to specific solubilisation effects.

5.3 Summary figure for thesis



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Posters and Papers

Sep. 2013 APS International PharmSci Conference
Heriot Watt University, Edinburgh
Poster & Presentation

The use of statistical design approaches to investigate the impact of intestinal fluid composition on the equilibrium solubility of candidate drugs

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Abstract – Investigation of the relationships between simulated intestinal fluid media components and equilibrium drug solubility using a design of experiment protocol is reported. The results highlight expected, effects including media pH for an ionisable drug and micellar solubilisers for a neutral drug. However, analysis failed to show any effects for the concentration of phosphate buffer or of pancreatin and these components could be eliminated. This approach will be employed to rationalize simulated dissolution media and explore characteristics of a wider drug set.

INTRODUCTION

In vitro dissolution testing of solid oral dosage products is a key activity in pharmaceutical development but may poorly predict in vivo performance. The use of biorelevant media involving the utilization of simulated intestinal fluids has been adopted (Vertzoni *et al.*, 2004). These media are complex with variable recipes reflecting the true intestinal mileu as measured; simulated fasted intestinal fluid (FaSSIF) for example could consist of up to ten ingredients. The physicochemical relationships between FaSSIF components and its effect on drug solubility are therefore important attributes. In initial steps to probe these relationships we have studied equilibrium solubility in FaSSIF using a design of experiment (DOE) technique based around seven literature components or conditions, sodium taurocholate, lecithin, sodium phosphate, sodium chloride, pH, pancreatin and sodium oleate. Three common drugs covering acidic (ibuprofen, IBU), basic (mebendazole, MBZ) and neutral (fenofibrate, FFB) compounds have been screened.

MATERIALS AND METHODS

A ¼ factional factorial DOE with 7 factors and 2 levels was constructed using MiniTab and required 64 different experiments. The required fluid composition was mixed from stock solutions to provide a final volume of 4 mL in a 15 mL centrifuge tube

and pH adjusted to 5 or 7 using 0.5 M HCl or 0.5 M KOH. An excess of solid drug was added and the tubes placed in an orbital shaker for 24 h at 37 °C and 240 rpm. After incubation tubes were centrifuged (10,000 rpm, 15 min), 500 µL of supernatant removed and the concentration determined by HPLC.

RESULTS AND DISCUSSION

For IBU, pH is the predominant factor influencing solubility, however salt concentration also affects the result, with a significant interaction between the parameters. Maximum solubility was determined at pH 7 (highest level) and 68 mM salt (lowest level).

MBZ (pKa ~4) solubility was low in all samples and indicated that pH and other components had no influence. However, this also reduced the reliability of the statistical analysis.

FFB solubility was positively affected by sodium oleate and lecithin concentrations with the former more influential. Four significant interactions were noted (in decreasing order): salt*pH, bile salt*sodium oleate, bile salt*pH and salt*sodium oleate. Maximum solubility was at high concentrations of sodium oleate, bile salt, lecithin and low salt concentration. Although FFB is non-ionised interactions with bile salt, salt and pH were evident with a higher solubility in pH 5 media than pH 7.

CONCLUSIONS

These data indicate that a DOE can be employed to examine simulated intestinal fluids and to explore the relationships between components and effect on drug solubility. Obvious relationships such as pH on ionisable compounds (IBU) or “micelle” systems (lecithin/bile salt/sodium oleate) for neutral compounds are demonstrated but adoption of this statistical methodology permits the detection of more subtle interactions between components and allows reduction to the simplest compositions for biorelevant media.

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Poster & Presentation

Investigations of solubility profiles of poorly soluble drugs by varying ratios of biorelevant surfactants

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Abstract - Investigation of the mixture ratio of three biorelevant surfactants, bile salt (BS), phospholipid (PL) and fatty acid (FA) to the equilibrium solubility of insoluble basic and neutral drugs was conducted. Results highlight the importance of surfactant proportions to design simulated small intestinal fluids (SIF), which should consider the lipophilicity, molecular shape and ionisation of the drugs. This study can be used to further explore situations with food digests and indicates whether the individual variability of lipid composition may affect drugs' bioavailability to a large extent.

INTRODUCTION

Poor solubility with respect to dose is a recognised issue hindering drug development and oral bioavailability (Stegemann *et al.*, 2007). Food in gastrointestinal tract can play a significant role in the bioavailability of orally administered drugs through its effects on the tract contents and milieu affecting drug solubility and dissolution. Fasted and fed SIF were introduced containing physiologically relevant levels of BS, PL and FA with fixed ratios of surfactants employed. However, studies of aspirated gastrointestinal media indicate a large degree of individual variability in the fluid composition (Bergstrom *et al.*, 2014).
Statistical evaluation of a simulated fasted fluid composition indicated that biorelevant surfactants exerted a significant influence on the solubility of neutral and basic drugs (Khadra *et al.*, 2015). A phase diagram (PD) based study employing a constant total molar concentration of BS, PL and FA was therefore conducted to investigate interactions and their impact on drug solubility.

MATERIALS AND METHODS

A mixture design of PD was constructed using Minitab® 16.0 containing 15 compositions in the ternary contour plot. A medium total amphiphile concentration (11.7 mM) was chosen for the PD study. The required fluid composition was mixed from stock solutions to provide a final volume of 4 ml in a 15 ml centrifuge tube with pH adjusted to 7 using 1 M HCl or 1 M KOH. An excess of solid drug was added

and the tubes were shaken for 24 h at 37 °C. After incubation, tubes were centrifuged; 0.5 ml of supernatant removed and the concentration was determined by HPLC.

RESULTS AND DISCUSSION

Except for two drugs (griseofulvin and spironolactone), solubility across the PD varies markedly with up to a 30-fold increase (i.e. carvedilol). The lipophilicity of a drug can affect how much the drug engages with the lipid-rich micelles, which explains the insensitivity of spironolactone (log P 2.78) and griseofulvin (log P 2.18) due to comparatively lower log P.

In addition, drugs react diversely to the three surfactants. For example, the solubility of zafirlukast only increases when the proportion of PL increases (coefficient determination r2=0.96). This might be attributed to its planar and rotatable structure which could fit into the two hydrophobic chains of lecithin. While for fenofibrate and felodipine, an appropriate share of all the three surfactants would provide better solubility. Carvedilol favours a more negatively charged system with higher sodium oleate (25% PL, 75% FA) (Fig. 1), which implies that for moderately hydrophobic and ionic compounds, electrostatic attraction may also affect the drug-micelle interaction.

CONCLUSIONS

Results confirm the importance of considering the mixture of digestion-related lipids in SIF and additionally raise the issue of mixture ratios. It also gives insight of interaction within the mix micelles. Five out of seven drugs present diverse solubility profiles in the PD, implying that the total solubilisation capabilities are not a simple accumulation of the three biorelevant surfactants.

The lipophilicity of the drugs, molecular shape, charge and structures may show specific drug-surfactant affinity. Therefore, targeting the analysis on the media in a more physiological related and compacted ratio range of BS, PL and FA would provide more comprehensive information on the sensitivity of the drugs to combination of different amphiphiles.

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Oct. 2015 American Association of Pharmaceutical Scientists (AAPS) Annual Meeting and Exposition
Orlando, Florida, 2 Posters

Investigations of Solubility Profiles of Poorly Soluble Drugs by Varying Ratios of Biorelevant Surfactants

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Purpose

Poor solubility with respect to dose is a recognised issue hindering drug development and oral bioavailability. Food in gastrointestinal tract can play a significant role in the bioavailability of orally administered drugs through its effects on the tract contents and milieu affecting drug solubility and dissolution. Fasted and fed simulated small intestinal fluids (SIF) were introduced containing physiologically relevant levels of bile salt (BS), phospholipid (PL) and fatty acid (FA) with fixed ratios of surfactants employed. However, studies of aspirated gastrointestinal media indicate a large degree of individual variability in the fluid composition. Statistical evaluation of a fasted SIF composition indicated that biorelevant surfactants exerted a significant influence on the solubility of neutral and basic drugs. A phase diagram (PD) based study employing a fixed total concentration of BS, PL and FA was therefore conducted to investigate interactions and ratio impact on drug solubility.

Methods

A mixture design PD was constructed using Minitab 16.0 containing 15 compositions in the ternary contour plot. A medium total amphiphile concentration (11.7 mM) was chosen for the PD study. The required fluid composition was mixed from stock solutions to provide a final volume of 4 ml in a 15 ml centrifuge tube with pH adjusted to 7 using 1 M HCl or 1 M KOH. An excess of solid drug was added and the tubes were shaken for 24 h at 37 oC. After incubation, tubes were centrifuged; 0.5 ml of supernatant removed and the concentration was determined by HPLC.

Results

Except for two drugs (griseofulvin and spironolactone), solubility across the PD varies markedly with up to a 30-fold increase (i.e. carvedilol). The lipophilicity of a drug can affect how much the drug engages with the lipid-rich micelles, which explains the insensitivity of spironolactone and griseofulvin due to comparatively lower log P. In addition, drugs react diversely to each surfactant. For example, the solubility of zafirlukast (Fig. 1) only increases when the proportion of PL increases (coefficient determination r2=0.96). This might be attributed to its planar and

rotatable structure which could fit into the two hydrophobic chains of lecithin. While for fenofibrate and felodipine, an appropriate share of all the three surfactants would provide better solubility. Carvedilol favours a more negatively charged system with higher sodium oleate (25% PL, 75% FA) (Fig. 1), which implies that for moderately hydrophobic and ionic compounds, electrostatic attraction may also affect the drug-micelle interaction.

Conclusion

Results highlight the importance of surfactant ratios to design SIF, which should consider the lipophilicity, molecular shape and ionisation of the drugs. Five out of seven drugs present diverse solubility profiles in the PD, implying that the total solubilisation capabilities are not a simple accumulation of the three biorelevant surfactants. Therefore, targeting the media designed in a more physiological related and compacted ratio range of BS, PL and FA would provide more comprehensive information on the sensitivity of the drugs to the variability of lipid composition.

Impact of Fasted Intestinal Fluid Composition on the Dissolution Rate of BCS II Drugs

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Purpose

In vitro gastrointestinal (GI) dissolution test is a major method to evaluate the formulation and adsorption of solid oral drugs. Fasted and fed simulated intestinal fluids (SIF) were employed for in vitro solubility and dissolution studies since 1998 (Dressman *et al.*, 1998) to correlate results with human intestinal fluids. Miniaturised version of rotating disk and powder dissolution apparatus has been utilised and monitored in real time with in situ UV dip-probe. However this technique relies on a clear dissolution media for accurate measurement of the UV signal. In this study, off-line sampling was conducted to avoid interference of the turbid media to drug measurement, by using fasted SIF designed in a previous Design of Experiment (DoE) solubility study, which proved the multivariate effects of different biorelevant media on solubility of BCS II drugs (Khadra *et al.*, 2015). This study further explore whether these effects are also exhibited during dissolution.

Methods

Two different dissolution media were chosen representing the highest and middle equilibrium drug solubility among all thirty three kinds of DoE media. They were freshly prepared from corresponding powder and pH adjusted. Dissolution was

performed in 40-50 ml media using Sirius inForm with built-in pH control and constant stirring at 25 °C. Off-line sampling was taken every 10 min up to two hours through a 96-well plate filter and filtrate analysed in HPLC. The intrinsic dissolution rate is expressed as below: IDR= V (dc/dt) (1/S) Where V is the dissolution media volume, c is the concentration, t is the time and S is the surface area of the powder. For fast dissolved compounds (reach maximum within 10 min), the dissolution rate were calculated as an average rate in 10 min
(dc/dt = maximum concentration/10 min).

Results

Phenytoin, tadalafil, piroxicam and griseofulvin can achieve their maximum concentration in the first 10 min. The dissolution rates correlated well with the DoE solubility, which is media with high solubility resulted in high dissolution rates and low solubility resulted in low dissolution rates (Figure 1). Results support that dissolution rates highly depend on the nature of the dissolution media under the same dissolution condition (temperature, particle surface area, stirring rate etc.) and can be predicted from the equilibrium solubility.

Conclusion

It has been long recognised that dissolution rates can control the rate of absorption and bioavailability if the absorption in GI tract is rapid. Therefore in vitro dissolution test is of great importance to establish bioequivalence related to formulation design, salt formation and quality control. This study addresses the diverse effects of media components, not only to the solubility of the drug, but also the intrinsic dissolution rate, and the importance to account for this dissolution variability when modelling absorption and build in vitro-in vivo correlation.

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Statistical investigation of simulated intestinal fluid composition on the equilibrium solubility of biopharmaceutics classification system class II drugs

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ABSTRACT

A drug's solubility and dissolution behaviour within the gastrointestinal tract is a key property for successful administration by the oral route and one of the key factors in the biopharmaceutics classification system. This property can be determined by investigating drug solubility in human intestinal fluid (HIF) but this is difficult to obtain and highly variable, which has led to the development of multiple simulated intestinal fluid (SIF) recipes. Using a statistical design of experiment (DoE) technique this paper has investigated the effects and interactions on equilibrium drug solubility of seven typical SIF components (sodium taurocholate, lecithin, sodium phosphate, sodium chloride, pH, pancreatin and sodium oleate) within concentration ranges relevant to human intestinal fluid values. A range of poorly soluble drugs with acidic (naproxen, indomethacin, phenytoin, and piroxicam), basic (aprepitant, carvedilol, zafirlukast, tadalafil) or neutral (fenofibrate, griseofulvin, felodipine and probucol) properties have been investigated. The equilibrium solubility results determined are comparable with literature studies of the drugs in either HIF or SIF indicating that the DoE is operating in the correct space. With the exception of pancreatin, all of the factors individually had a statistically significant influence on equilibrium solubility with variations in magnitude of effect between the acidic and basic or neutral compounds and drug specific interactions were evident. Interestingly for the neutral compounds pH was the factor with the second largest solubility effect. Around one third of all the possible factor combinations showed a significant influence on equilibrium solubility with variations in interaction significance and magnitude of effect between the acidic and basic or neutral compounds. The least number of significant media component interactions were noted for the acidic compounds with three and the greatest for the neutral compounds at seven, with again drug specific effects evident. This indicates that a drug's equilibrium solubility in SIF is influenced depending upon drug type by between eight to fourteen individual or combinations of media components with some of these drug specific. This illustrates the complex nature of these fluids and provides for individual drugs a visualisation of the possible solubility envelope within the gastrointestinal tract, which may be of importance for modelling *in vivo* behaviour. In addition the results indicate that the design of experiment approach can be employed to provide greater detail of drug solubility behaviour, possible drug specific interactions and influence of variations in gastrointestinal media components due to disease. The approach is also feasible and amenable to adaptation for high throughput screening of drug candidates.

1. Introduction

The preferred route of administration for medicinal products is the oral route where the use of tablets and capsules accounts for seventy percent or more of the marketed products available. It is accepted the solid drug must dissolve from the medicinal product in the fluids of the gastrointestinal tract, remain in solution and then permeate through the gut wall in sufficient quantities to reach the portal and then systemic circulation. The mechanistic concept of the two stage process was first formalised in the biopharmaceutics classification system (BCS), which teaches that drug solubility and gastrointestinal permeability are two key factors controlling oral bioavailability (Amidon et al., 1995). The

Abbreviations: BCS, biopharmaceutics classification system; DoE, design of experiment; FASSIF, fasted simulated intestinal fluid; FESSIF, fed simulated intestinal fluid; IVIVC, *in vitro in vivo* correlation.

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bioavailability of BCS class II drugs (low solubility and high permeability) *in vivo* is predominantly dissolution-limited and controlled by the behaviour within the gastrointestinal tract (Klein, 2010). A low solubility, with respect to dose (Muenster et al., 2011) can result in incomplete absorption of the administered drug and therefore solubility and rate of dissolution of the active pharmaceutical ingredient is a pivotal characteristic controlling its drug like properties (Lipinski, 2000) and biopharmaceutical characteristics (Sugano et al., 2007).

The solubility and dissolution of a drug substance is influenced by a range of factors such as the drug's chemical structure, presence of ionisable groups, crystal form and particle size, formulation, pH of the dissolution medium and the presence of surfactants to list but a few (Wurster and Taylor, 1965). A compound's p*K*a for example can induce extreme effects due to the variation of pH in the intestine (5–7) compared to the stomach (1.5–2). This can greatly affect solubility of for example weakly basic compounds (Gould, 1986) which could rapidly dissolve in the stomach yet precipitate in the higher pH environment of the intestine. In addition non-pH related factors for example the presence of intestinal solubilising agents such as bile salts and lecithin can increase the aqueous solubility of non-ionisable drugs (Mithani et al., 1996; Naylor et al., 1993; Pedersen et al., 2000a). The gastrointestinal tract's main role is nutrition not drug delivery and the influence of food on drug absorption has been evident since the early 1960s (Crounse, 1963). These results are part of a wide extant literature (Augustijns et al., 2014; Dressman et al., 2007; Kleberg et al., 2010b; Reppas and Vertzoni, 2012), which illustrate that the composition of dissolution media is important and influences the solubility, dissolution and therefore bioavailability of poorly soluble drugs and that the physicochemical relationships between media components and their effect on drug solubility are critical attributes. The challenge to accurately predict a drugs biopharmaceutical performance and relate *in vitro* drug dissolution data against *in vivo* drug profiles is an important one (Gowthamarajan and Singh, 2010), since around forty percent of new chemical entities are rejected in the early stages of drug development usually because of low aqueous solubility. Though this in itself should not be sufficient to disregard these drugs from further development as low solubility in aqueous buffer systems does not always correlate with low intraluminal solubility (Clarysse et al., 2009) and evidence has shown some low water soluble drugs to exhibit higher gastro-intestinal solubility and associated increased bioavailability (Persson et al., 2005). The *in vitro* solubility and dissolution testing of solid drugs and oral dosage forms is therefore a key activity in pharmaceutical development but may poorly predict *in vivo* performance.

The most obvious solvent for the *in vitro* study of drug solubility and dissolution with respect to oral administration is therefore human gastric (HGF) (Finholt and Solvang, 1968) or intestinal fluids (HIF) (Dressman et al., 2007). However, it is not practical to obtain the quantities that would be required for large scale drug development studies (Finholt and Solvang, 1968), the composition of HIF is variable (Lindahl et al., 1997) and dependent upon the donor's fasted or fed state and collection technique employed (Bergstrom et al., 2013). To circumvent the issues associated with HIF availability and variability, extensive work has been conducted on the design of simulated biorelevant media (Tenhoor et al., 1991) with Galia et al. (1998) using systems designed to reflect the *in vivo* pH as well as incorporate biological compounds such as bile salts and phospholipids to mimic fasted and fed intestinal fluid (fasted simulated intestinal fluid (fastedsimif) and fed simulated intestinal fluid (fedsimif)) (Diakidou et al., 2009; Jantratid et al., 2008b; Kalantzi et al., 2006; Vertzoni et al., 2004). Subsequent work has expanded and refined these initial simulated media in an attempt to optimise the composition and as such the constituents can be complex especially where the inclusion of food based components to mimic the fed state is required (Kleberg et al., 2010a; Reppas and Vertzoni, 2012). Therefore multiple recipes reflecting the intestinal milieu as measured have been presented (Table 1) and for example could consist of up to ten ingredients (Fagerberg et al., 2010; Ilardia-Arana et al., 2006; Jantratid et al., 2008a; Kleberg et al., 2010a; Luner and Vander Kamp, 2001; Marques et al., 2011; Persson et al., 2007; Soderlind et al., 2010; Sunesen et al., 2005; Vertzoni et al., 2004). In this paper the term fastedsimif will be employed to generically refer to all simulated fasted intestinal fluid recipes irrespective of composition. Several if not all of the studies characterise the influence of component concentration (Mithani et al., 1996) on solubility and Clarysse et al. (2009) have employed multiple linear regression to determine the importance of various media factors on the solubility of five drugs in HIF. This group have extended this (Clarysse et al., 2011) to perform a principal component analysis and a comparison of HIF, fastedsimif and tocopherol polyethylene glycol surfactant system. However, as far as we can ascertain in the literature there has not been a systematic investigation into the effects of combining the multiple components present in simulated media on drug solubility.

Design of experiment (DoE) is widely employed in complex systems to observe the effects of multiple factors, their interactions on the system response and optimise the conditions for a desired result, with minimal experimental effort. Factors can be quantitative (i.e. pH) or qualitative (i.e. base/acid). The most commonly applied design has two levels of each factor, which are the low and the high level. In a full factorial design, all the possible combinations of each factor level are conducted, but when the factors are numerous or replicates are needed, a fractional factorial design is a more practical choice (Myers et al., 2009). However, when only part of the full experimental set is conducted, some of the effects will be confounded and the effects cannot be estimated separately, which may require further targeted experiments. Therefore, the fraction must be chosen carefully and the conclusion of those confounded results should be drawn with due consideration.

Therefore in order to probe the influence of media composition on solubility we have applied a DoE based protocol to investigate the effect of seven factors or components previously employed in simulated fasted media variants. These are sodium taurocholate (bile salt), lecithin (phospholipid), sodium phosphate (buffer), sodium chloride (salt and osmotic adjustment), pH, pancreatin (enzyme content) and sodium oleate (digestion product) (see Table 2) at upper and lower levels based upon analysis of those concentrations described previously in literature (see Table 1). Simulated fasted media has been chosen as a starting point since it simpler than fed simulated media with the aim of determining if it is feasible to conduct these experiments and identify those components or interactions between components relevant to drug equilibrium solubility. In addition a range of common poorly soluble (BCSII) drugs with acidic (naproxen, indomethacin, phenytoin, and piroxicam), basic (aprepitant, carvedilol, zafirlukast, tadalafil) or neutral (fenofibrate, griseofulvin, felodipine and probucol) properties, which have previously been extensively employed in the literature will be used to further expand the scope of the study. This study is being conducted as part of a wider investigation of oral absorption under the auspices of a European Union Innovative Medicines Initiative on the Development of new Oral Biopharmaceutical Tools (OrBiTo), see acknowledgements.

2. Materials and methods

2.1. Materials

Hydrochloric acid (HCl), potassium hydroxide (KOH), acetic acid, sodium taurocholate, pancreatin from porcine, monosodium

Table 1
Sample literature recipes for fasted simulated intestinal fluid (FaSSIF).

Component/parameter and concentration (mM)	Dressman et al. (1998)	Galia et al. (1998)	Pedersen et al. (2000a,b)	Vertzoni et al. (2004)	Sunesen et al. (2005)		Jantratid et al. (2008a,b)	Brinkmann-Trettenes and Bauer-Brandl (2014)
					Low	High		
Sodium TC	5	3	0	3	2.5	6.3	3	3
Sodium GC			3.7					
Lecithin	1.5	0.75	0.9	0.75	0.5	1.25	0.2	1.5
NaH_2PO_4	(K) 29	(K) 28.6	50	28.66	(K) 29	(K) 29		32.9
NaOH				~13.8			34.8	98
NaCl	(K) 220	(K) 103.3	150 (total Na)	106	No salt (KCl)	No salt (KCl)	68.62	105
Pancreatin							100 U/mL	3.2 mg/mL
Tris/maleic acid							19.12	
Osmolality (mOsmol)	280–310	270 ± 10		270 ± 10			180	
pH	6.8	6.5	6.5	6.5	6.8	6.8	6.5	6.5

phosphate (NaH_2PO_4), sodium chloride (NaCl), chloroform, naproxen, fenofibrate, griseofulvin, phenytoin, piroxicam, and indomethacin were purchased from Sigma–Aldrich, Poole, Dorset UK. Lecithin S PC (phosphatidylcholine from Soybean "98%") was sourced from Lipoid, Germany. Aprepitant, carvedilol, felodipine, probucol, tadalafil and zafirlukast were kindly provided through OrBiTo (see acknowledgements) by Dr. R. Holm Head of Preformulation, Lundbeck, Denmark. Sodium oleate was from BDH Chemical Ltd. Poole England. All water was ultrapure Milli-Q water. Methanol, acetonitrile were of HPLC grade (VWR, UK). Others were trifluroacetic acid (Merck Schuchardt OHG, Germany) and ammonium acetate (Merck, Germany).

2.2. Design of experiment and data analysis

A quarter of the full factorial DoE with 7 factors (either a component concentration or a system parameter such as pH) and 2 levels (upper and lower limits) was constructed and analysed using Minitab® 16.0 (Minitab Inc.). Minitab generated 66 different experiments by various combinations of the upper and lower limits of these 7 factors based on Table 2 (32 experiments by upper/lower limits and 1 centre points, each condition measured in duplicate). A few assumptions were made before the DoE was analysed. 1. Only main effects and 2-way interactions are considered in the analysis, 3-way (or more) interactions are not determined. 2. Confounded Interactions. All the main effects are only confounded with 3-way/4-way interactions, which were not considered. There are a few 2-way interactions, which are confounded with another 2-way interactions for example, buffer and pH with pancreatin and sodium oleate, buffer and pancreatin with pH and sodium oleate, buffer and sodium oleate with pH and pancreatin. In these instances if the results indicate for example that buffer and pH is a significant interaction, this interaction might be caused by buffer and pH or pancreatin and sodium oleate or both. In these situations any conclusions must be drawn with caution. 3. The main effect can be positive (+) or negative (−), but when it is involved in interaction, the conclusion will be considered with the interactions (+/−).

2.3. Equilibrium solubility measurements

Stock solutions of the various components of the simulated intestinal media were freshly prepared, pH adjusted using 0.5 M HCl or 0.5 M KOH and aliquoted (4 ml) into vials for freeze-drying. Each vial was reconstituted with water to 4 ml and transferred to 15 ml Corning® tubes before use. The media components were based on the DoE generated via Table 2. A visual excess amount of solid drugs were added into each tube. The tubes were capped and placed into a shaker (OS 5 basic Yellowline, IKA, Germany) for 1 h and the final pH was re-adjusted to 5, 6 or 7 as required using the previously indicated method. The tubes were shaken for another 24 h at 320 rpm in the constant temperature room at 37 °C and the pH checked. Following incubation, 1 ml of the upper solution in each tube was transferred to a 1.5 ml Eppendorf® tube and centrifuged at 15,000 rpm for 5 min. The supernatant was transferred to HPLC vials for drug solubility analysis by HPLC using an Agilent Technologies 1260 Series Liquid Chromatography system controlled by Clarity Chromatography software, individual HPLC conditions are presented in Table 3. A validation study (results not shown) for each drug was conducted at the DoE mid-point to ensure that equilibrium solubility was attained after 24 h and that experimental technique induced analytical variation was not present.

3. Results and discussion

3.1. Equilibrium solubility measurements

The equilibrium solubility measurements from each drug's individual DoE experiment are presented in Fig. 1. The DoE's two level construction is instantly apparent in the results for the acidic drugs where two groupings of solubility measurements related to the two pH levels of determination are easily visualised. The exception being phenytoin, which has a reported p*K*a value of 8.1 and is therefore largely un-ionised through the DoE pH range. For the remaining drugs this pH based demarcation is not evident and a broad range of solubility values are obtained with a possible variation between the minimum and maximum of two to three orders of magnitude depending upon the drug. For comparison, single point literature solubility values, where available (Augustijns et al., 2014), for nine of the drugs in fastedsimif or HIF have been superimposed in Fig. 1. In all cases these results are comparable and within the measured range of DoE solubility values reported in this study. In addition it is apparent that multiple factors are influencing drug solubility with some drugs zafirlukast and fenofibrate for example exhibiting a wide solubility variation whilst griseofulvin and tadalafil show greater consistency. This property is recognised in the literature where the reported standard deviation for fenofibrate solubility in fasted HIF is one hundred and thirty-two percent of the mean value (Clarysse et al., 2011) whilst for griseofulvin a standard deviation of twenty-nine percent of the mean is measured (Annaert et al., 2010). These results indicate that the measured equilibrium solubility values and drug related solubility variability is consistent with previous literature studies on both fastedsimif and HIF and that the DoE is operating and covering the required solubility space.

3.2. Solubility influence of individual DoE factors

For each DoE the measurements were analysed using Minitab to calculate an individual factor's standardised effect on measured equilibrium solubility, this provides a value for the magnitude

Table 2
Composition and concentration of fasted simulated intestinal media employed in design of experiment.

Parameter	Substance	Lower limit	Centre point	Upper limit
Bile salt (mM)	Sodium TC	1.5	3.7	5.9
Lecithin (mM)	Egg PL	0.2	0.6	1
Buffer (mM)	NaH_2PO_4	15	30	45
Salt (mM)	NaCl	68	87	106
pH	NaOH/HCl	5	6	7
Enzyme (U/ml)[a]	Pancreatin	270	465	660
Fatty acid (mM)	Sodium oleate	0.5	5.25	10

TC: taurocholate, PL: phosphatidylcholine.
[a] Concentration based on Armand et al. (1996).

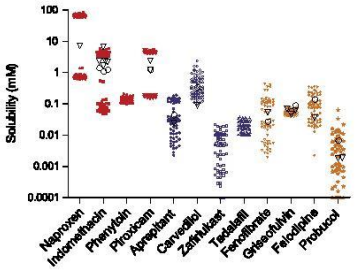

Fig. 1. Design of experiment equilibrium solubility measurements. Equilibrium solubility measurements for each drug based in DoE media compositions detailed in Table 2. ○ reported solubility values for individual drugs in fastedsimif media, ▽ reported solubility values for individual drugs in HIF, all values from Augustijns et al. (2014).

and direction of the factor and permits comparison between different factors and drugs (Fig. 2a–g). In Fig. 2h the mean of the absolute value of the standardised effect grouped for acidic, basic or neutral compounds is presented in order to summarise the previous figures but note that this masks the direction of the factor's effect providing information only on magnitude of effect. With the exception of pancreatin and salt for acidic compounds all of the factors have a statistically significant influence on solubility for all of the drugs. However, the magnitude of the factor's effect varies between groups of drugs and individual drugs within a group emphasising the complex nature of the interactions between individual drugs and media systems.

For acidic drugs not unsurprisingly pH has the greatest magnitude of effect with increasing pH, increasing solubility and the influence of pH on the solubility of acidic drugs in HIF has been previously reported (Clarysse et al., 2009). In terms of magnitude of effect (Fig. 2h) sodium oleate, bile salts and buffer concentrations are almost equivalent but approximately one tenth of the effect of pH, a result, which correlates well with the multiple linear regression determination of Clarysse et al., for indomethacin (Clarysse et al., 2009). Interestingly lecithin is only just significant for acids a feature that may have been overlooked in previous systems which consider total concentrations of bile salts and lecithin as a single factor (Clarysse et al., 2009), or examine a single pH, see discussion on factor interactions below.

The basic and neutral drugs display similar profiles (Fig. 2h) with sodium oleate the factor with the greatest magnitude of effect with pH only slightly less, which is in marked contrast to the acidic drugs. Bile salt and lecithin have comparable magnitudes of effect with buffer and salt effects significant but approximately one third of the magnitude of sodium oleate and pH. Interestingly bile salt has an unusual and significant negative effect on carvedilol solubility (Fig. 2d), which mirrors literature results indicating that anionic surfactants retard solubility (Chakraborty et al., 2009). Overall no individual factor therefore dominates but pH and "solubilising" capacity (combination of sodium oleate, bile salt and lecithin) are clearly important, a combination that the multiple linear regression analysis of Clarysse et al. (2009) indicated for the basic compounds diazepam and ketoconazole but pH did not feature for the neutral compound danazol. The influence of pH on the solubility of neutral compounds has not really been investigated since the majority of studies focus on the influence of "solubilising" capacity (combination of sodium oleate, bile salt and lecithin) at a single media pH (Kleberg et al., 2010a; Pedersen et al., 2000b; Soderlind et al., 2010). However, the influence of pH on the solubility of hydrocortisone in samples of HIF has been reported in the literature (Pedersen et al., 2000a). This effect cannot be due to changing drug ionisation and therefore is attributable to changing ionisation of the media components and potentially masked by the use of media systems with single pH values, see discussion on factor interactions below.

The single factor analysis highlights that whilst general trends within the drug groups are evident (Fig. 2h) modification to accommodate an individual drugs characteristics will be required.

Table 3
HPLC Assay Conditions.

Column	Drug	Mobile phase	Flow rate (ml/min)	Injection volume (μL)	Detection (nm)	Retention time (min)	R^2	RSD (%)
1	Aprepitant	ACN:0.02 M phosphate buffer (50:50) pH 3	1	10	220	3	1.0000	1.9
1	Carvedilol	Methanol:water:OPA (60:35:5)	1	10	243	3	0.9993	0.2
1	Felodipine	Methanol:water (75:25, v/v)	1	20	260	2.7	0.9999	0.4
1	Fenofibrate	ACN:water (70:30 v/v)	1	100	291	3	1.0000	0.4
2	Griseofulvin	ACN:water (50:50 v/v)	0.5	10	291	3.7	0.9970	0.4
2	Indomethacin	ACN:0.05 M acetate buffer (60:40) pH 4.5	1	10	254	4	0.9997	0.1
2	Naproxen	ACN:50 mM ammonium acetate (60:40 v/v) pH 4.5	1	10	254	4	1.0000	0.2
2	Phenytoin	Methanol:20 mM phosphate buffer pH 6 (55:45)	0.8	5–10	205	4	0.9978	0.3
1	Probucol	MeOH:ACN:water = 45:45:10	1	100	220	3.5	0.9997	0.7
2	Proxicam	Methanol:20 mM phosphate buffer pH 6 (55:45)	1	10	254	2.2	0.9992	0.2
2	Tadalafil	ACN: 20 mM phosphate buffer pH 7 70:30	1	10 or 50	290	2	0.9996	0.9
1	Zafirlukast	ACN:10 mM phosphate buffer pH 6 50:50	1	10	245	2.2	0.9989	1.6

Apparatus agilent technologies 1200 series liquid chromatography.
Software clarity chromatography data system.
Column 1 speck & burke ODS-H optimal 150 × 30 mm id 5 μm.
Column 2 Agilent Polaris 5 C18-A 150 × 4.6 mm id 5 μm.
R^2, Linear regression coefficient of calibration curve, all $n = 6$ concentrations. RSD, average relative standard deviation based on 3 injections of the same standard concentration.
ACN: acetonitrile, TFA: trifluoroacetic acid, OPA: orthophosphoric acid.

Amongst the acidic drugs phenytoin for example is obviously different with respect to the order of magnitude for the various factors (Fig. 2a–g) and unusual in registering a statistically significant effect of lecithin on solubility. This is relatively easily ascribed, as discussed above, to phenytoin's p*Ka*, however with only four drugs per category more subtle interactions between p*Ka* and log*P* for example will be difficult to discriminate. The mean analysis in Fig. 2h is only therefore a gross overview, which has to be interpreted with caution in combination with the results for each individual drug.

3.3. Solubility influence of combinations of DoE factors

The statistical analysis of the DoE also considers interactions between factors and determines the magnitude and statistical significance of the interaction, these results are presented for each individual drug in Fig. 3a–r. In Fig. 3s the mean of the absolute value of the standardised effect grouped by drug category is presented in order to summarise the previous figures. For the combinations of factors with the individual drugs statistical significance is only present in approximately one third of the possible interactions in marked contrast to the individual factor analysis where every factor, except pancreatin was significant. Where a significant interaction is present the magnitude of the effect is generally slightly less than single factor values, with again high values associated with factor combinations, incorporating pH. Comparison of Figs. 2 and 3 indicates that there is a more complex pattern evident within the drug categories with some combinations only significant for a single drug (e.g. aprepitant with buffer * sodium oleate), almost all drugs (pH * sodium oleate + buffer * pancreatin) or a mixture from each category (e.g. bile salt * pH). Indicating that the combination interactions hint at a degree of specificity related to the individual drug properties. Some combinations do not provide any statistically significant interactions, for example

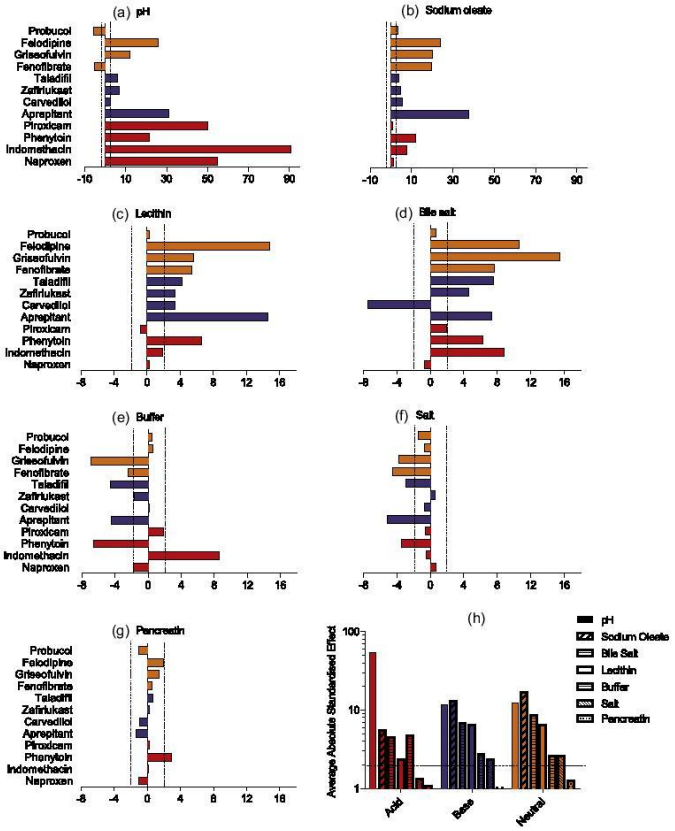

Fig. 2. Standardised effect values for individual DoE factors on equilibrium solubility. DoE standardised effect values (*x*-axis) for individual factors (as listed in figure titles) on equilibrium solubility. Vertical black lines indicate statistical significance ($P < 0.05$), bar direction indicates direction of effect, to the right of 0 on *x* axis is positive effect on solubility, bar length indicates the magnitude of the effect. Fig. 2h the average value of the absolute standardised effect for each factor grouped by drug category, note that this removes direction of effect information.

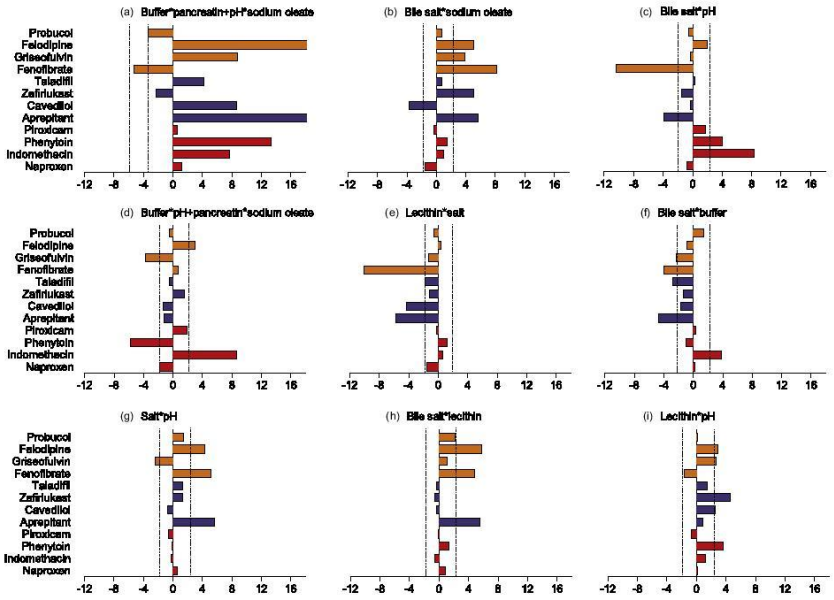

Fig. 3. Standardised effect values for interactions between DoE factors on equilibrium solubility. DoE standardised effect values (*x*-axis) for interactions between factors (as listed in figure titles) on equilibrium solubility. Vertical black lines indicate statistical significance ($P < 0.05$), bar direction indicates direction of effect, to the right of 0 on *x* axis is positive effect on solubility, bar length indicates the magnitude of the effect. Fig. 3s the average value of the absolute standardised effect for each factor grouped by drug category, note that this removes direction of effect information. Factor interactions with a – at the end are cofounded, see text for explanation.

pancreatin with bile salt, lecithin and buffer or sodium oleate with lecithin and buffer. Therefore as for the individual factors the mean analysis in Fig. 3s only provides a gross overview, which has to be interpreted with caution in combination with the results for each individual drug.

Due to the structure of the DoE possible interactions between pancreatin, pH, sodium oleate, and buffer are confounded, meaning that the model does not possess enough statistical power to differentiate between interactions of any combinations of these factors. For example, the interaction between the combinations of pH with sodium oleate cannot be separated from the interaction between pancreatin and buffer, with a similar restriction on other possible combinations from this group. However, pancreatin, which as a single factor showed no statistically significant influence on solubility, also exhibits no significant interactions when assessed in combination with the other non-confounded factors (bile salt, lecithin or salt). Since pancreatin is not significant in these cases it is reasonable to remove the pancreatin combination from the confounded pair and assign significance to the other factor pairing.

For acidic drugs only three significant interactions are present in Fig. 3s and all include pH, with either sodium oleate, buffer or bile salt. The interaction of pH with sodium oleate and bile salt is not surprising since the p*Ka* value of these acids is approximately 5 (Holm et al., 2013) and the DoE pH range is 5–7, thus introducing a change in the percentage ionisation of both factors. Indicating that this has a statistically significant influence on the solubility of the acidic drugs at a constant sodium oleate or bile salt concentration. Interestingly the DoE does not find a significant interaction for the acidic drugs between sodium oleate with bile salt or either component with lecithin (Soderlind et al., 2010), possibly indicating that the influence of pH on solubility is dominant masking lower magnitude effects which might only be present at constant pH. The interaction between pH and buffer is again not surprising and indicates a salt effect.

Basic drugs have a more complicated set of interactions with six significant combinations, pH with sodium oleate, bile salt with sodium oleate, lecithin with salt, bile salt with buffer, salt with pH, and lecithin with pH. The interaction of pH with sodium oleate for the basic compounds is the highest magnitude of effect on solubility for all the two factor interactions (Fig. 3s) and potentially arises through the p*Ka*/pH effects discussed above. There is a significant interaction between sodium oleate and bile salt but again no significance between bile salt and lecithin, see discussion above. The remaining interactions are of a lower magnitude but indicate a complex series of interactions influencing the solubility of the basic drugs is present, lecithin and salt for example could be ascribed to the ability of salt to alter the critical micelle concentration and therefore solubilisation.

Neutral drugs have the most complex pattern with eight significant interactions, pH with sodium oleate, bile salt with sodium oleate, bile salt with pH, lecithin with salt, bile salt with buffer, salt with pH, bile salt with lecithin and salt with sodium oleate. The interaction of pH with sodium oleate has the greatest magnitude of effect on solubility and potentially arises through the p*Ka*/pH effects discussed above and obviously not ionisation of the neutral drugs. A similar although lesser magnitude interaction is evident with bile salt and salt with pH indicating that for neutral compounds investigation of pH induced solubility changes will be important. For the neutral compounds interactions between solubiliser combinations bile salts with sodium oleate and bile salt

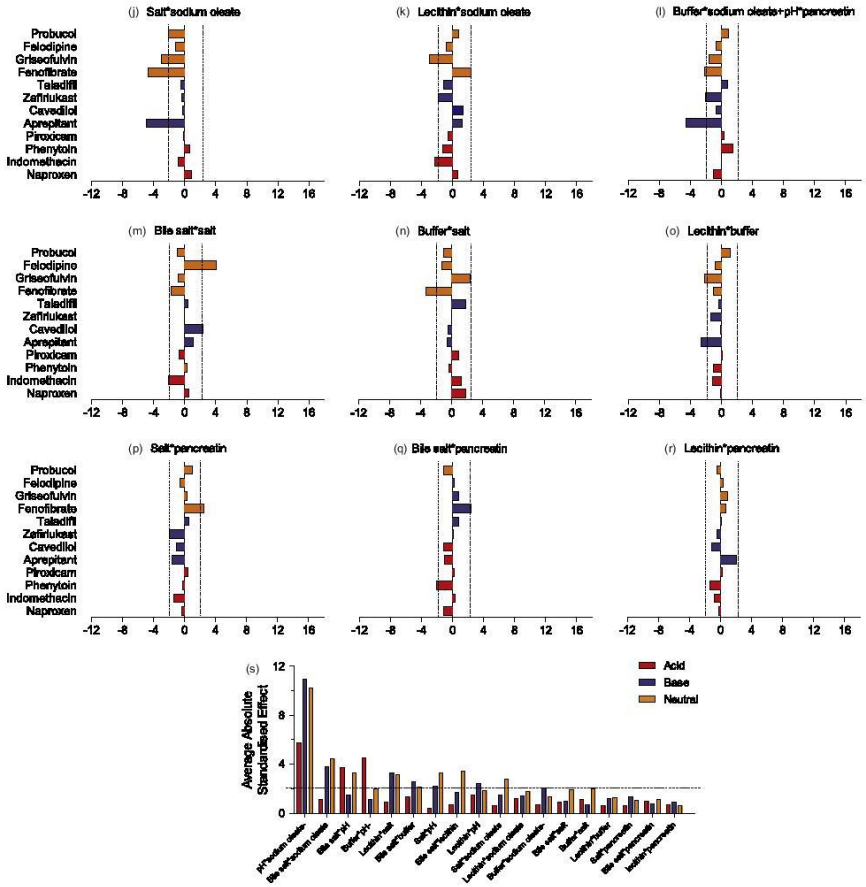

Fig. 3 (*continued*)

with lecithin are significant but low magnitude. This is comparable with literature information on the synergy of bile salts and lecithin on the solubility of neutral compounds (Soderlind et al., 2010) and the low magnitude of the effect in this system compared to the literature is possibly due to the swamping magnitude of pH mediated solubility effects. It is notable that the interaction between bile salts and lecithin is only significant for the neutral compounds in Fig. 3s, although examination of individual results indicates that this is only the case for felodipine and fenofibrate and the basic drug aprepitant, pointing to drug specific features for these interactions and their influence on solubility.

In general the effects and interactions determined for the factors are comparable to those presented in the literature, however some differences are evident. For example bile salt and lecithin are usually considered together as a solubilising system in the literature for neutral drugs (Clarysse et al., 2009; Pedersen et al., 2000b) but in this experiment this interaction in terms of magnitude of effect was only seventh for the neutral drugs. The difference may be due to the fact that some of these studies have been conducted at single pH values, whilst in this study a pH and sodium oleate range has been employed which may swamp the "signal" from these components. This highlights that whilst the DoE provides detailed information it is dependent upon the factors and ranges chosen. Further studies will be required to determine the influence of bile salt composition (Zughaid et al., 2012), interactions between solubilisers (Soderlind et al., 2010) and food degradation products (Kleberg et al., 2010a) for example.

3.4. Solubility media factor patterns and principal component analysis

The DoE provided sixty-four equilibrium solubility measurements consisting of thirty-two pairs of conditions in duplicate and a centre point, which was also measured in duplicate. Fig. 1 presentation of solubility results *en mass* does not permit

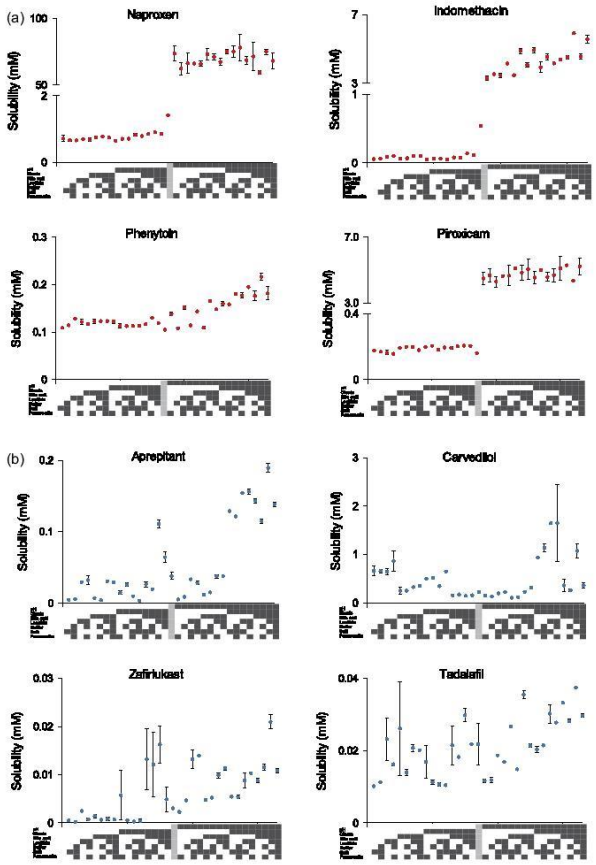

Fig. 4. Variation of equilibrium solubility in relation to media components. Fig. 4a acidic compounds, Fig. 4b basic compounds Fig. 4c neutral compounds. Solubility value mean ± standard error of the mean ($n = 2$); □ Factor at low level in DoE; ■ factor at high level in DoE; ▩ factor at central point in DoE.

discrimination of the individual pairs of measurements, or the visual assessment of the factors (with the exception of the acids already discussed) on solubility. The standardised effect analysis (see above) indicates that the individual factors are significant along with certain combinations of factors and maybe properties specific to the drugs employed in the study. In order to examine these effects we have performed multivariate and principal component analysis on the solubility data incorporating drug physicochemical parameters, however, this did not produce a greater understanding of the results (results not shown). Probably due to the low numbers of drugs studied with respect to oral drug space (Lindenberg et al., 2004). We have therefore also presented (Fig. 4) the solubility data ranked according to the two levels of the DoE and the magnitude of effect of the individual factors (Fig. 1h). This highlights the previous standardised effect analysis but also indicates where drug specific effects may be in operation.

For the acidic compounds as previously discussed the two level nature of the DoE is easily visible for all drugs with the exception of phenytoin. The higher pH systems do show a level of measurement variation but this is probably due to the difficulty of adjusting pH to a constant value during set up as the acidic drugs continued to dissolve, rather than the effect of the other components. For phenytoin the highest solubilities are determined when the highest concentrations of sodium oleate, bile salts and lecithin are present an effect due to micellar solubilisation of the un-ionised drug in combination with the presence of ionised species.

For basic compounds the two level pH effect is not as evident and a visual comparison of the figures highlights variations between the drugs. For example, the influence of the solubiliser (sodium oleate, bile salt and lecithin) concentrations on aprepitant solubility at the high pH level is greater than at the low pH level whilst zafirlukast exhibits a similar effect but with an increased variability at the low pH level. Carvedilol exhibits a similar

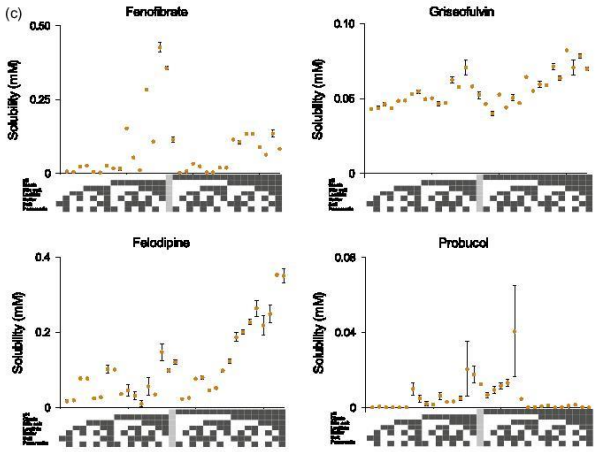

Fig. 4 (*continued*)

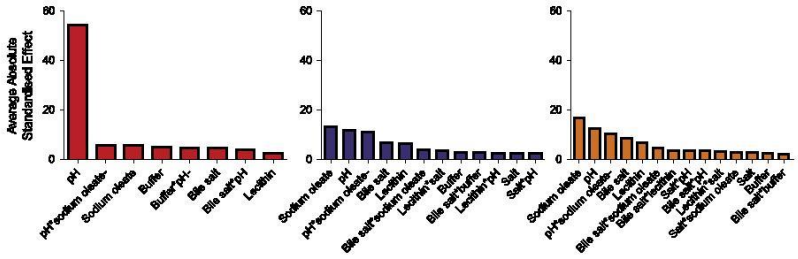

Fig. 5. Average significant absolute standardised effect values. Average significant DoE standardised effect values for individual factors and factor interactions (as listed in figure x-axis) on equilibrium solubility grouped by drug category. Factor interactions with a – at the end are cofounded, see text for explanation.

phenomenon but some combinations reduce solubility indicating that the ratio of sodium oleate, bile salt and lecithin is crucial, and Fig. 3b indicates that for this drug bile salt and sodium oleate have a significant and negative effect on solubility as do lecithin and salt.

The neutral compounds are similar to the basic in terms of the interactions between pH and solubilisers (bile salt, sodium oleate and lecithin). These interactions are usually synergistic as at higher pH, surfactants have better solubilisation effect on drugs (Fig. 3a, c and i). The graphs in Fig. 4 (aprepitant, felodipine and griseofulvin) display an ascending pattern at both low and high pH with increasing solubiliser concentration, although fluctuations are evident (griseofulvin). Probucol and fenofibrate follow similar interactions, but to the opposite direction with respect to media composition (Fig. 3a, and c), which indicates that drug specific parameters are present for these two compounds.

An interesting feature of Fig. 4 is the level of solubility variability within duplicate media conditions that is apparent for particular combinations of drug and media, for example zafirlukast and felodipine. The fact that these variations are not entirely random might indicate that as the concentration or more critically the ratio of media components changes through the DoE "phase changes" (Madenci et al., 2011), which influence solubility and are possibly specific to the individual drugs arise. Investigation of this would require more detailed solubility/phase studies around these specific ratios but this might be an interesting and intrinsic feature of these simulated media systems.

4. Conclusions

The purpose of this study was to determine the feasibility of applying the design of experiment technique to investigate the influence of gastrointestinal media components on the equilibrium solubility of BCS II drugs. The results clearly indicate that this approach is feasible and provides data that is comparable to previous literature studies on both sampled and simulated intestinal fluids in terms of the magnitude and variability of drug solubility (Fig. 1). The expansion of the DoE with different BCS class drugs would be useful to determine if the large variations in solubility determined are an inherent property of these complex media and therefore the gastrointestinal tract or an artifact of the sample compounds employed in this study. The former seems likely since in this study only two drugs (phenytoin and griseofulvin) out of

twelve show little solubility variation with media components and literature comments with respect to high variability abound, see (Annaert et al., 2010; Dressman et al., 2007) for example.

Six factors (pH, sodium oleate, lecithin, bile salt, buffer and salt) were found individually to exert a statistically significant influence on equilibrium drug solubility with one (pancreatin) not significant. Statistically significant interactions between factors were only present in around one third of all possible combinations and the DoE provided interesting insights not previously highlighted in the literature. Some of these effects and the evidence of drug specific influences on solubility have been reported (Kleberg et al., 2010a,b) indicating that the DoE is capable of replicating simulated media performance and providing increased insight and quantification of the complex solubility properties of these media. Modification of the DoE factors to remove non-significant or change the analytical emphasis for example differences between bile salts or use of a constant pH would permit increased information around a drug's sensitivity to media components. The results also indicate that synthetic media based around surfactants (Aburub et al., 2008; Lehto et al., 2011) might require individual confirmation of equivalence to "natural" complex media incorporating the known variations in component concentrations (Bergstrom et al., 2013; Lindahl et al., 1997).

Collection of the average significant factors and interactions for each group of drugs and sorting in order of magnitude (Fig. 5) highlights the difference between the groups and indicates that anywhere between eight to fourteen factors or interactions between factors can be contributing to the measured equilibrium solubility. This may indicate that analysis of the individual components of HIF as single parameters with mean and standard deviation (Kalantzi et al., 2006) maybe limited and that determination of the linkage between combinations or a population distribution envelope for combinations for example bile salt concentration with respect to pH, might be required in order to fully understand and replicate behaviour. In addition this could also be important for age and disease states where the ratios of gastrointestinal components might change (Vanbergehenegouwen et al., 1976).

A further aim of this study was to determine if the multiple recipes present in the literature for simulated intestinal fluids (Jantratid et al., 2008b; Sunesen et al., 2005) could be rationalised. This is a seductive goal, which makes logistic and comparative sense for drug development and testing within the pharmaceutical industry. It has long been recognised that the gastro-intestinal tract is a dynamic system, the composition of its fluid content is highly variable (Bergstrom et al., 2013) along with chemistry of the drugs administered by this route (Lindenberg et al., 2004; Takagi et al., 2006) and therefore it is not surprising that the solubility variability already noted in the literature (Augustijns et al., 2014) and drug specific effects are replicated by the DoE. A single media will therefore only provide information on one set of conditions and the DoE indicates that changing a single factor, whilst keeping others constant may induce, depending upon the factor, drug category and drug itself, significant changes or very little effect. To permit full interpretation will require information on the sensitivity of the compound's solubility behaviour to variation in media components. The results in this paper indicate that the DoE approach can provide this information and could be applied during drug development to assess a candidate's solubility sensitivity to the gastro-intestinal milieu. The presentation of solubility data with respect to media composition (Fig. 4) visualises this effect across the entire DoE and provides a "solubility envelope" which if extended to cover all conditions could be applied to provide limits for modelling possible drug solubility behaviour within the gastro-intestinal tract.

Finally the DoE with 66 samples was constructed with a view to future application via high throughput robotic systems and 96 well plate technology. The results indicate that this is feasible and could be manipulated to provide within a single plate a representative picture of a drug's solubility envelope and possible interactions within the gastrointestinal tract. However, conduct of this study indicates that the technology and equipment to perform the pH adjustment stage required for each sample is at this juncture not available for 96 well plates. One system is capable of handling this on a miniature scale (up to 5 mL) (Etherson et al., 2014; Volgyi et al., 2010) and is capable of handling excipient interactions as well but would require adaptation.

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