Statistical investigation of simulated intestinal fluid composition on the equilibrium solubility of Biopharmaceutics Classification System Class II drugs.

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solubility; biorelevant; design of experiment; naproxen; indomethacin; phenytoin; piroxicam; aprepitant; carvedilol; zafirlukast; tadalafil; fenofibrate; griseofulvin; felodipine; probucol

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;

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 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 pKa 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 1960's (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 (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., (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 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 trifluoroacetic 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 hours 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 Figure 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 pKa 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 Figure 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 and direction of the factor and permits comparison between different factors and drugs (Figure 2a-g). In Figure 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 (Figure 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 (Figure 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 (Figure 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 (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 (Figure 2h) modification to accommodate an individual drugs characteristics will be required. Amongst the acidic drugs phenytoin for example is obviously different with respect to the order of magnitude for the various factors (Figures 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 pKa, however with only four drugs per category more subtle interactions between pKa and log P for example will be difficult to discriminate. The mean analysis in Figure 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 Figure 3a-r. In Figure 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 Figures 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 pancreatin with bile salt, lecithin and buffer or sodium oleate with lecithin and buffer. Therefore as for the individual factors the mean analysis in Figure 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 Figure 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 pKa value of these acids is approximately 5 (Holm et al., 2013) and the DoE pH range is 5 to 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 (Figure 3s) and potentially arises through the pKa/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 pKa/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 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 Figure 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. The Figure 1 presentation of solubility results *en mass* does not permit 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 (Figure 4) the solubility data ranked according to the two levels of the DoE and the magnitude of effect of the individual factors (Figure 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 phenomenon but some combinations reduce solubility indicating that the ratio of sodium oleate, bile salt and lecithin is crucial, and Figure 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 (Figure 3a, 3c and 3i). The graphs in Figure 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, 3c), which indicates that drug specific parameters are present for these two compounds.

An interesting feature of Figure 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 (Figure 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; Kleberg et al., 2010b) 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 (Figure 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 (Figure 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 modeling 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 5mL) (Ethersen et al., 2014; Volgyi et al., 2010) and is capable of handling excipient interactions as well but would require adaptation.

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Figure Legends

Figure 1.

Title: Design of Experiment Equilibrium Solubility Measurements

Legend: Equilibrium solubility measurements for each drug based in DoE media compositions detailed in Table 2. $\circ$ reported solubility values for individual drugs in fastedsimif media, ∇ reported solubility values for individual drugs in HIF, all values from (Augustijns et al., 2014).

Figure 2.

Title: Standardised Effect Values for Individual DoE Factors on Equilibrium Solubility.

Legend: 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. Figure 2h the average value of the absolute standardised effect for each factor grouped by drug category, note that this removes direction of effect information.

Figure 3.

Title: Standardised Effect Values for Interactions between DoE Factors on Equilibrium Solubility.

Legend: 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. Figure 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.

Figure 4.

Title: Variation of Equilibrium Solubility in relation to media components.

Legend: Figure 4a Acidic Compounds, Figure 4b Basic Compounds Figure 4c Neutral Compounds

Solubility value Mean $\pm$ 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.

Figure 5.

Title: Average Significant Absolute Standardised Effect Values.

Legend: 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.

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Figure 1

Figure 3a

Figure 3a. Buffer*pancreatin+pH*sodium oleate

Figure 3b. Bile salt*sodium oleate

Figure 3c. Bile salt*pH

Figure 3d. Buffer*pH+pancreatin*sodium oleate

Figure 3e. Lecithin*salt

Figure 3f. Bile salt*buffer

Figure 3g. Salt*pH

Figure 3h. Bile salt*lecithin

Figure 3i. Lecithin*pH

Figure 3j

Figure 3s

Figure 4a

Figure 4b

Figure 4c

Figure 5

Figure 2

Table 1

Table 1.

Title: Sample literature recipes for Fasted Simulated Intestinal Fluid (FaSSIF).

Component/Parameter and Concentration (mM)	Dressman, Amidon et.al., 1998	Galia, Nicolaidis et.al., 1998	Pedersen, Brondsted et.al., 2000	Vertzoni, Fotaki et.al., 2004	Sunesen, et.al., 2005	Pedersen	Jantratid, Janssen et.al., 2008	Brinkmann- Trettenes & 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
NaH2PO4	(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							100U/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

Table 2.

Title: 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 ₂ PO ₄	15	30	45
Salt (mM)	NaCl	68	87	106
pH	NaOH/HCl	5	6	7
Enzyme (U/ml) ¹	Pancreatin	270	465	660
Fatty acid (mM)	Sodium oleate	0.5	5.25	10

TC: taurocholate, PL: phosphatidylcholine

1: Concentration based on Armand, M et.al., 1996

Table 3

Table 3.

Title: 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 150x30 mm id 5 µm
Column 2 Agilent Polaris 5 C18-A 150x4.6 mm id 5 µm
R², 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