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Dual Level Statistical Investigation of Equilibrium Solubility in Simulated Fasted and Fed Intestinal Fluid

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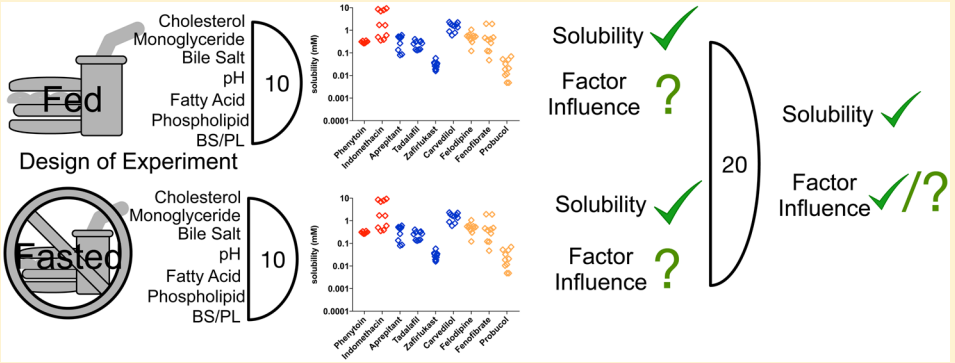

ABSTRACT: The oral route is the preferred option for drug administration but contains the inherent issue of drug absorption from the gastro-intestinal tract (GIT) in order to elicit systemic activity. A prerequisite for absorption is drug dissolution, which is dependent upon drug solubility in the variable milieu of GIT fluid, with poorly soluble drugs presenting a formulation and biopharmaceutical challenge. Multiple factors within GIT fluid influence solubility ranging from pH to the concentration and ratio of amphiphilic substances, such as phospholipid, bile salt, monoglyceride, and cholesterol. To aid in vitro investigation simulated intestinal fluids (SIF) covering the fasted and fed state have been developed. SIF media is complex and statistical design of experiment (DoE) investigations have revealed the range of solubility values possible within each state due to physiological variability along with the media factors and factor interactions which influence solubility. However, these studies require large numbers of experiments (>60) and are not feasible or sensible within a drug development setting. In the current study a smaller dual level, reduced experimental number (20) DoE providing three arms covering the fasted and fed states along with a combined analysis has been investigated. The results indicate that this small scale investigation is feasible and provides solubility ranges that encompass published data in human and simulated fasted and fed fluids. The measured fasted and fed solubility ranges are in agreement with published large scale DoE results in around half of the cases, with the differences due to changes in media composition between studies. Indicating that drug specific behaviors are being determined and that careful media factor and concentration level selection is required in order to determine a physiologically relevant solubility range. The study also correctly identifies the major single factor or factors which influence solubility but it is evident that lower significance factors (for example bile salt) are not picked up due to the lower sample number employed. A similar issue is present with factor interactions with only a limited number available for study and generally not determined to have a significant solubility impact due to the lower statistical power of the study. The study indicates that a reduced experimental number DoE is feasible, will provide solubility range results with identification of major solubility factors however statistical limitations restrict the analysis. The approach therefore represents a useful initial screening tool that can guide further in depth analysis of a drug's behavior in gastrointestinal fluids.

KEYWORDS: *design of experiment, fasted state, fed state, gastrointestinal fluids*

INTRODUCTION

The worldwide demand for new drug therapies is growing, rapidly driven by aging of populations increasing stratification of diseases,[1] leading to the growth of drug discovery research. The oral dosage form is optimal[2] as it is the most convenient, cost-effective route of administration with the highest patient compliance. For oral dosage forms to attain the required systemic exposure the drug needs to dissolve in the gastro-intestinal fluid, which can be influenced by its variable composition.[3] For poorly water-soluble drugs, low solubility coupled with low dissolution rate can result in limited and variable absorption. Studying drug solubility is therefore of critical significance in order to understand the behavior of low solubility drugs in the gastrointestinal

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Table 1. Fasted and Fed Media Components and Concentration Levels

component	MW (g/mol)	substance	fasted state		fed state	
			lower	upper	lower	upper
bile salt	515.70	sodium taurocholate	1.5 mM	5.9 mM	3.6 mM	15 mM
lecithin	750.00	phosphatidylcholine	0.2 mM	0.75 mM	0.5 mM	3.75 mM
fatty acid	304.44	sodium oleate	0.5 mM	15 mM	0.8 mM	25 mM
monoglyceride	358.57	glyceryl mono-oleate	0.1 mM	2.8 mM	1 mM	9 mM
cholesterol	386.65	cholesterol	0.1 mM	0.26 mM	0.13 mM	1 mM
pH	sodium hydroxide/hydrochloric acid		5	7	5	7
BS:PL ratio			7.5	7.9	7.2	4

tract (GIT) and thus improve drug absorption and bioavailability.[2,4] The biopharmaceutics classification system (BCS)[5] categorizes drugs into four groups based on a combination of their solubility and GIT permeability characteristics. Drugs with a low solubility (Class II or IV) represent an interesting challenge during pharmaceutical development.

Gastrointestinal Solubility Factors. Several drug specific factors, for example, pK_a, logP, chemical structure, and properties (i.e., acidic, basic, or neutral), are known to affect aqueous solubility generally and also in intestinal media. In addition, multiple factors constitutively present in GIT media, such as bile salts, buffer capacity, and food composition,[6] can further influence drug solubility. In the fasted state, bile salt and lecithin concentrations are lower than in the fed state, where their concentrations are increased due to the ingestion of food and the presence of associated lipid digestion products.[7] The formation in GIT fluid of mixed micelles consisting of "bile salts, lecithin, and lipolytic products" tends to have a solubilizing ability for poorly soluble drugs.[4]

Gastrointestinal Media. Multiple studies have been published, directed at achieving an improved understanding of drug solubility in the GIT and its impact on oral bioavailability.[8] The obvious media to employ is human intestinal fluid (HIF) samples, aspirated either from the fasted or fed state[3] however, HIF is difficult to obtain (requiring human volunteers or patients), variable, and therefore not ideal for routine solubility studies.[9,10] To avoid the issues associated with human sampling, research has been performed to provide in vitro derived media which simulates and resembles HIF by containing all of the components that are known to play a role in drug solubility, such as bile salt, buffer, lecithin, and lipid degradation products.[11] Thus, fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF) have been developed. Further research has extended these initial media with the addition of food based constituents, for example cholesterol[12] and multiple media recipes, are now available for both fasted[13] and fed[14] states.

Statistical Investigation of Simulated Intestinal Media. Two recent studies have applied a structured statistical design of experiment (DoE) approach to examine the significance of media components individually and in combination in fasted[13] and fed[14] simulated media on the equilibrium solubility of a range of acidic, basic, and neutral BCS class II and IV drugs. The results indicated that an individual drugs solubility could vary over 3 orders of magnitude in either the fasted or fed state, solubility in fasted media was lower than fed and published literature solubility values in either HIF or simulated media were in agreement.[8] For acidic drugs in fasted or fed simulated media pH was the most important individual solubility driver with only minor contributions from sodium oleate, bile salt, and lecithin, significant combinations of factors were limited to pH either with oleate or bile salt. For basic drugs pH was an important individual solubility driver in both fasted and fed media systems but the magnitude of the effect was equivalent to sodium oleate, bile salt, and lecithin. Interactions between media factors were slightly greater in number and again involved the factors which were individually significant. For neutral drugs in both media systems pH, sodium oleate, bile salt, and lecithin were roughly equivalent as single factors with a lower significance for monoglyceride. Since these drugs are nonionizable the impact of pH must be mediated through ionization of media components and this is evident in an increased number of significant factor interactions influencing solubility. Both DoE studies illustrated the applicability of this statistical method for determining the media factors affecting drug solubility and the possible range of solubility values that might arise. However, the fasted DoE required 60 six individual media experiments and the fed 90 two, an experimental load that separately or in combination is resource intensive and not suited to early development studies where drug availability may be limited.

Dual Range Design of Experiment Study. In this article a dual range DoE covering fasted and fed states in a smaller single experiment with biorelevant factor levels (see Table 1) was applied to determine the equilibrium solubility of BCS class II compounds. This was achieved through removing salt and buffer as media factors since they were not statistically significant,[13,14] adding cholesterol and monoglyceride as new factors[15,16] in both fasted and fed states, resulting in a media consisting of seven factors (bile salt, lecithin, sodium oleate, monoglyceride, cholesterol, pH, and bile salt phospholipid molar ratio (BS/PL)). A 1/16 of the full factorial DoE design with two levels (upper and lower) was constructed separately for the fasted and fed states (8 experiments with upper and lower levels and 2 center points in each state) then the two experimental tables were employed as an input for a factorial custom DoE which combined the fasted and fed data into a single DoE. The DoE therefore has three arms, two small arms of 10 experiments each covering fasted and fed, with a third arm based on the combination of fasted and fed. This has the advantage of examining both fasted and fed states within the same experiment coupled with the ability to combine the data to provide an overall solubility assessment for both states. The equilibrium solubility of nine (for consistency and to aid the presented comparisons, drug classification is based on our original paper[13]) BCS class II drugs was investigated: two acids (phenytoin and indomethacin), four bases (aprepitant (aprepitant with a reported pK_a of 9.7[17] at the pH values in this study it will be predominantly un-ionized), tadalafil (tadalafil with a reported pK_a value of 15[18] at the pH values in this study it will be predominantly un-ionized), zafirlukast (zafirlukast with a reported pK_a value of 4[19] will in this system behave as an acidic

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Table 2. Stock Mixture Concentrations (15× Lower, Mid, and Upper Limits)

	fasted state			fed state		
component	lower	middle	upper	lower	middle	upper
bile salt	22.5 mM	55.5 mM	88.5 mM	54 mM	139.5 mM	225 mM
lecithin	3 mM	7.125 mM	11.25 mM	7.5 mM	31.8 mM	56.25 mM
monoglyceride	1.5 mM	21.75 mM	42 mM	15 mM	75 mM	135 mM
cholesterol	1.5 mM	2.7 mM	3.9 mM	1.95 mM	8.475 mM	15 mM

Table 3. Fatty Acids Volumes (5× Upper Limit)

	fasted state			fed state		
component	lower	middle	upper	lower	middle	upper
sodium oleate	16 μL	248 μL	480 μL	25.6 μL	412.8 μL	800 μL

drug), carvedilol), and three neutral drugs (felodipine, fenofibrate, probucol) and compared to the published fasted and fed DoE studies. The same samples of compounds were employed in the cited published DoE studies[13,14] thus eliminating any potential issues associated with the solid state during comparisons.

MATERIALS AND METHODS

Materials. Sodium taurocholate (>97%), monosodium dihydrogen phosphate (100%), ammonium formate (>99.995%), formic acid (98−100%), sodium chloride (NaCl), potasium hydroxide (KOH, > 85%), hydrochloric acid solution (HCl, analytical grade), cholesterol (>99%), chloroform (99.5%), fenofibrate, indomethacin, and phenytoin were purchased from Sigma-Aldrich, Poole, Dorest, UK. Lecithin S PC (phosphatidylcholine from soybean 98%) was supplied from Lipoid, Germany. Sodium oleate (technical grade) was from BDH chemical Ltd. Poole, England. Monoglyceride (glyceryl monooleate, > 92% monoester, and 88% oleic acid) was kindly supplied from CRODA. The BCS class II compounds felodipine, aprepitant, tadalafil, carvedilol, and zafirlukast were provided through OrBiTo by Dr. R Holm, Head of Preformulation, Lundbeck, Denmark. All water used was ultrapure Milli-Q water. Methanol and acetonitrile were purchased from VWR Prolabo Chemicals, UK.

Dual Level Design of Experiment and Data Analysis. For each media parameter (bile salt, lecithin, sodium oleate, monoglyceride, cholesterol, pH and BS: PL ratio) lower and upper limit concentration values for fasted and fed states were defined, Table 1. Using Minitab 17.2.1 and a custom experimental design, a 1/16 of the full factorial DoE with the seven factors and two levels (lower and upper limits) was constructed (8 experiments around the upper and lower levels plus two center points) separately for the fasted and the fed states. These two tables were then applied as an input for a factorial custom design of experiment which combined the fasted and fed using all 20 data points to provide an overall analysis. The study therefore consists of three arms, two smaller (10 data point) fasted and fed arms, which are then merged into a larger (20 data point) combined arm.

When designing and analyzing the DoE, only a factor's main effects and 2 way interactions have been considered and 3 way interactions or more were not included. For each DoE the magnitude for each factor's effect on equilibrium solubility was determined by the standardized effect value for all of the individual factors and the significant 2-way interactions. This value was used to articulate whether these factors are increasing or decreasing drug solubility. Due to the design and the low number of experiments, the standardized effect values calculated for the smaller fasted and fed state arms indicate a significant increase in drug solubility when it is greater than +4 and a decrease when it is less than −4. For the combined fasted and fed state arm the value of the standardized effect is considered to indicate a significant increase in drug solubility when it is greater than +2 and a decrease when it is less than −2. Finally, two way interactions could only be determined for the combined DoE arm with the larger number of data points.

The Kolmogorov normality test was used in Minitab to assess the normality distribution of each data set. A Mann−Whitney test was used to evaluate the median between two data sets (not normally distributed) and the two-sample *t*-test was used to evaluate the mean of two data sets (normally distributed).

Equilibrium Solubility Measurement. The concentration of each stock solution has been designed to be 15 times greater than the upper limit concentration value required for the DoE with the exception of oleate where only a 5 times concentration was possible (Table 2 and 3).

Preparation of Stock Systems. Preparation of Lipid Suspension. Sodium taurocholate, monoglyceride, lecithin, and cholesterol were weighed and transferred into a flask then 2 mL of chloroform was added to dissolve all the solid material. A stream of nitrogen gas was applied in order to remove the chloroform and to ensure the formation of a dried film. Water was added to reconstitute the dried film and mixed to obtain a homogeneous suspension, transferred to a 5 mL volumetric flask and brought to volume with water.

Preparation of Sodium Oleate Solution. Sodium oleate (1.90 g) was weighed into a 50 mL volumetric flask, dissolved in water, with the assistance of gentle heating (37 °C) to aid dissolution and then made up to volume with water and kept under heat to aid solubilization.

Preparation Buffer Solution. A concentration of 0.3 M monosodium dihydrogen phosphate buffer was prepared by adding 20.4 g into a 500 mL volumetric flask and making up to volume with water. This is split into two and the pH adjusted to 5 and 7 using aqueous 0.5 M HCl or 0.5 M KOH.

Preparation of Experimental Measurement Solutions. Preparation of Individual Design of Experiment Solutions. The solution was prepared by the addition of an excess amount (above the estimated solubility) of solid for each compound investigated to a centrifuge tube (15 mL Corning Centristar cap, polypropylene RNase/DNase free, nonpyrogenic) followed by the addition of each component of the simulated intestinal fluid media according to the run order generated by the DoE. After all of the media components were added, pH was adjusted to 5, 6, or 7 according to the run order using 0.1 M HCl or 0.1 M

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Table 4. HPLC Analysis Conditions[a]

drug	mobile phase	flow rate (mL/min)	injection volume (μL)	detection (nm)	retention time (min)	R2**
phenytoin	mobile phase A: ammonium formate 10 mM pH 3.0 in H_2O; mobile phase B: ammonium formate 10 mM pH 3.0 in ACN/H_2O (9:1 v/v)	1	10	260	2.3	0.9998
indomethacin		1	10	254	2.5	0.9999
aprepitant		1	100	254	2.7	0.9992
tadalafil		1	10	291	1.7	0.9996
zafirlukast		1	10	260	3.1	0.9996
carvedilol		1	10	254	1.2	0.9989
felodipine		1	10	260	3.1	1.0000
fenofibrate		1	10	291	3.6	0.9999
probucol		1	10	254	4.3	0.9995

[a]Apparatus Agilent Technologies 1260 Series Liquid Chromatography system with clarity Chromatography software. Gradient method: time 0, 70% A:30%B, 3 min 0%A:100%B, 4 min 0%A:100%B, 4.5 min 70% A:30%B. Total run 8 min. Column X Bridge C18 column/186003108/50 mm × 2.1 mm id. 5 μ. **R2 Linear regression coefficient curve, n = 6 or more. ACN: acetonitrile.

KOH and tubes were capped and placed on an orbital shaker (OS 5 basic Yellowline, IKA, Germany) for 1 h after which the pH was readjusted if required. The 20 different tubes were then shaken in a tube rotator for 24 h at 40 rpm at 37 °C to simulate intestinal fluid conditions. After 24 h, a 1 mL amount was taken from each of the 20 tubes and transferred to a 1.5 mL Eppendorf tube then centrifuged at 15 000 rpm for 5 min. Following centrifugation 0.5 mL of the supernatant solution was transferred to an HPLC vial to analyze drug solubility using HPLC (Table 4). This protocol has previously been demonstrated to successively permit the determination of equilibrium solubility.[13]

RESULTS

Equilibrium Solubility Measurements. The results of all the equilibrium solubility measurements have been presented in Figure 1, and illustrate that a broad range of solubility values have been observed depending on the drug and the media state (fasted or fed) investigated. For comparison available literature solubility values for the drugs in simulated intestinal fluid (SIF) and/or human intestinal fluid (HIF) in both fasted and fed states[8] have been plotted in Figure 1. The results also indicate that drug specific factors are influencing solubility, Tadalafil has a smaller solubility variation than fenofibrate for example, a feature that has been previously reported[13,14] for these types of studies. In Figure 2a–c the dual level equilibrium solubility results for the fasted and fed states have been presented alongside a box and whisker plot of published fasted[13] and fed[14] measurements along with a statistical comparison of the distributions. It is important to note that slightly different levels of factors have been used in this dual design when compared to the published fasted and fed data. The results indicate that fasted solubility is in the majority of cases lower than fed solubility and that the solubility values from the dual level study are comparable, with some exceptions (tadalafil fasted for example) to the published data.

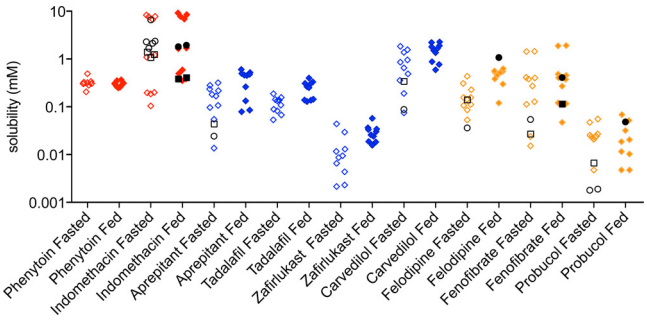

Figure 1. Design of experiment equilibrium solubility measurements. Equilibrium solubility measurements for each drug in DoE media compositions detailed in Table 1. Red data points for acidic drugs, blue basic drugs, and yellow for neutral drugs—open symbols for fasted media conditions, closed symbols for fed media conditions. O reported solubility values for individual drugs in fasted (open symbol) simulated intestinal fluid and fed (closed symbol) simulated intestinal fluid media, respectively, □ reported solubility values for individual drugs in fasted (open symbol) human intestinal fluid and fed (closed symbol) human intestinal fluid, respectively, all values from ref 8.

Statistical Comparison. For the current dual level DoE, statistical examination indicated that nine out of a possible 18 data sets had a normal distribution. This is in marked comparison to the published data where all 18 data sets had non-normal distribution. A statistical comparison between the published fasted[13] and fed[14] data indicates that for all nine drugs there is a significant difference with fasted solubility lower than fed. Statistical comparison of the current dual level fasted against fed data indicates that there was a significant difference in only four (tadalafil, zafirlukast, carvedilol, and felodipine) out of the nine drugs tested and in these cases the fasted solubility was lower than the fed. Finally, comparison of the dual level with the published data indicates that for fasted six (phenytoin, aprepitant, tadalafil, felodipine, fenofibrate, and probucol) out of the nine results were significantly different and for the fed the value is four significantly different (phenytoin, aprepitant, carvedilol, and fenofibrate) out of nine.

Influence of Individual DoE Factors on Solubility in Fasted and Fed Study Arms. The standardized effect value for each factor in the fasted and fed study arms have been presented in Figure 3. Due to the small experimental data set a value of greater than ±4 is significant and two way factor interactions cannot be determined. Out of the possible 126 values only 29 (around 23%) were significant, and drug dependent behavior was evident since some drugs (tadalafil and carvedilol) have no significant factors, while felodipine has eight out of a possible 14 (around 62%). A comparison with published significant effect values in larger fasted[13] and fed[14] studies has been presented in Table 5. In these studies (where comparable) out of a possible 81 values, 64 (around 80%) were significant, indicating that the current study has found a lower incidence of significant factors. Agreement between this study and the published data arises in 32 out of the 64 (50%) possible comparisons (Table 5), with the level varying between the

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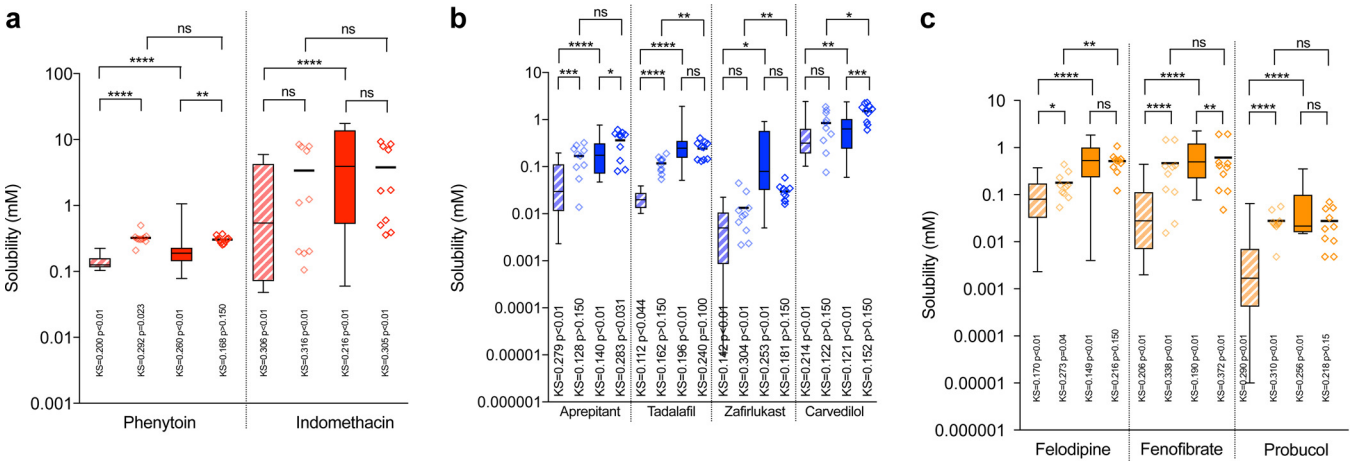

Figure 2. Statistical comparison of design of experiment equilibrium solubility measurements. Box and Whisker plots, published fasted[13] and fed[14] design of experiment solubility data. Scatter plots separate fasted and fed design of experiment equilibrium solubility data current study, bar indicates arithmetic mean. KS Kolomogrov normality test on the data set, $p < 0.05$ indicates the distribution is not normal. Comparison bars Mann−Whitney test, not significant (ns) if $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; and **** $p \leq 0.0001$. (a) Comparison of acidic drugs. Published fasted (red and white box) and fed (red box) DoE equilibrium solubility data. Current study fasted (light red diamonds) and fed (dark red diamonds) equilibrium solubility data. (b) Comparison of basic drugs. Published fasted (blue and white box) and fed (blue box) DoE equilibrium solubility data. Current study fasted (light blue diamonds) and fed (dark blue diamonds) equilibrium solubility data. NB fasted and fed tadalafil were both normally distributed so two-sample t-test was used to compare the mean of the two groups. (c) Comparison of neutral drugs. Published fasted (orange and white box) and fed (orange box) DoE equilibrium solubility data. Current study fasted (light orange diamonds) and fed (dark orange diamonds) equilibrium solubility data.

factors for example, pH seven out of 18 agreed, lecithin 11 out of 18, but for bile salt only two out of 18 agreed. Further comparison indicates that where the factor is significant in the published studies the current study only agreed in around 28% of cases, but if the published data indicated that the factor is not significant the agreement is around 61%.

For the acidic compounds (Figure 3a and b) pH was the most significant factor in both fasted and fed states, which is identical to the two previously reported DoE studies. Indomethacin matched the previous studies with respect to pH, but phenytoin was contrasting as pH showed a negative effect on solubility. The effect of pH on indomethacin is attributable to drug ionization ($pK_a = 4.5$) in the experimental pH range. The negative pH effect on phenytoin ($pK_a = 8.1$), which will be predominantly un-ionized in the experimental pH range, must be related to changes in the media composition (most notably the incorporation of cholesterol) between experiments impacting on media behavior and solubility. For example, the significant negative solubility effect of cholesterol for phenytoin has not been previously reported. Sodium oleate, cholesterol, and the BS/PL ratio showed significant effects in fasted phenytoin, but not with the fed state and all other factors showed no significant influence on solubility.

For all the basic compounds (Figure 3c−f), there were no significant factors influencing solubility in the fed state and for tadalafil and carvedilol there were no significant factors influencing solubility in both states. Only aprepitant and zafirlukast showed an influence by the media factors in fasted state with sodium oleate, lecithin, and monolyceride for aprepitant and pH, cholesterol and monoglyceride for zafirlukast significant. This low incidence of significant factors is in marked contrast to the published studies (see Table 5), where pH, sodium oleate, and lecithin have previously been shown to be significant. The positive effect of cholesterol on zafirlukast solubility has not been previously reported in the literature, nor the negative effect of monoglyceride. However, both of these factors have not been previously studied in fasted DoE systems.

For the neutral compounds (Figure 3g−i), sodium oleate was significant for all drugs in both fasted and fed state which is in compliance with the published fasted[13] and fed[14] studies. Lecithin was significant in both fasted and fed states in the case of felodipine and fenofibrate, which is in compliance with the published studies but did not agree for probucol. pH was significant in both fasted and fed states for fenofibrate and fasted for felodipine, but not for probucol, which was significant in the published studies. The effect of pH on the solubility of the neutral compounds must be through an indirect effect on ionization of the different media components. Bile salt had no significant impact on solubility in the fasted state, which is at variance with the literature for felodipine and fenofibrate but not probucol. Cholesterol which has not been previously studied did not show a significant impact on solubility. Monoglyceride showed a positive effect on felodpine solubility in the fasted and fed state, which is not in agreement with published data.

Influence of Individual DoE Factors and Factor Interactions on Solubility in the Combined Study Arm. The standardized effect value for each factor and factor interactions in the combined arm (fasted + fed data) have been presented in Figure 4, due to the larger experimental data set a value of greater than ±2 is significant and eight two way factor interactions can be determined. Out of the possible 63 values only 16 (around 25%) were significant and drug dependent behavior is evident since some drugs (phenytoin and zafirlukast) have no significant factors, while fenofibrate has three out of a possible seven (43%). No similar DoE studies covering fasted and fed states in this manner have been published, the overall significance level appears low when compared to the previous published larger fasted[13] and fed[14] studies where around 80% are significant, indicating that this study is finding a lower number of significant factors.

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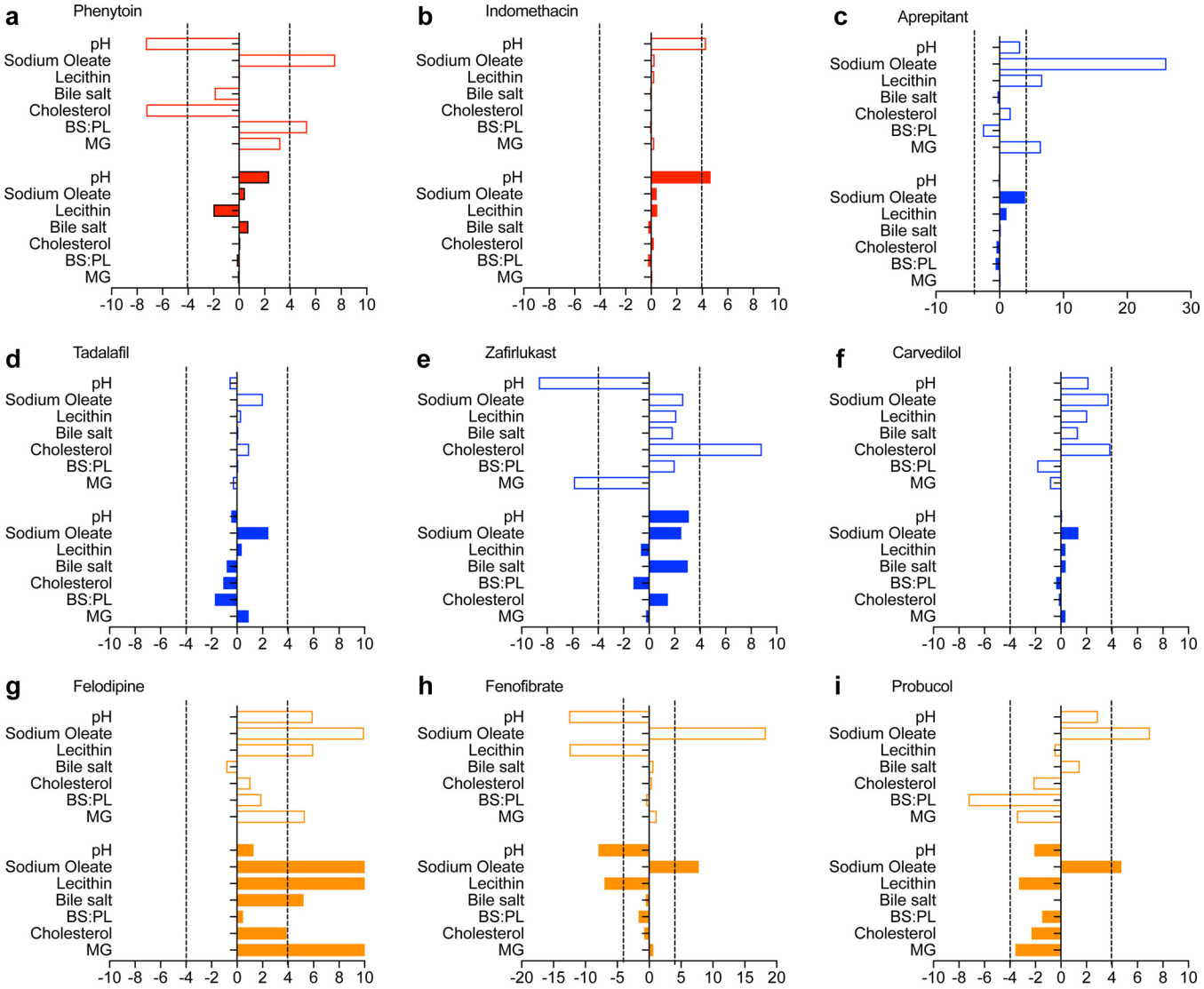

Figure 3. Standardised effect values for DoE factors on equilibrium solubility in fasted and fed study arms. DoE standardized effect values for factors (as listed in figure *y*-axis) on equilibrium solubility. Separated fasted result empty histogram bar, separated fed result closed histogram bar. Vertical black lines indicate statistical significance ($P < 0.05$ NB, significance value = ± 4 due to small sample number in separate fasted and fed study), horizontal bar direction indicates direction of effect, to the right of 0 on axis is positive effect on solubility, bar length indicates the magnitude of the effect.

For the acidic drugs (Figure 4a,b), pH was the only significant factor with an effect on indomethacin solubility which can be attributed to the ionization of the compound over the pH of the DoE, see above.

For basic drugs (Figure 4c–f), sodium oleate was significant for three (aprepitant, tadalafil, and carvedilol) out of the four drugs with in these cases a positive solubility impact which agrees with the previous published fasted and fed data. No significant effect was determined for pH, which based on published results[13,14] was unexpected as was the low significance of lecithin (significant for aprepitant only) and bile salt.

The neutral drugs (Figure 4g–i) exhibited a more complicated pattern since for each drug at least three or four factors were significant encompassing all seven factors in the DoE pH, sodium oleate, lecithin, bile salt, cholesterol, BS:PL ratio, and monoglyceride. Sodium oleate was the factor with the highest magnitude of effect in all 3 drugs and always positive, followed by lecithin, monoglyceride, and then pH, with bile salt and cholesterol only significant for felodipine. This multifactorial result is in agreement with the published fasted[13] and fed[14] studies where for neutral drugs multiple factors contributed to solubility.

The increased number of data points available by combining the fasted and fed arms permits the determination of two way interactions and these have been presented in Figure 4. Only three out of the nine drugs (phenytoin, zafirlukast, and probucol) exhibited significant interactions with an overall rate of around 32% of significant interactions out of the total possible. This overall rate is similar to the previous fasted[13] and fed[14] studies which for the two way interactions matched with this study had significance rates of 33% and 28%, respectively. However, the significant interactions were not restricted to the three above-noted drugs, for example bile salt*oleate significantly increased the solubility of felodipine and fenofibrate in the fasted study and felodipine and probucol in the fed study, a result not matched in the current study.

Statistically Significant Solubility Factor and Factor Interactions. The mean of the absolute value of all standardized

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Table 5. Comparison of the Statistical Significance of DoE Factors across Studies

	factor																							
	pH				sodium oleate				lecithin				bile salt				cholesterol				BS:PL				monoglyceride	
	fasted		fed		fasted		fed		fasted		fed		fasted		fed		fasted		fed		fasted		fed	
drug	current[a]	published[b]	current[a]	published[c]	current[a]	published[b]	current[a]	published[c]	current[a]	published[a]	current[a]	published[c]	current[a]	published[b]	current[a]	published[c]	current[a]	published[b]	current[a]	published[c]	current[a]	published[b]	current[a]	published[c]
phenytoin	S^d	S^d	NS^{eg}	S^{dg}	S^d	S^d	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	NS^e	NS^e	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	S^d	f	NS^e	f	S^d	f	NS^e	f	NS^{eg}	S^{dg}
indomethacin	S^d	S^d	S^d	S^d	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	NS^e	NS^e	NS^e	NS^e	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	NS^e	f	NS^e	f	NS^e	f	NS^e	f	NS^e	NS^e
aprepitant	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	S^d	S^d	NS^{eg}	S^{dg}	S^{dg}	NS^{eg}	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	NS^e	f	NS^e	f	NS^e	f	S^d	f	NS^e	NS^e
tadalafil	NS^{eg}	S^{dg}	NS^e	NS^e	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	NS^e	NS^e	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	NS^e	f	NS^e	f	NS^e	f	NS^e	f	NS^e	NS^e
zafirlukast	S^d	S^d	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	NS^e	NS^e	NS^{eg}	S^{dg}	NS^e	NS^e	NS^{eg}	S^{dg}	S^d	f	NS^e	f	NS^e	f	NS^e	f	S^d	f	NS^e	NS^e
carvedilol	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	NS^e	NS^e	NS^{eg}	S^{dg}	NS^e	f	NS^e	f	NS^e	f	NS^e	f	NS^e	f	NS^{eg}	S^{dg}
felodipine	S^d	S^d	NS^{eg}	S^{dg}	S^d	S^d	S^d	S^d	S^d	S^d	S^d	S^d	NS^{eg}	S^{dg}	S^d	S^d	NS^e	f	NS^e	f	NS^e	f	S^d	f	S^{dg}	NS^{eg}
fenofibrate	S^d	S^d	S^{dg}	NS^{eg}	S^d	S^d	S^d	S^d	S^d	S^d	S^d	S^d	NS^{eg}	S^{dg}	NS^e	f	NS^e	f	NS^e	f	NS^e	f	NS^e	f	NS^{eg}	S^{dg}
probucol	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	S^d	S^d	S^{dg}	NS^{eg}	NS^e	NS^e	NS^{eg}	S^{dg}	NS^{eg}	S^{dg}	NS^e	f	NS^e	f	S^d	f	NS^e	f	NS^e	f	NS^{eg}	S^{dg}
total significant	5	9	2	7	5	9	3	8	3	6	2	4	0	8	1	9	2	f	0	f	2	f	0	f	1	4

[a]Current = current study results, Figure 3. [b]Fasted published = data from ref 13, Figure 2. [c]Fed published = data from ref 14, Figure 2. [d]S = factor statistically significant in design of experiment study. [e]NS = factor not statistically significant in design of experiment study. [f]- = comparison not possible. [g]No consistent result between studies.

effect values in the three arms of the study arranged by drug group is presented in Figure 5 in order to summarize the experimental results. Note that this removes the factor's direction of effect information.

For acidic drugs (Figure 5a,b) the only significant single factor is pH in the fasted and combined arms a result that was not surprising based on the published data for acidic drugs in fasted[13] and fed[14] media. In the published DoEs sodium oleate, lecithin, and bile salt were also significant, although that result is not reflected in this study. All the two way interactions investigated were significant a result that is due to the impact of phenytoin, since indomethacin had no significant interactions.

For basic drugs (Figure 5c,d) sodium oleate was the only significant single factor in the fasted, fed, and combined arms. This was also the most significant factor for basic drugs in the fasted[13] and fed[14] studies, however in these studies other factors for example pH, bile salt, and lecithin were also significant although with a marginally lower magnitude. No two way interactions were significant in this study, which is at variance with the published studies since bile salt*oleate was significant in the fasted state and lecithin*oleate in the fed.

For neutral drugs (Figure 5e,f) in the fasted arm pH, sodium oleate and lecithin were significant, with sodium oleate, lecithin, and monoglyceride in the fed and pH, sodium oleate, and lecithin in the combined. This is in close agreement with the published fasted[13] where pH, sodium oleate, bile salt, and lecithin were approximately equally significant and the fed[14] where the four aforementioned factors were significant with sodium oleate dominant. No two way interactions were significant in this study, which is at variance with the published studies since bile salt*pH, bile salt*oleate, and bile salt*lecithin was significant in the fasted state and bile salt*oleate, bile salt*MG, lecithin*oleate, and bile salt*lecithin in the fed.

DISCUSSION

Equilibrium Solubility Measurements. The equilibrium solubility results in either arm (fasted or fed) of this study are presented in Figures 1 and 2 indicate that the measurements are in broad agreement with available published equilibrium solubility data in fasted and fed HIF, simulated intestinal fluids,[8,20] and published DoE studies in fasted[13] and fed[14] simulated intestinal media systems. In addition, the results demonstrate individualistic drug behavior, with some drugs providing a low solubility variability for example phenytoin and others large variability for example probucol. A feature that was evident in previous fasted[13] and fed[14] DoE studies. This indicates that the current study is investigating a similar solubility space to previous simulated studies and comparable to sampled HIF.

Statistical Comparisons of Solubility. The generation of a solubility data set for each drug permits a statistical comparison with published data and this is presented in Figure 2. Examination of the published fasted[13] and fed[14] data indicates that for all of the systems the solubility distribution is non-normal, an unexpected result based on the number of data points in each system (fasted 66, fed 92). This analysis is evident but not replicated by the results in this current study where around 50% of the measured distributions in the fasted and fed arms are non-normal. This result may arise through the non-normal sample pattern induced by the DoE structure, the fact that drug solubility is not normally distributed in the sample space or that the sample is not sufficiently large. The former explanation is visually evident in the indomethacin

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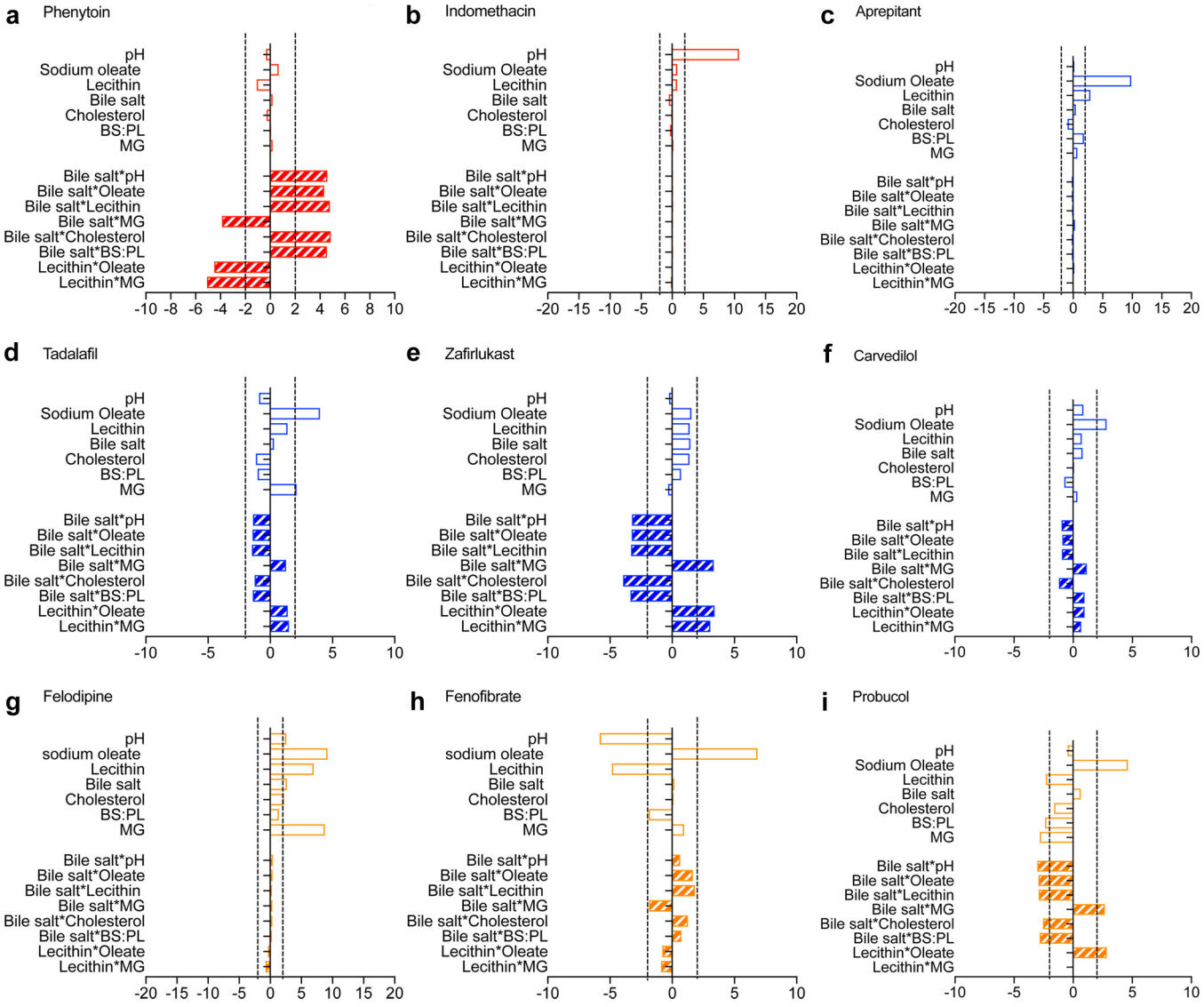

Figure 4. Standardized effect values for DoE factors and factor interactions on equilibrium solubility in combined study arm. DoE standardized effect values for factors and factor interactions (as listed in figure *y*-axis) on equilibrium solubility. Combined fasted and fed result empty histogram bar, combined factor interactions histogram bar. Vertical black lines indicate statistical significance ($P < 0.05$ NB, significance value = ± 2 due to larger sample number when compared to separate fasted and fed study, Figure 3), horizontal bar direction indicates direction of effect, to the right of 0 on axis is positive effect on solubility, bar length indicates the magnitude of the effect.

fasted and fed data in this study (Figure 2a) where the impact of the three pH levels (Table 1, midpoint pH 6 not shown) on solubility creates a non-normal distribution. This stratified variability is likely to be induced by all factors and therefore a non-normal distribution is sensible, although further sampling studies would be required to investigate this phenomenon.

The comparison of the published fasted and fed DoE results indicate that in all cases the fed solubility is statistically significantly higher than the fasted which is in agreement with the literature data[4,8,21] and indicates that these published DoEs[13,14] have investigated different solubility spaces. A comparison of the current study fasted and fed arms indicates that in four (tadalfil, zafirlukast, carvedilol, and felodipine) out of the nine cases the fasted is statistically significantly different from the fed which has a higher solubility and therefore in agreement with the cited literature. However, in five (phenytoin, indomethacin, aprepitant, fenofibrate, and probucol) of the cases in this study there is no statistically significant difference between the fasted and fed arms. For the acidic drugs Figure 2a indicates that in the case of phenytoin this is related to the narrow solubility distribution, which when coupled with the small sample number is not sufficient to discriminate between the arms. While for indomethacin, since pH is the major factor influencing solubility (see Figure 3b) in both fasted and fed states and is identical in the fasted and fed states the lack of a statistically significant difference is understandable. For the basic drug aprepitant (Figure 2b) while the mean fasted solubility is lower the range of solubility overlaps with the fed and coupled with the small sample number is not sufficient to discriminate between the arms. For the neutral compounds both fenofibrate and probucol (Figure 2) have no significant difference between the fasted and fed arms, which appears to be due to the inability of the fasted arm to measure the lower solubility values evident in the published fasted results, see next paragraph.

Comparison of the fasted arm with published fasted results indicates that in six (phenytoin, aprepitant, tadalfil, felodipine,

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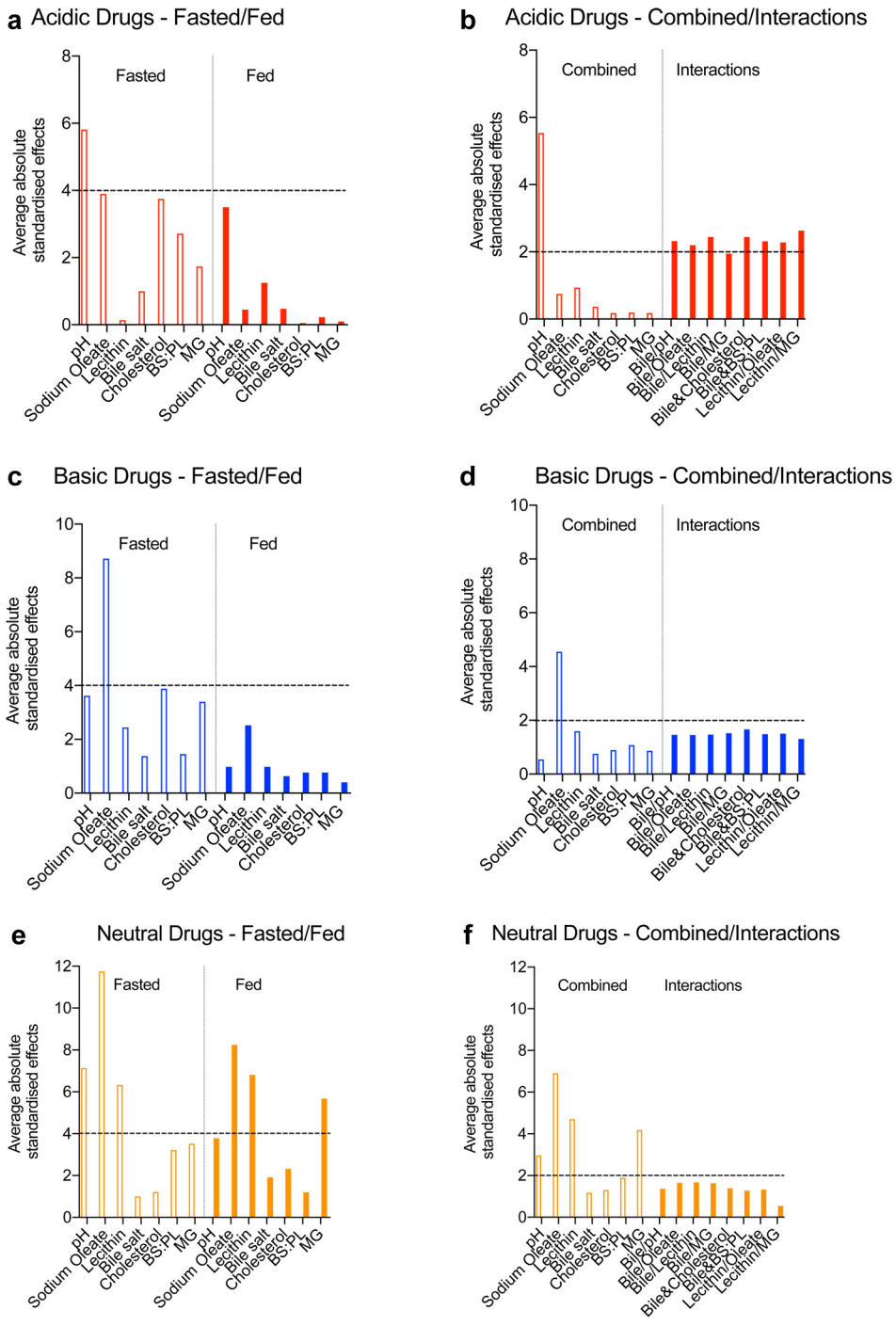

Figure 5. Average absolute standardized effect values for DoE factors on equilibrium solubility in fasted, fed, and combined arms. Average absolute (NB this removes direction of effect information) standardized effect values for individual factors on equilibrium solubility grouped by drug category. Horizontal black line indicates statistical significance ($P < 0.05$).

fenofibrate, and probucol) out of the nine cases there is a statistically significant difference between the solubility data sets with the current fasted arm having a higher solubility. Visual examination of Figure 2 indicates that this appears to be due to the inability of the fasted arm to measure the lower solubility values evident in the published fasted results. There is a subtle difference in the media compositions, since in this study cholesterol and monolglyceride were included at low levels (Table 1) based on current literature[16] and recent proposed changes to the composition of fasted state simulated media.[15] Both of these factors were not employed in the fasted DoE[13] or the original fasted simulated intestinal fluid recipes.[11,22] This is re-enforced by the literature data included in Figure 1, where for example the fasted value for probucol, determined in fasted media without cholesterol or monolyceride,[23] is below the values determined in this study. In addition, in fed media it has been demonstrated that increasing the total "surfactant" concentration, which included monoglyceride, increases the solubilization of fenofibrate.[10] Figure 3 indicates that cholesterol positively impacts zafirlukast solubility and negatively impacts phenytoin, while monoglyceride positively impacts the solubility of aprepitant and felodipine. The solubility difference therefore

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is probably due to the presence of cholesterol and monoglyceride in the current fasted media system which increases the amphiphilic phase components by 0.2 mM (around 8% of total content) at the lower and 3.06 mM (around 12%) at the higher level increasing overall solubilization capacity.

Comparison of the fed arm with published fed results[14] indicates that in four (phenytoin, aprepitant, carvedilol, fenofibrate) out of the nine cases there is a statistically significant difference between the solubility data sets with the current fed arm generally higher (with the exception of fenofibrate). A similar explanation to that presented above for phenytoin is applicable and for aprepitant and carvedilol the differences seem to be due to a higher solubility than the published range, while for fenofibrate it appears to be due to a marginally increased solubility range. Although the overall number of significant differences is smaller a similar explanation to that presented above for the fasted media appears to be applicable. The current study factor ranges are different to the published data set (sodium oleate (current 0.8−25 mM vs published 0.8−52 mM), bile salt (3.6−15 mM vs 3.6−24 mM), lecithin (0.5−3.75 mM vs 0.5−4.8 mM), monoglyceride (1−9 mM vs 1−6.5 mM)), and cholesterol (0.13−1 mM) is included as an additional component in the current media.

Standardized Effect Values. The determined standardized effect values presented in Figures 3 and 4 and summarized in Table 5 for the fasted and fed states indicates that in this setting very few factors have a statistically significant impact on solubility. In the current fasted, fed, and combined arms factors were significant in only 46 (around 24%) out of 189 possible cases, which is around one-quarter of the incidence determined from the previous fasted[13] and fed[14] DoE studies. Interestingly the fasted study employed a quarter and the current study employs a 16th of the full factorial DoE (the fed is not comparable since it employed a D-optimal design) indicating that reducing the number of data points in the study limits the ability to determine significant factors. However, based on the comparison in Table 5 and Figure 5 the current study has correctly identified the factors with the highest magnitude of effect (for example pH for acidic drugs, sodium oleate for basic drugs and pH, sodium oleate and lecithin for neutral drugs) on solubility. Interestingly though the current study suggests that bile salt has no significant impact on solubility a result that is not in agreement with the literature[23−25] but a reflection of the reduced statistical power of the current study. Indicating that small scale studies will have inherent statistical limitations.

The use of small numbers in DoE reduces the ability to determine higher level interactions between the factors and in this study only eight could be determined. The level of significant interactions at around 32% of the total possible is similar to the previous studies[13,14] but is restricted to only three (phenytoin, zafirlukast, and probucol) out of the nine drugs, which is a lower incidence. In the previously published studies factor interactions generally had a lower standardized effect value to their single factor counterparts and in the current study none of the interactions are on average significant for the basic or neutral drugs, indicating that the argument presented above with respect to the reduced statistical discrimination due to the lower sample number in this study is also active for factor interactions.

CONCLUSIONS

The results indicate that a reduced experimental number design of experiment covering both fasted and fed simulated media states in a single study is feasible and provides equilibrium solubility data and drug related behaviors that are similar to previous studies. The study will provide for a drug, equilibrium solubility values that are comparable to published individual solubility measurements in either fasted or fed sampled human intestinal or simulated media systems. The results indicate that changes in the media composition will impact the solubility ranges determined and when coupled with the reduced number of data points will determine a smaller solubility range than larger scale studies. However, the study does provide lower and upper solubility values for the compounds along with an estimate of the mean solubility determining a solubility window that can be applied to drug development studies.

The system will be able to establish the simulated media factors with the largest influence on equilibrium solubility but due to the reduced experimental number and therefore statistical power, factors with a lower influence will not be revealed. In the current study for example bile salt paradoxically has no significant effect on equilibrium solubility.

In conclusion it is feasible to apply a small scale DoE to determine the equilibrium solubility range for a drug in either fasted or fed simulated intestinal fluids, this will also indicate the major factors influencing solubility but the statistical limitations of the approach must also be considered.

ASSOCIATED CONTENT

Accession Codes

Chemical compounds studied in this article: felodipine (Pubchem CID: 3333); fenofibrate (Pubchem CID: 3339); probucol (Pubchem CID: 4912); indomethacin (Pubchem CID: 3715); phenytoin (Pubchem CID: 1775); aprepitant (Pubchem CID: 6918365); tadalafil (Pubchem CID: 110635); carvedilol (Pubchem CID: 2585); zafirlukast (Pubchem CID: 5717).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS:

BCS, biopharmaceutics classification system; DoE, design of experiment; FASSIF, fasted simulated intestinal fluids; FESSIF, fed simulated intestinal fluid; BS/PL, bile salt to phospholipid ratio

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