SCHOOL OF PHARMACY

Inter Subject Variability in Oral Drug Absorption

Sarit Cohen Rabbie

This thesis is submitted in partial fulfillment of the requirements for the degree of the Doctor of Philosophy in Pharmaceutics
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UCL - School of Pharmacy
29-39 Brunswick Square
London
WC1N 1AX

Plagiarism Statement

This thesis describes research conducted in UCL- School of Pharmacy between 2010 and 2015 under the supervision of Prof. Abdul Basit. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

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Signature Date

“Out of clutter, find simplicity.

From discord, find harmony.

In the middle of difficulty lies

opportunity”

Albert Einstein

Dedications

To,

My parents, Shoshana and Shmuel Cohen who placed a high value on knowledge and education, supporting me through life; with their love, blessings and guidance.

My husband, Gil Rabbie: Thank you for your endless support, for believing in me and for enabling me to complete this work without compromise. Most of all, I thank you for our two lovely girls and more that will come. Your dedication to our family is admirable and the manner in which you follow your dreams, inspires me most of all.

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Abstract

A low and highly variable bioavailability is often the main reason for the failure of the development of a drug intended for oral delivery. Focusing on absorption instead of bioavailability from oral administration enables the identification and understanding of key causes of low and erratic absorption to improve drug performance in early development.

In the work carried out as part of this thesis, the *in silico* estimation of drug absorption (f_a*f_g) was carried out. The use of a population pharmacokinetic approach was proposed, as implemented in NONMEM, to estimate f_a*f_g and variability from phase I clinical studies (AstraZeneca database). This work enabled the identification of the rate limiting step in oral drug absorption, and allowed for comparisons of f_a*f_g and inter-subject variability for different drug formulations.

Solubility/dissolution and permeability were investigated *in vitro* in terms of their variability for two model drugs – dipyridamole and furosemide. Physiological parameters such as bile salt concentrations and pH were simulated *in vitro* to understand their effects on the absorption process. Dipyridamole saturated solubility and dissolution are pH and bile salt dependent. However, when both dissolution and permeability were tested simultaneously, it was found that pH plays an important factor in the permeation of dipyridamole rather than bile salt concentration. This can explain to some extent the variability between individuals in the absorption of dipyridamole. Furosemide solubility experiments showed that pH, buffer capacity and, to a lesser extent, bile salt concentration affect its saturated solubility. Surprisingly, almost complete drug release was observed under all simulated conditions with a clinical dose. Similarly, the permeation of furosemide did not differ under different conditions. It was suggested that with this clinical dose, other physiological parameters contribute to variability in furosemide absorption, such as gastric emptying time.

Moreover, the efficacy of three formulations (solid dispersion, Self Micro Emulsifying Drug Delivery systems and nano-particles) in increasing solubility\ dissolution *in vitro* and *in vivo* in the rat model was compared. Lack of *IVIVC* was observed. It was suggested that the missing link is the human absorption estimation that can be resolved by the proposed population pharmacokinetics approach presented herein.

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Abbreviations and Acronyms

ACAT - Advance CAT model

ADAM - Absorption, distribution and metabolism model

ADME – Absorption, distribution, metabolism and elimination

API - Active pharmaceutical ingredient

AUC - Area under the curve in plasma concentration vs. time curve

AZ - AstraZeneca

BCS - The biopharmaceutics drug classification scheme

$C_{b/p}$ - Blood to plasma concentration ratio

CAT - Compartmental absorption and transit model

C_{max} - Maximum concentration in plasma concentration vs. time curve

CV - Coefficient of variation

CYP - Cytochrome P45O enzyme system

D/P system - Dissolution/permeation system

ER- Extended release formulation

F - Bioavailability

f_a - fraction of drug absorbed

FaSSGF - Fasted simulated gastric fluids

FaSSIF - Fasted simulated intestinal fluids

f_g - fraction of drug escaping gut wall metabolism

f_h - fraction of drug escaping liver metabolism

FOCE - First-order conditional estimation

GI tract - Gastrointestinal tract

GOF - Goodness-to-fit plots

HBD - Hydrogen bond donors

IBD - Inflammatory bowel disease

IIV - Inter-individual variability

IOV - Inter-occasion variability

IR - Immediate release formulation

IV - Intravenous

IVIVC - *In vitro in vivo* correlations

*m*Hanks - Modified Hanks buffer system

MW - Molecular weight

NONMEM - NONlinear Mixed Effects Modelling

OFV - Objective function value

PBPK - Physiologically based pharmacokinetics models

PD - Pharmacodynamics

P_{eff} (cm* s^{-1}) - Effective permeability

P-gp - P-glycoprotein transport

PK - Pharmacokinetic

PO- Per os

PR- Prolonged release formulation

PSA - Polar surface area

RV - Residual variability

SMEDDS - Self Micro Emulsifying Drug Delivery System

T_{max} -Time to reach maximum concentration in plasma concentration vs. time curve

VPC - Visual predictive checks

Chapter 1- Inter-subject Variability in Oral Drug Absorption

1.1 Overview

Oral drug delivery is the preferred route of drug administration. However, it is a multi-factorial process and the performance of any dosage form is the result of complex interplay between the drug, formulation and GI (gastrointestinal) physiology. Often the variability in gut physiology is underestimated, with only one or two variables being considered in formulation design and drug targeting (McConnell *et al*., 2008a). Therefore, formulation development research is required to take into account the very variable nature of the gastrointestinal tract to achieve dosage form optimisation.

The work in this thesis focuses on drug absorption and inter-subject variability. The *in silco, in vitro and in vivo* approaches commonly used in drug development have been investigated herein in relation to gastrointestinal physiology to identify the factors which contribute to low and erratic oral absorption.

The sections below provide an overview of the physiological processes in the GI tract and their influence on drug absorption and inter-subject variability in relation to the measurement of absorption and bioavailability.

1.2 Bioavailability and Absorption

The terms absorption (f_a) and bioavailability (F) are often used interchangeably (Chiou, 2001). Oral bioavailability is primarily a function of oral absorption and first pass hepatic elimination which represents the fraction of a dose administered that reaches the systemic circulation (Figure 1.1). f_a is the fraction of a dose entering the cellular space of the enterocytes from the gut lumen (the drug may not be released from the formulation and remain in solid form, the drug may also be lost by decomposition in the gut lumen, or it may become soluble in the gut lumen but fail to permeate through the gut wall). The elimination phase is represented by f_h (the fraction of drug entering the liver that escapes first pass hepatic metabolism and biliary secretion, thus entering the systemic circulation) and f_g (the fraction of drug entering the enterocytes that escapes first pass gut wall metabolism and enters the portal vein) (Huang *et al.*, 2009). Therefore, low oral bioavailability may be attributed to poor absorption and/or extensive first pass elimination (Equation 1.1).

$$F = f_a * f_g * f_h$$

Equation 1.1: Oral bioavailability

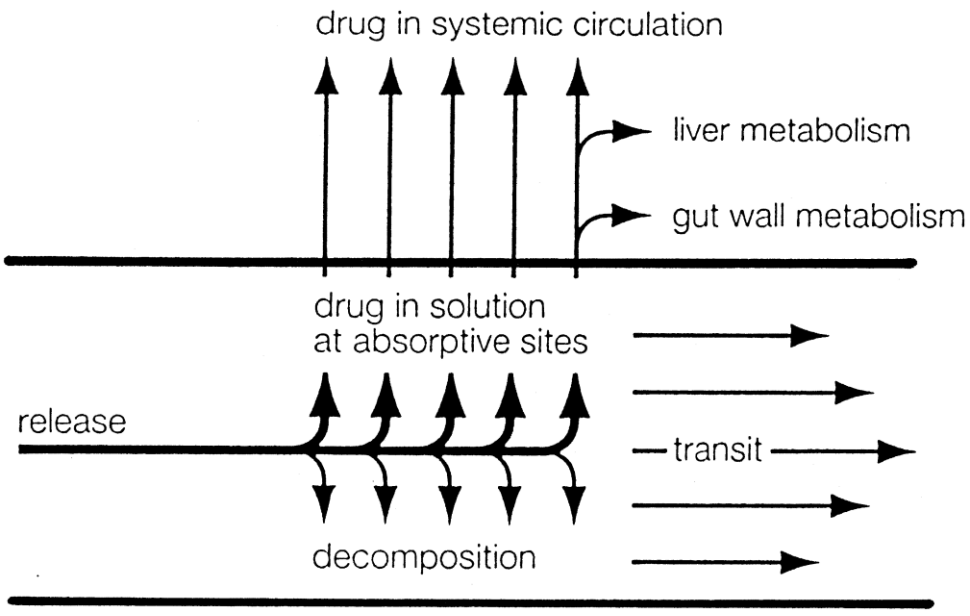

Figure 1.1: Events in the gastrointestinal tract following administration of an oral dosage form (Dressman and Reppas, 2000).

1.3 Bioavailability and Inter-subject Variability

An important problem identified during drug development and therapy is inter-subject variability. The result is that a standard dosage regimen of a drug may prove therapeutic in some patients, ineffective in others, and toxic in others. The need to adjust the dosage regimen of a drug for an individual patient is evident, and this need is clearly greatest for drugs that have a narrow therapeutic window, that exhibit a steep concentration-response curve, and that are critical to drug therapy.

Hellrigel *et al*. (1996) examined the relationship between absolute oral bioavailability and inter-subject variability. Their results clearly showed a significant relationship between the absolute bioavailability of an oral dosage form and its inter-subject coefficient of variation (CV). Drugs with low bioavailability will have greater inter-subject variability in bioavailability, and vice versa (Figure 1.2). These results have a significant clinical implication and could have an impact on how bioavailability and bioequivalence studies are designed and interpreted.

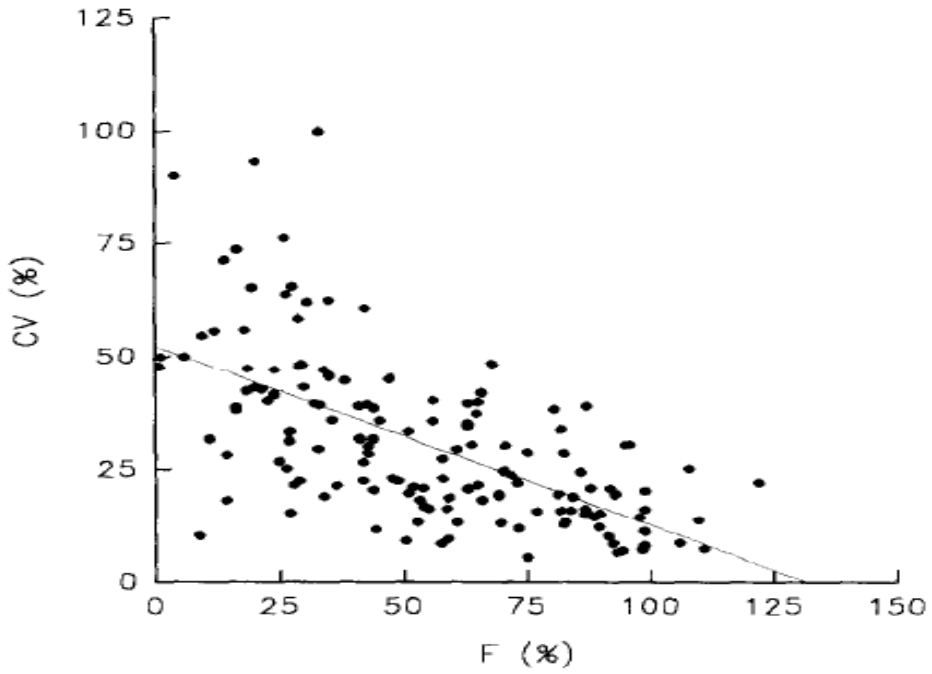

Figure 1.2: Relationship between absolute bioavailability (F) and inter-subject variability (CV) in absolute bioavailability (Hellrigel *et al*. 1996).

All the components of bioavailability (f_a, f_g and f_h) are sensitive to inter-subject difference (Jamei *et al*., 2009a). The factors which contribute to inter-subject variability in f_a are the formulation aspects (disintegration and particle size), physicochemical attributes of the drug (dissolution and solubility) and variation in GI physiology which is represented by pH, stomach emptying time, and transit time varying with age, gender, and diseases. Factors include food, alcohol, or concomitant medications that may also affect the dissolution of the drug or GI function (Figure 1.3). f_g is sensitive to the abundance and the regional distribution of drug metabolizing enzymes which could be influenced by genetics and diet. Variation in blood flow to the gut, and disease states can also add variability in oral drug absorption. Efflux pump, i.e. P-glycoprotein (P-gp), as well as influx and efflux by other transporters may be subject to inter-subject variations affecting transporters abundance and/or activity. The final element that determines inter-subject variability in bioavailability is the first pass metabolism of the drugs by the liver (f_h). Hepatic clearance of drugs which are inefficiently extracted from the blood is sensitive to changes in the activity of drug metabolizing enzymes in the liver. Environmental substance or toxins as well as genetic makeup (polymorphism) contributes to inter-subject differences in drug metabolism. Other factors affecting inter-subject variability in hepatic clearance are related to age, ethnic groups and gender. Another aspect of variability is the intra-subject variability, any of the factors mentioned before is additionally subjected to intra-subject variability. In particular, intra-subject variability in absorption is affected by diurnal factors, changes in blood flow, body position, and volume of fluid and food intake.

1.4 The Requirement for Good Prediction of Gastrointestinal Absorption

Focusing on f_a instead of bioavailability, enables the identification and understanding of the key causes of low absorption and consequently of inter-subject variability in absorption. The first step will be to obtain a preliminary account of the extent of oral absorption (f_a) and variability instead of bioavailability (F). Requirement for this is that accurate methods for prediction or estimation of the gastrointestinal fraction absorbed (f_a), are available and applied. Further on, the factors that determine GI absorption and inter-subject variability need to be well understood and considered. Finally, investigating different formulation approaches to increase absorption and reduce inter-subject variability is required. Potential benefits of high absorption are less inter-subject variation in systemic exposure, smaller dosage forms and lower material costs. Thus, it is desirable to find candidate drugs with sufficiently high absorption.

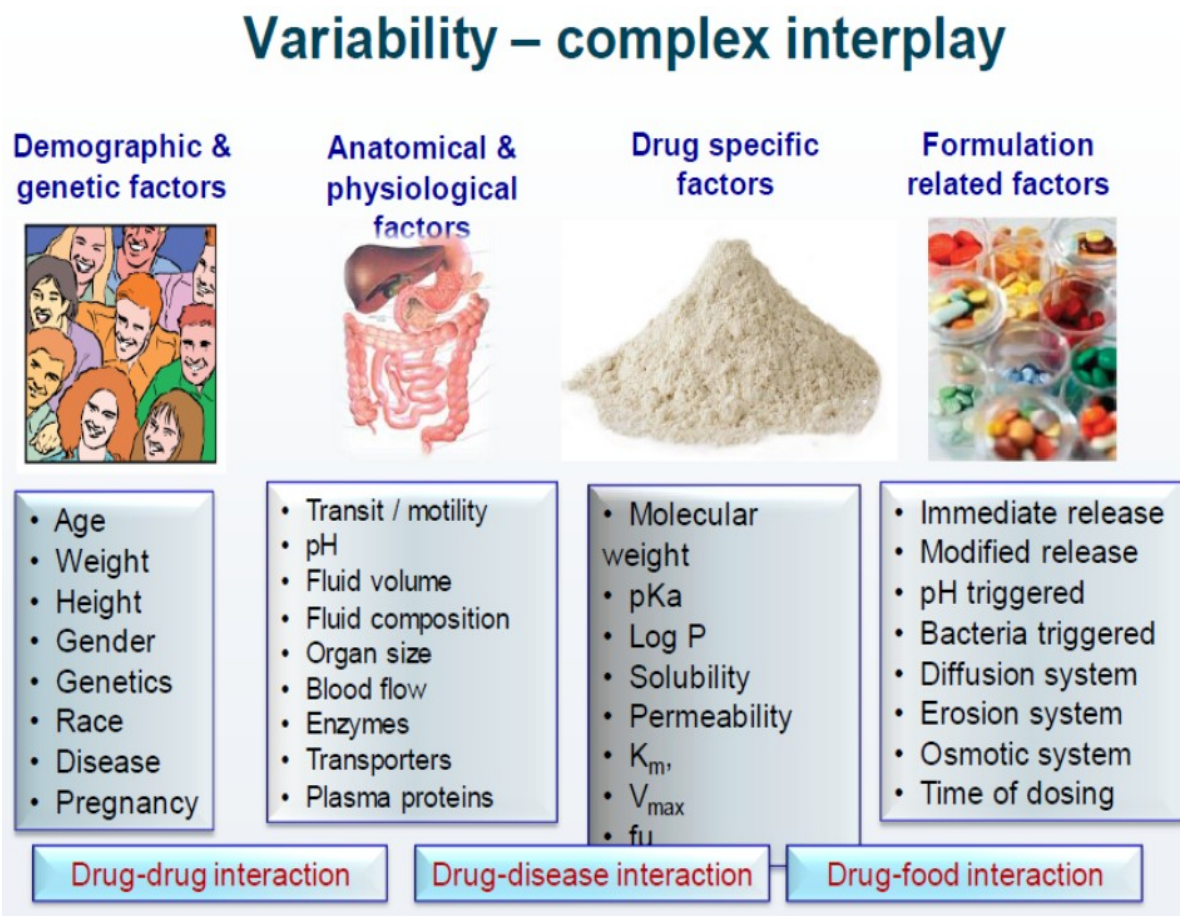

Figure 1.3: Factors contributing to inter-subject variability in oral absorption

1.5 Physiological Factors Affecting Drug Absorption

Each compartment of the GI tract features distinctive physiological and morphological characteristics capable of influencing drug pharmacokinetics (Table 1.1). Key challenges typically faced by drugs on oral administration are: the dynamic luminal conditions (changes in pH along the gut, gastrointestinal fluids composition and microbiota rich colon), the complex gut wall (enzymes and transporters) and highly variable gastric emptying time and gut motility (Pocock et al., 2013).

Table 1.1: Characterisation of different parts of the GI tract (Washington *et al*. 2001)

Region	Length (m)	Surface area (m^2)	pH	Residence time	Microorganism
Oesophagus	0.3	0.02	6.8	<30 seconds	Unknown
Stomach	0.2	0.2	1.8-2.5	1-5 hours	$\leq 10^2$
Duodenum	0.3	0.02	5-6.5	<5 minutes	$\leq 10^2$
Jejunum	3	100	6.9	1-2 hours	$\leq 10^2$
Ileum	4	100	7.6	2-3 hours	$\leq 10^7$
colon	1.5	3	5.5-7.8	15-48 hours	$\leq 10^{11}$

1.5.1 Gastrointestinal Fluids Volume and Composition

Gastrointestinal fluid is complex dynamic and fluctuating and essential for disintegration, dispersion, solubility/dissolution of drugs. Like other GI parameters, the volume of liquids in various compartments can vary within and between individuals. It is affected by the amount of liquid ingested, the volume of gastric and pancreatic secretion, gastric emptying rate, intestinal transit time, as well as uptake and efflux of liquids along the GI membrane.

Post mortem studies in humans have shown the presence of fluids in the stomach and the small intestine are 118mL and 212mL, respectively (Gotch *et al*., 1957; McConnell *et al*., 2008a). Cummings *et al*. (1990) measured the large intestine fluid which was 187mL mean value. The fluidity of the caecum and ascending colon is slowly reduced as the water is reabsorbed. The reduction in the water content means that there is less mixing in the bulk phase and therefore less access to the mucosal surface, along with less water available for drug dissolution. Gas bubbles present in the colon also will reduce contact of the drug with the mucosa (Johnson and Gee, 1981). These values represent the total water including that bound to GI mass and therefore may not be a very relevant factor in drug dissolution.

Schiller *et al*. (2005) investigated the GI transit of sequentially administered capsules in relation to the free water along the intestinal lumen by magnetic resonance imaging and found that fluid is not homogeneously distributed along the gut, which likely contributes to the inter-subject variability of drug absorption (Table 1.2).

Table 1.2: Water sensitive magnetic resonance imaging (MRI) in 12 healthy volunteers (Schiller *et al*. 2005)

	Fasted		**Fed (1 hour before imaging)**	
	Mean (mL)	Median (mL)	Mean (mL)	Median (mL)
Stomach	45	47	686	701
Small intestine	105	83	54	39
Large intestine	13	8	11	18

The composition of the GI fluids varies according to the stimulus and the secretion rate. The gastric juice is a mixture of water, hydrochloric acid, electrolytes and organic substance. The main electrolytes in gastric secretion are: H^+, Cl^- , K^+, Na^+, Mg^{2+}, and Ca^{2+} (Hirschowitz, 1961). The composition of the fluids in the upper small intestine includes chyme from the stomach, as well as secretions from the liver, the pancreas, and the wall of the small intestine. Composition is affected by fluid compartmentalisation, mixing patterns, absorption of fluid into the intestinal wall, and transit down the intestinal tract. Secretions from the pancreas include bicarbonate as well as proteases, amylases, and lipases. The liver secretes bile which contains bile salts, phospholipids, bicarbonate, cholesterol, bile pigments and organic waste. The wall of the small intestine secretes mineral ions such as bicarbonate, sodium and chloride, as well as water. Lindahl *et al*. (1997) chemically characterised the upper GI tract fluids in the fasted state. It was concluded that the chemical characteristics of the GI fluids not only varied between individuals, but also showed a pronounced day to day variation in the same individual, which might be crucial for the overall rate and extent of drug absorption. As food intake triggers many of the secretions in the small intestine, the composition of fed state intestinal fluid can vary greatly from fasted state (Kalantzi *et al*., 2006).

1.5.2 Gastrointestinal pH

1.5.2.1 Gastric pH

The fasting gastric pH has been studied in depth. Using a pH sensitive radiotelemetry capsule, the records measured for gastric pH were highly acidic (range 1.0-2.5) (Dressman *et al.*, 1990; Evans *et al.*, 1988; Fallingborg *et al.*, 1990). Dressman *et al.* (1990) have investigated the changes in pH due to the buffering effect of food. The authors found that when a meal was administered the gastric pH climbed temporarily from 1.7 in fasted state to average peak value of 6.7, then declined gradually back to the fasted state value over a period of less than two hours (Figure 1.4). In addition, it was proven that the pH is not uniform in the stomach, due to the differences in the distribution of parietal cells, and the different patterns of motility in various regions of the stomach (McLauchlan *et al.*, 1989).

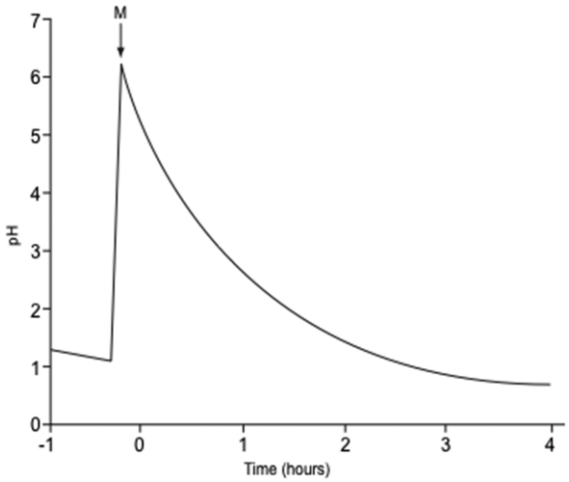

Figure 1.4: Approximation of a typical pH profile in the stomach. The letter "M" denotes food intake (Dressman *et al.* 1990)

In the same article, considerable differences in the pH between subjects were reported in the fed and fasted state. Gastric pH is sensitive to increasing age, pathological conditions and drug induced changes. Although the majority of elderly people exhibit gastric pH profiles similar to younger people, 10-20% of the elderly population exhibit either diminished (hypochlorhydria) or no gastric acid secretion (achlorhydria), leading to basal gastric pH values >5.0 (Holt *et al.*, 1989; Sievers, 1966).

1.5.2.2 Intestinal and Colon pH

The range of intestinal pH profiles were investigated widely in a representative group of normal subjects. The mean pH in the proximal small intestine lies within the range 5.5-7.0, gradually increasing by about 1 pH unit to 6.5-7.5 in the distal. There was a sharp fall in pH to a mean of 6.4 (range 5.5-7) as the capsule passed into the caecum. pH then rose progressively from the right to the left colon with a final mean value of 7.0 (Evans *et al*., 1988; Fallingborg *et al.*, 1989; Fallingborg *et al.*, 1990; Nugent *et al.*, 2001).

The duodenal pH is directly influenced by a meal. In the fed state, the small intestine pH first decreases in response to a meal with the arrival of acidic chyme from the stomach but later the fasted state pH is re-established as a result of pancreatic bicarbonate secretion. Dressman *et al*. (1990) measured the median fasting duodenal pH as 6.1. During the meal, a brief period of elevated duodenal pH was observed, as the median pH value was 6.3. The pH in the postprandial phase in the duodenum is considerably lower than in the fasted state around 5.4 (Hörter and Dressman, 2001).

One of the most important messages from studies is that the pH shows huge inter-subject variability between people, and an outstanding example of this is demonstrated in the pH profiles measured by Fallingborg *et al*. (1989) in 39 healthy individuals in which there can be over two pH units difference at the same site. Similar results were recorded by Annaert *et al*. (2010) (Figure 1.5). In addition to inter-subject variability, there are also potentially marked differences within individuals on different occasions; previous work showed substantial differences in gastrointestinal pH profiles measured one week apart, under the same feeding conditions for the same subject (Ibekwe *et al*., 2008).

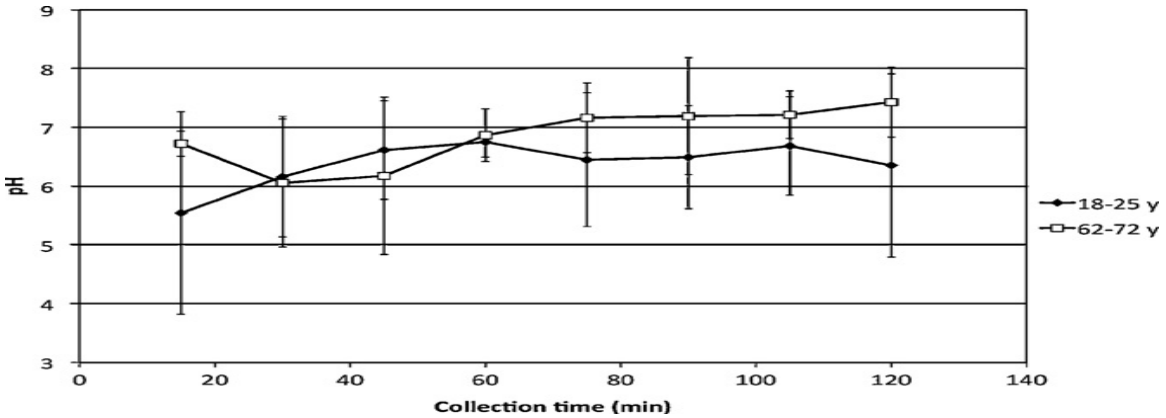

Figure 1.5: Profiles of luminal pH in the duodenum as a function of collection time. Values for two age groups: 18–25 years and 62–72 years (Annaert *et al*. 2010)

Considering the pH changes along the GI tract and between subjects, weak acid and base solubility will be highly affected. It is important that poorly water-soluble weakly basic drugs dissolve rapidly in the stomach because dissolution of undissolved drug in the intestine may be too low to permit complete absorption. Moreover, weak bases will be less soluble in the stomach if given immediately after food intake because the gastric fluids are less acidic. Poorly soluble weak acids with pKa values less than six are relatively insoluble in the preprandial gastric juice and dissolution occurs first in the upper small intestine. However, in the case of very weak acids the variations in pH in the gastrointestinal tract are irrelevant to the solubility because these compounds are always in the free acid form over the physiological pH range (Hörter and Dressman, 2001).

1.5.3 Gastrointestinal Motility and Transit

Gastric emptying time and transit time along the intestine are absolutely crucial when considering the dissolution rate of poorly soluble drugs, as it reflects the time available for dissolution. Since the stomach is an important site for the dissolution of weak bases, a shorter gastric emptying time will decrease the time for the weak base to dissolve, and hence less of the drug in solution form will transfer to the intestine. Shorter or erratic transit time in the intestine will also impact considerably on the dissolution rate for poorly-soluble drugs with small absorption windows. If the dissolution rate is low and the oral drug delivery system moves rapidly through the intestine, a much lower proportion of the drug will be available for absorption. Transit time will also affect the boundary layer thickness: the contractions which create the motility of the intestine and stomach also contribute to the mixing of the luminal contents. Consequently, in the fasted state when only short bursts produce motor activity, the boundary layer will be wide. However, this might be compensated for by a longer transit time. The contrary will happen in the fed state – that is, more contraction, increasing motility and mixing - hence increasing the dissolution rate.

1.5.3.1 Gastric Motility

Many factors affect the residence time of a solid or dissolved drug in the stomach. Thus, factors influencing the rate of gastric emptying may alter the rate or extent of absorption of most if not all orally administered drugs. The most important factors include stomach fullness, frequency of feeding, and the composition of the chyme in stomach and intestine or even the anticipation of food (Olsson and Holmgren, 2001).

The gastric emptying of tablets, pellets and liquids is variable whereas solutions empty from the stomach quite rapidly and are not greatly affected by the digestive state of the individual. There are conflicting reports as for the emptying time of solid dosage forms from the stomach. Kaniwa *et al*. (1988) found that small pellets with the size below 1mm empty from the stomach more quickly than large pellets and tablets. However, Clarke *et al*. (1993) found that pellets of two size 0.5 and 4.75mm have the same gastric emptying rate. Newton (2010) showed that the issue of how pellets empty from the stomach is not well supported by the pharmaceutical literature and claims that pellets less than 2mm will empty from the stomach as if they were liquids in the fed state, can be contradicted easily by examining the study protocol and/or the analysis of the data.

Many tools have been developed in order to characterise the inter- and intra-subject variability in gastric emptying. Intra-subject variation of drug absorption rates appeared to be due to variations in gastric emptying rates (Levy and Hollister, 1965). Petring and Flachs (1990) tried to determine the extent of inter- and intra-subject variability in the gastric emptying of semisolids and liquids. The results showed that the intra-subject variability was not statistically significant for any absorption parameters, (this may be due to the use of a small semi-solid test meal) while the inter-subject variability was significant for all parameters.

1.5.3.2 Small Intestine Transit Time

The small intestine transit time of dosage forms is almost invariably quoted at 3–4 h, and a meta-analysis of transit data in the small intestine showed no difference between tablets, pellets and liquids (Davis *et al*., 1986). In other measurements of the small intestine transit time, it was found that the small intestinal transit was 8h, and colonic transit time was 17.5h (median values) (Fallingborg *et al.*, 1989). However, those are mean values from pooled data with different methodologies. It is, therefore, safe to assume that the actual values ranged from 0.5 to ~9.5 h. Figure 1.6 demonstrates the high inter-subject variability in the Davis study. It can be noted that individual small transit values appeared quite variable and the range is quite large.

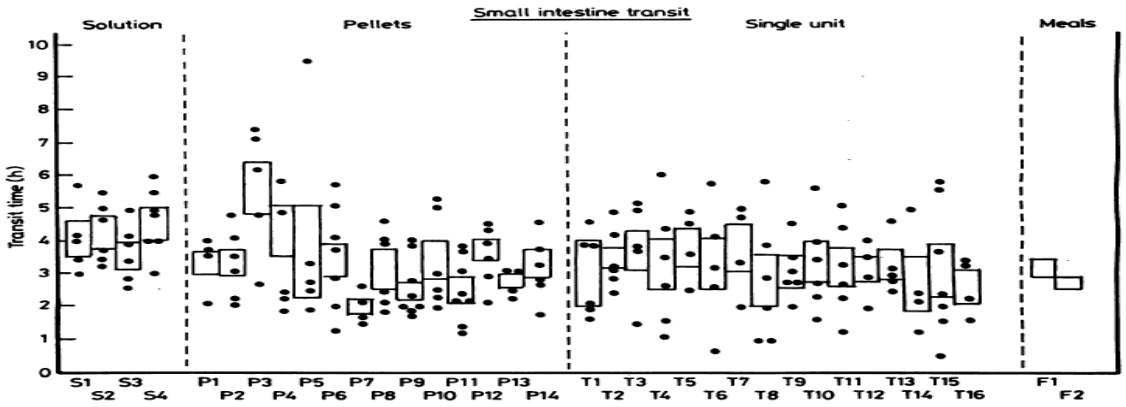

Figure 1.6: Small intestine transit of pharmaceutical dosage forms. Mean ± SEM (Davis *et al*. 1986).

Fadda *et al*. (2009a) examined how the timing of tablet and food administration can affect the small intestine transit time. The results are shown in Table 1.3. The small intestinal transit times of tablets after the fasted and fed dosing regimen were similar while with the pre-fed (when tablet was administered 30 min prior to a meal) dose, small intestinal transit time was significantly shorter than in the fasted or fed state. This can influence drug bioavailability. The explanation of this phenomenon is the increasing peristaltic activity of the small intestine in response to the intake of a meal.

Table 1.3: Transit time of non-disintegrated tablet in fasted and different fed states (Fadda *et al*. 2009)

	Small intestine transit time (min)		
	Fast	Fed	Pre-fed
Median	204	210	141
Range	167-521	198-226	115-188

1.5.3.3 Colon Transit Time

Whole bowel transit time is generally between 24 and 36 hours in healthy individuals, but values ranging from 0.4 to 5 days have been reported in the literature (Abrahamsson *et al*., 1988; Arhan *et al.*, 1981). Eating and morning awakening appear to be the major stimuli in eliciting colonic motility. Transit through the large bowel is highly influenced by the pattern of daily activity. The highest calorie intake in the western world occurs in the evening and colonic motility decrease at night. Dietary fibre, in the form of bran and wholemeal bread, fruit and vegetables, increases faecal weight by acting as a substrate for colonic bacterial metabolism. This increased faecal bulk is associated with a reduced colonic transit time, although the mechanism is uncertain (Cummings *et al.*, 1978). Irregular motility and lack of bile salts in the colon can affect the solubility and dissolution of some compound.

To conclude, there is high inter-subject variability of motility along the GI tract. Figure 1.7 demonstrates this. There is no doubt that when analysing data, one should exercise caution in interpreting the mean value as a definite value.

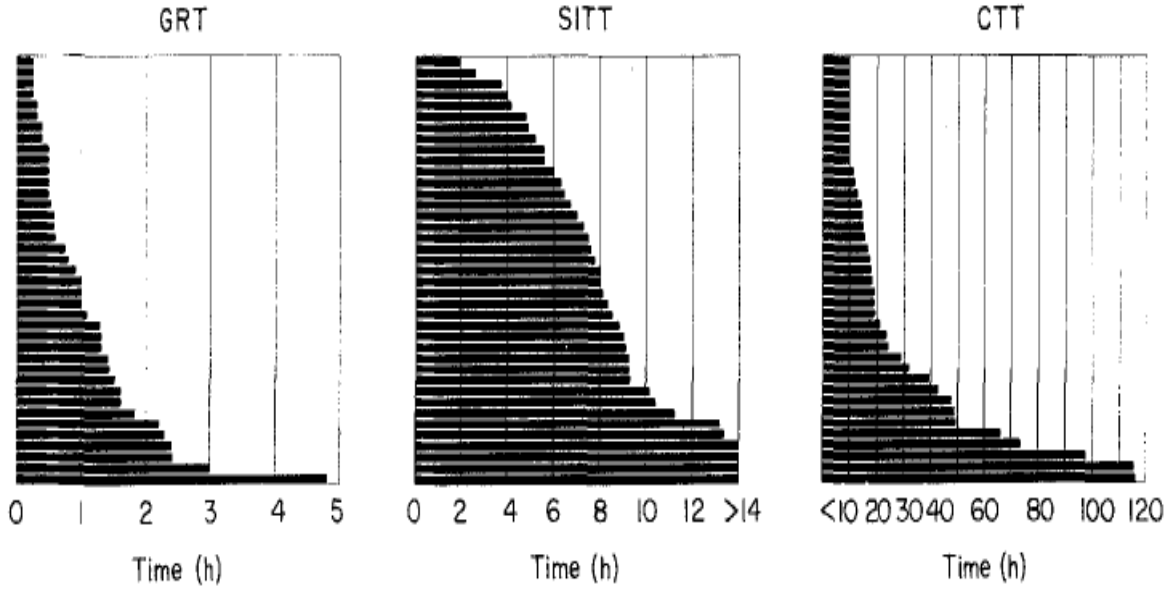

Figure 1.7: Regional GI transit times of a non disintegrating capsule in 39 subjects (GRT=gastric residence time, SITT= small intestine time, CTT= colonic transit time) (Fallingborg *et al*. 1989).

1.5.4 Gut Metabolism and Transporters

The mucosal enzymes and transporters are very important and manipulate drug bioavailability. The enzymes (e.g. CYP3A), metabolise the substance crossing the mucosa, whereas efflux transporters (e.g. P-gp) transfer the drug back into the lumen, and influx transporters (e.g. PEPT1) which can enhance absorption.

Lin and Lu (2001) indicated that the intestinal mucosa is the most important extra-hepatic site of drug biotransformation. Hence, potential exists for significant pre-systemic metabolism and as a result enhanced reduction in bioavailability as the drug passes, consecutively, through the small intestine and liver. In the small intestine as well as in the liver, CYP3A is the most abundant P450 subfamily expressed, with an average specific content representing 50-70% of spectrally determined P450 content (Paine *et al.*, 1997; Watkins *et al.*, 1987). Like hepatic CYP3A, enteric CYP3A is localised within the mature absorptive columnar epithelial cells (enterocytes) that largely compose the mucosal lining (Kolars *et al.*, 1994).

Total P450 content in GI mucosa microsomal fraction varies with anatomical region. Even within the small intestine, proximal mucosal P450 content is generally higher than the P450 content of more distal mucosa. Transition from ileal to colonic mucosa again results in a further drop in total P450 content (Waziers *et al.*, 1990; Zhang *et al.*, 1999). Not only might CYP3A reduce the oral bioavailability, but it may also be a major source of inter-subject variability in blood level and drug response as a consequence of variable constitutive enzyme expression and drug-drug interaction (Paine *et al.*, 1997).

P-gp acts as part of a detoxification and excretion pathway in the gastrointestinal tract. P-gp shows extremely broad substrate specificity with a tendency towards lipophilic, cationic compounds (Chan *et al.*, 2004). Intestinal P-gp is localised to the villus tip enterocytes, which is the main site of absorption for orally administered drugs (DeVita *et al.*, 1991). It has been found that P-gp not only limits drug absorption by efflux but also increases the access of drug to metabolism by mucosal enzymes through repeated cycles of absorption and efflux (Benet *et al.*, 2004).

1.6 Solubility/Dissolution and Permeability

The limiting steps of oral absorption can be categorised into three types: permeability, dissolution rate and solubility (Figure 1.8). The oral absorption is 'dissolution rate limited' if the permeation rate is much larger than the dissolution rate; the dissolved drug instantly disappears from the intestinal fluid. In the case of 'permeability limited' the permeation is slow and dissolution is fast. The dissolved amount accumulates in the intestinal fluid. The third classification is the case where the concentration of the drug in the intestine reaches the maximum solubility in GI fluids. Therefore, the solid drug can no longer dissolve into the intestinal fluids and the oral absorption is 'solubility limited' (Sugano et al., 2007).

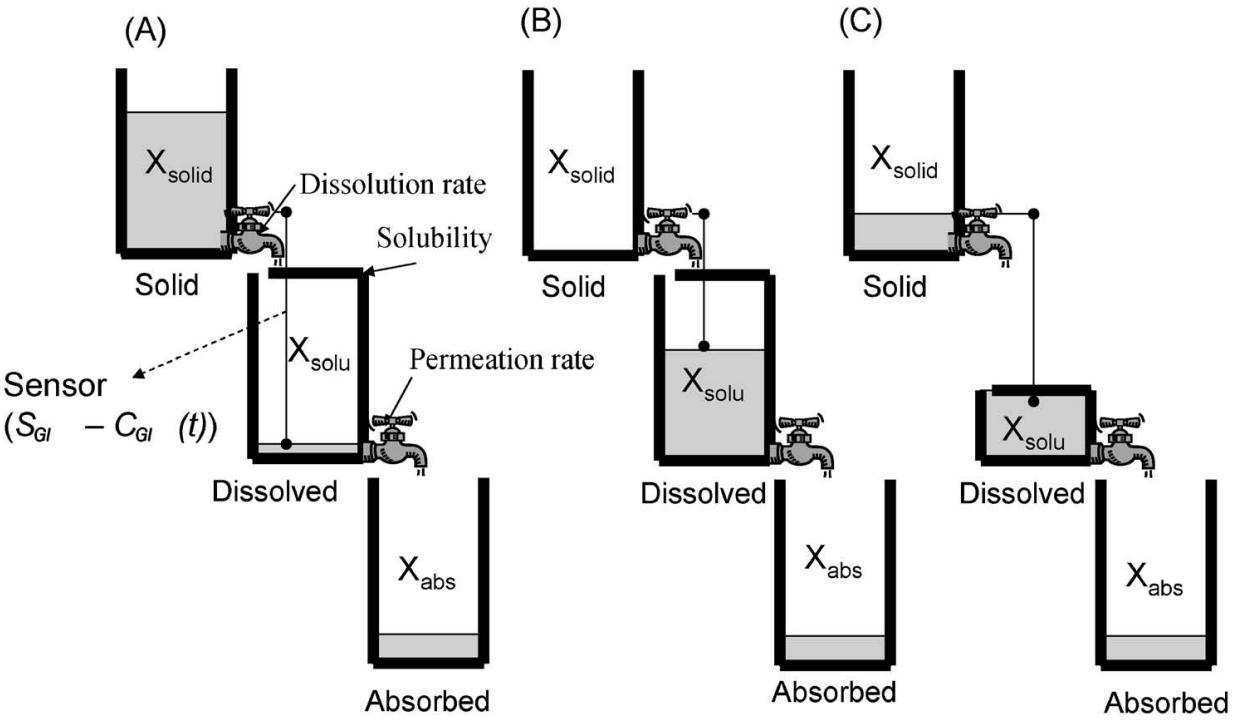

Figure 1.8: Bucket presentation of oral absorption. (A) Dissolution rate limited absorption. (B) Permeability limited absorption. (C) Solubility limited absorption (Sugano *et al*. 2007).

1.6.1 Dissolution

The dissolution of a drug is a prerequisite for it be absorbed. Important factors which influence the kinetics of drug dissolution can be identified through modification of the Noyes-Whitney equation (Equation 1.2):

$$DR = \frac{dx}{dt} = \frac{A*D}{h} * \left(C_S - \frac{X_d}{V}\right)$$

Equation 1.2: Modified Noyes-Whitney equation (Hörter and Dressman, 2001)

Where DR is the dissolution rate, A is the surface area available for dissolution, D is the diffusion coefficient of the drug, h is the thickness of the boundary layer adjacent to the dissolving drug surface, C_S is the saturation solubility of the drug, X_d is the amount of dissolved drug and V is the volume of dissolution media (Hörter and Dressman, 2001).

An important factor determining the dissolution rate is the particle size of the drug. The dissolution rate is directly proportional to the surface area of the drug, which increases with the decrease in particle size. However, decreasing particle size might also become a limitation when the wetting capacity of the buffer is very poor, resulting in particulate agglomeration (Solvang and Finholt, 1970).

The contact angle at the liquid-solid interface can give estimation of the buffers' wetting capacity: when the angle is high, the wetting properties of the buffer are poor. There are several factors that can decrease the angle and hence increase wet-ability; among them the native surfactant in the gastric and intestinal fluids. Bile salt concentration in the fasted state in the small intestine can vary between 1 to 6mM. Differences in bile salts concentration between individuals can also affect the contact angle, thus increasing or decreasing the fluid wetting capacity (Bakatselou *et al*., 1991).

According to the Noyes-Whitney equation, dissolution rate is also affected by drug diffusivity (D). The Stokes -Einstein equation (Equation 1.3) states that diffusivity is inversely dependent on the fluid viscosity (η):

$$D = \frac{k * T}{6\eta\pi r}$$

Equation 1.3: The stokes- Einstein equation

Viscosity (η) of the GI fluids may vary between individuals as the function of food intake and secretion to the gastrointestinal luminal. The food effect is highly dependent on the food components and the volume of co-administered fluids. Furthermore, the diffusion coefficient is reduced by micellar solubilisation (Hörter and Dressman, 2001).

1.6.2 Permeability

Overall permeability can be considered as the sum of passive (diffusion driven) and active (transporter mediated) processes. The latter can affect both influx and efflux of a drug (i.e. P-glycoprotein). Molecular properties relevant to intestinal absorption include lipophilicity, molecular size and charge, and hydrogen bonding, and importantly, most of these properties are also intrinsically dependent on one another.

Depending on the mechanism of transport, the drug flux through the intestinal mucosa (J) can be described with the following equations (Equation 1.4 and Equation 1.5):

$$J = C_W * P_W$$

Equation 1.4: Drug flux for passive transport

$$J = \frac{J_{max} * C_W}{C_W + K_M}$$

Equation 1.5: Drug flux for carrier mediated transport

Where P_w is the effective membrane permeability coefficient, C_w is the drug concentration, J_{max} is the maximum drug flux through the membrane and K_M is the Michaelis–Menten constant (Dressman and Lennernaes, 2000).

Any drug molecule that successfully overcomes the various biological membranes and reaches its site of action should feature a balance between its hydrophilic and lipophilic properties. According to Fick's first law of diffusion, passive drug transport across a membrane is proportional to the membrane-water partition coefficient. Since membrane-water partition coefficients are not readily available, partition coefficient between water and an organic solvent such as octanol are normally used. The octanol- water partition coefficient (P) is a physical property used extensively to describe a chemical's lipophilic or hydrophilic properties. It is the ratio of unionized compound in mutually saturated octanol and water. Since P values may range over several orders of magnitude, the logarithm (log P) is commonly used for convenience (Smith *et al.*, 1975). Partition coefficients that are measured

at a given pH are known as distribution coefficients (D), defined as the ratio of the concentration of compound in the organic phase to the concentration of both ionised and unionised species in the aqueous phase at a given pH (Scherrer and Howard, 1977).

The lipophilicity of a drug is the most-used physicochemical property to predict its permeation in biological systems. Molecules diffuse across the membrane in proportion to their concentration gradient across the membrane. and in proportion to their lipophilicity (Cao *et al.*, 2008). However, molecular size is yet another factor affecting drug permeability through the membrane, most conveniently defined by use of molecular weight (MW). However, this may not be sufficient, because MW, as such, contains no information about the actual three-dimensional (3D) shape of the molecules.

Another parameter used to describe permeation is hydrogen bonding. Ordered lipid layers provide a finite amount of hydrogen bonding groups. These groups, the majority of which are hydrogen bond acceptors, are located exclusively in the head group region of the lipids. In order to partition into the hydrocarbon region of the bilayer, the solute must be sufficiently lipophilic to overcome the energy losses that occur in breaking the hydrogen bonds with water or the lipid head groups. This step can thus present a considerable energy barrier for solutes, which exhibit strong hydrogen bonding (donor) tendencies. Accordingly, biological permeation can be expected to markedly depend on the hydrogen bonding capacity of the solute (Conradi *et al.*, 1991; Diamond and Wright, 1969; Tayar *et al*., 1991).

1.7 Prediction of Absorption

In a recent publication by Musther *et al*. (2014), an extensive analysis of the published literature data of human and animal (mouse, rat, dog and non-human primates) bioavailability was conducted. A database of 184 compounds was assembled. Linear regression for the reported compounds indicated no strong or predictive correlations to human data for all species, individually and combined.

1.7.1 Animals and Absorption in Human

Chiou and Barve (1998) investigated the correlation between f_a values in rats and humans (Figure 1.9). The extent of absorption which was reported in the literature or estimated by them was based on studies using radio-labelled compounds or based on pharmacokinetic methods. The authors found high correlation (R^2=0.97) between f_a values in rats and humans for 64 test substances.

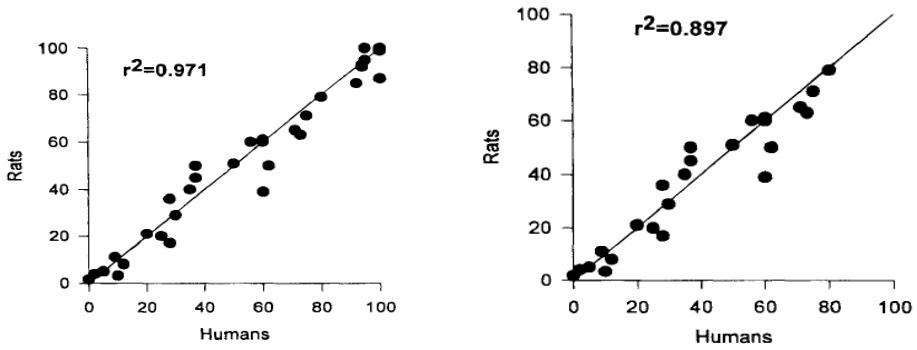

Figure 1.9: Correlation of fraction absorbed in humans VS rats for 64 drugs (right) and for 24 drugs with less than 90% absorption (left) (Chiou and Barve, 1998).

In a similar experiment, Zhao *et al*. (2003) collected data of 241 human, oral absorption from the literature. They identified a standard deviation of 11% between human and rat absorption. It was suggested that the absorption in rats could be used as an alternative method to human absorption in pre-clinical oral absorption studies.

In addition, Chiou *et al*. (2000) conducted a retrospective evaluation using dogs as an animal model to study f_a of 43 drugs in humans (Figure 1.10). The overall correlation was relatively poor (R^2=0.51) as compared to the earlier rat vs. human study. This suggests that caution must be exercised in the interpretation of data from dogs to humans, and may be related to "leakier" tight junctions found in dog jejunum.

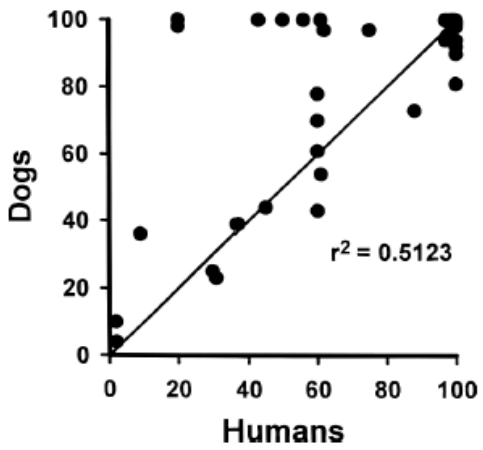

Figure 1.10: Correlation between fraction absorbed data in human vs. dogs for 43 drugs (Chiou *et al.*, 2000).

In a similar manner, Chiou and Buehler (2002) examined whether monkeys were an appropriate model to predict human data for the fraction absorbed, revealing a strong linear relationship between the f_a data of monkeys to that of humans (R^2=0.97).

Similar to the human study, a limitation of animal models is that they are unsuitable for high throughput screening. In addition, the absorption rate and absorption rate constant (k_a) are expected to differ between animals and humans due to the influence of intestinal radius and gastric emptying time. Absorption is expected to be slower in humans than in rats and other laboratory animals (Fagerholm, 2007).

1.7.2 Prediction of Absorption in Humans from Permeability Data

One of the major determinants for f_a is GI permeability (P_{eff}). The P_{eff} is defined as the speed ($cm*s^{-1}$) at which a molecule is transported (by passive diffusion and /or active transport) across a membrane, cell endothelium or epithelium.

Cao *et al*. (2006) tested the intestinal permeability of 14 drugs and three drug-like compounds with different absorption mechanisms in rat and human jejunum, determined by *in situ* intestinal perfusion. The authors showed that there was no correlation found in the bioavailability between rat and human, while a good correlation was observed between human and rat intestinal permeability of drugs with both carrier-mediated absorption and passive diffusion mechanisms (Figure 1.11). However, Fagerholm (2007) claimed that most of the compounds in this study were characterised by high permeability, which resulted in predicting complete f_a. The author suggested that perfusion methods are not sufficiently sensitive enough to measure the permeability of compounds with low or moderate P_{eff}. The

limits in this method are the comparably slow screening rate, and that this model is mainly useful only for predicting the active uptake potential.

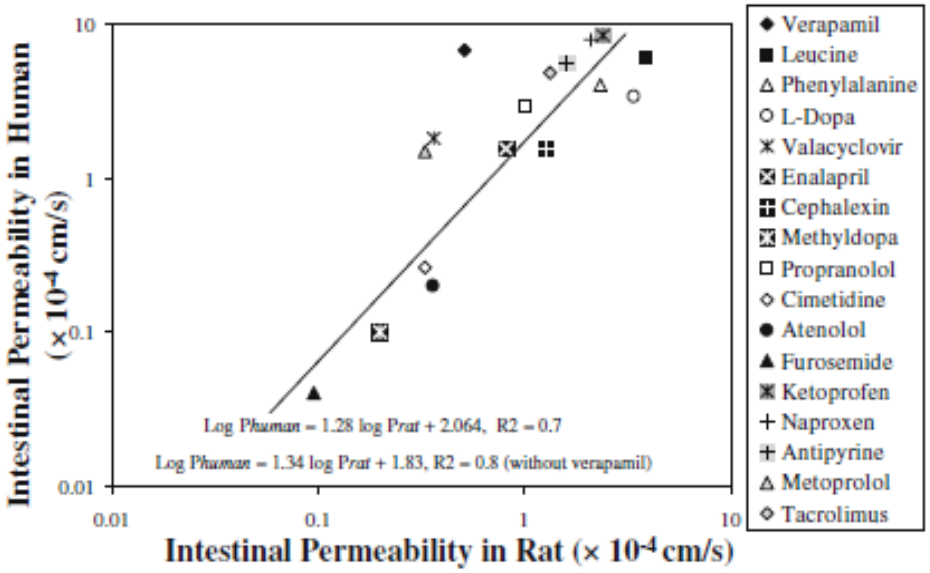

Figure 1.11: Correlation of drug permeability in rat jejunum and in human jejunum. Permeability coefficients (P_{eff}) were determined by in situ intestinal perfusion (Cao *et al*. 2006).

The Caco-2 cell model is used for screening/estimation of $P_{eff,}$ and consequently can be used to predict drug transporter by different pathways across the intestinal epithelium. Arguably, the best correlation is obtained for drugs that are transported by the passive transcellular route (Artursson *et al.*, 1996). However, as mentioned above, some of the disadvantages of this model such as differences in the composition of cell membrane, paracellular radius and transporter expression imply that the uptake characteristics of Caco-2 cells are different from that of the small intestine (Balimane and Chong, 2005). Lennernäs *et al*. (1996) indicated that Caco-2 monolayers can be used to predict passive drug transport in humans, while prediction of transport by carrier-mediated systems may require a scaling factor due to a low expression of carriers in this cell line.

Irvine *et al*. (1999) compared Caco-2 P_{eff} and human f_a data for around 40 passively absorbed compounds, and found a poor correlation between the two parameters. In addition, they related MDCK P_{eff} values and human f_a values, and found that the accuracy and precision were similar to that of the Caco-2 cell line. In turn, Matsson *et al*. (2004) compared the widely-used permeability model Caco-2 regarding ability to predict f_a after oral administration in humans. The authors showed relatively good correlation between model estimation of P_{eff} values and f_a for 14 compounds with mainly passive absorption. However,

as opposed to the study of Irrvin *et al*. (1999), only 14 compounds were tested. Thus, it can be assumed that the Caco-2 model generally works well to predict complete or near complete f_a for a highly permeable substance.

Stewart *et al*. (1995) compared the intestinal permeability obtained in three absorption models for consistency, and assessed the utility of the models in predicting the fraction absorbed in human studies. The methods compared are the rat *in situ* single pass intestinal perfusion method, the rat everted intestinal ring method, and Caco-2 cell monolayers. The authors found that Caco-2 cell monolayers and rat single pass intestinal perfusion combine the highest correlation between the systems, and correlate well with the fraction absorbed in humans. As most of the tests were done using small organic molecules, however, they suggested that caution was needed when extrapolating permeability data from those methods to complex molecule like peptidomimetics.

1.7.3 PBPK Models

Physiologically based pharmacokinetics models (PBPK) use the "bottom–up" approach. The bottom up approach is basically modelling and simulation of the ADME processes. The key element of this approach is the separation of information on the human body from that of the drug physicochemical characteristics and the study design. This 'Bottom up' approach allows easily changing the study design. Hence, the power of studies to recognise covariates can be investigated a priori with the aim of improved decision making (Jamei *et al*., 2009a). The first PBPK model was introduced in 1973 by Theorell *et al*. (1937a; 1937b) and since then, thanks to the better understanding of the body physiology and development of new *in vitro* tools to assess drug performance, great progress has been made in developing PBPK models (Kostewicz *et al.*, 2014).

1.7.3.1 CAT Model

Few models have been developed to mimic *in silico* the *in vivo* situation in the gut. One that laid the base for further developments is the CAT model. This model accounts for the transit in the stomach, duodenum, jejunum, and ileum, and the absorption in the duodenum, jejunum, and ileum The assumptions for the CAT model include: absorption from the stomach and colon is insignificant compared with that from the small intestine; transport across the small intestinal membrane is passive; dissolution is instantaneous and drug transit through the small intestine can be viewed as a process flowing through a series of segments, each described by a single compartment with linear transfer kinetics from one to next, and all compartments may have different volumes and flow rates, but having the same residence times (Figure 1.12).

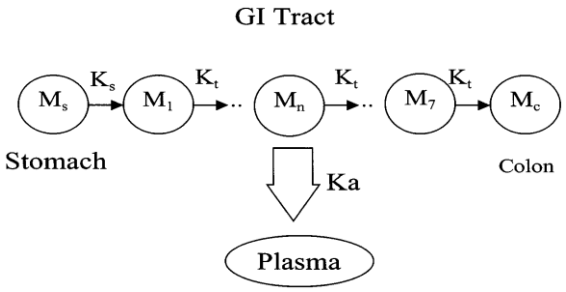

Figure 1.12: Schematic diagram of the CAT model with linear transit and passive absorption kinetics (Yu, 1999).

Jamei *et al*. (2004) have collected measures of variability for each of the physiological parameters relevant to the CAT model and have assessed the impact of these on the outcome of the modelling. This study was carried out for drugs with a wide range of permeability characteristics. The contribution of transit time, permeability and the radius of small intestine to the changes in f_a were 19%, 72% and 3.4%, respectively. They concluded that although permeability was the most influential factor determining f_a, individual parameters such as transit time and the radius of the small intestine were also important. The results confirmed the assertion that inter- and intra-variability of the parameters should be considered in any predictive PB modelling studies particularly when less permeable drugs are investigated.

1.7.3.2 ACAT model

Agoram *et al*. (2001) developed the ACAT model based on the CAT model, to include the dissolution rate, the pH dependence of solubility, absorption in the stomach or colon, metabolism in the gut or liver, degradation in the lumen, changes in the absorption surface such as transporter densities, efflux proteins densities, and other regional factors within the intestinal tract. Similar to the CAT model, the basic assumption of the ACAT model is that drug passing through the small intestine will have an equal transit time in each of the seven compartments. Addition of compartments corresponding to the enterocytes and surrounding tissues instead of treating the luminal barrier as a thin wall, add more compatibly to the real condition in the GI tract. Furthermore, the ACAT model uses the concentration gradient across the apical and basolateral membranes to calculate the rate of drug transfer into and out of an enterocyte compartment for each GI tract lumen compartment, whereas the CAT model assumed drug transfer to be unidirectional – luman to central compartment.

This model includes linear transfer kinetics and nonlinear metabolism/transport kinetics, six states of drug component (unreleased, undissolved, dissolved, degraded, metabolised, and absorbed), nine compartments (stomach, seven segments of small intestine, and colon), and three states of excreted material (unreleased, undissolved, and dissolved). It takes into consideration physicochemical factors (pKa, solubility, particle size, particle density, and permeability), physiological factors (gastric emptying, intestinal transit rate, first-pass metabolism, and luminal transport), and dosage factors (dosage form and dose) in predicting oral drug absorption (Figure 1.13).

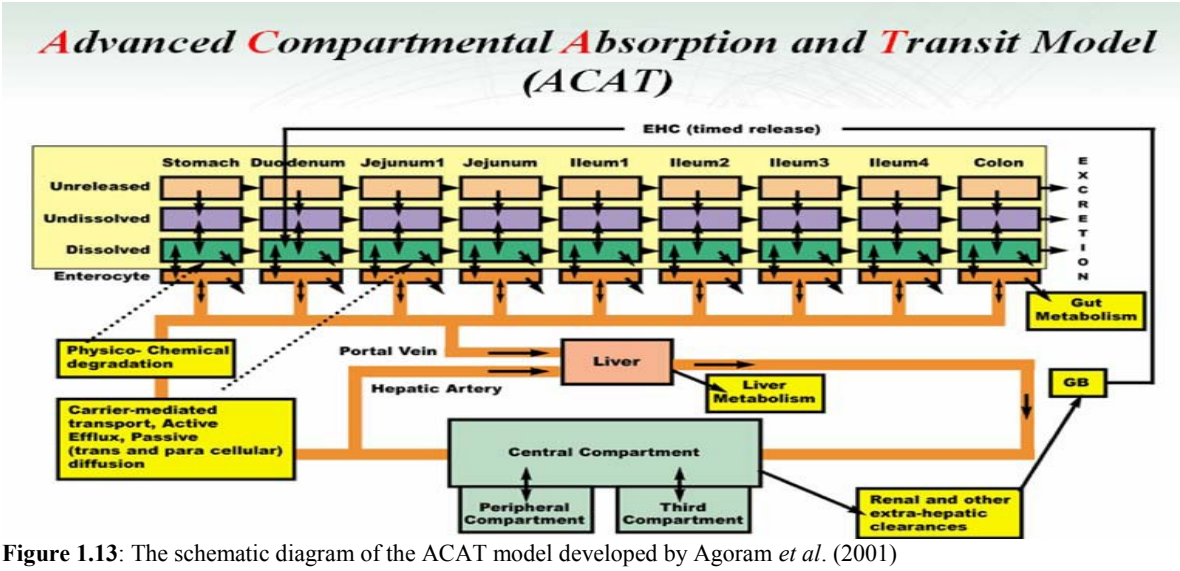

Figure 1.13: The schematic diagram of the ACAT model developed by Agoram *et al*. (2001)

The commercially available software, GastroPlus™, was developed based on the ACAT mode. This software has undergone several improvements with respect to its capability in predicting oral absorption of a variety of drugs in comparison to the original ACAT model. In addition to its use for predicting oral drug absorption in the GI tract, whole-body physiologically based pharmacokinetic and combined pharmacokinetic and pharmacodynamic models have been constructed within Gastroplus™ for predicting whole-body pharmacokinetic and pharmacodynamic characteristics in humans (De Buck *et al*., 2007; Tubic *et al.*, 2006).

The advantage of this software is that the combination of *in vitro*, *in vivo* or *in silco* parameters of the compound can be used to estimate drug performance. Moreover, investigation of different formulations (immediate release formulations, controlled release formulations or other forms of delayed release) based on the drugs physicochemical properties e.g., drug aqueous solubility–pH relationship, permeability, particle size distribution and formulation type can be carried out. Another important aspect of this software is that physiological parameters like GI transit time, pH, absorptive surface area, bile salt concentrations in different regions of the gut, pore size and density, compartment dimensions and fluid content are built into the model and can be modified to adjust to different population characteristics.

1.7.3.3 ADAM Model

Similar to the ACAT model, the ADAM model was developed based on the CAT model, and it is a compartmental transit model. It divides the GI tract into nine anatomically defined segments from the stomach through the intestine to the colon. Drug absorption from each segment is described as a function of release from the formulation, dissolution, precipitation, luminal degradation, permeability, metabolism, transport and transit from one segment to another. Furthermore, the ADAM model also considers the heterogeneity of the GI tract such as heterogeneous distribution of enterocytic blood flow and enzymes in the gut wall. Food effects such as the impact of changes in gastric emptying, splanchnic blood flow, and luminal pH are also taken into consideration and simulated. This model was incorporated in a software called SimCyp population based ADAM simulator

As in the previous model, some assumptions have been made. First, it is assumed that the absorption from the stomach is insignificant compared to that from the small intestine. Second, the movement of liquid and solid drug through each segment of the GI tract may be described by first order kinetics. Third, it is also assumed that drug metabolism in the colon is negligible.

One main advantage of the ADAM model as incorporated in the SimCyp simulator is that it is capable of capturing the likely inter-subject variability in oral bioavailability as it conditioned by age, sex, race, genetics and disease of the patient, and by the intake of food. Jamei *et al*. (2009b) investigated inter-subject variability in the bioavailability of four drugs covering a wide range of permeabilities. The predicted median values of f_a and their inter-subject variability were calculated and observed (mean) and predicted (mean and range) values were compared. While predicted and observed mean values were similar for the three most permeable compounds, there was a greater discrepancy for the drug which is the least permeable. This was associated with a greater predicted variability in the value of f_a of that drug. Therefore, they indicated that inconsistency between point predictions of f_a and observed values from small clinical studies may be expected to the extent that the latter may not capture the full extent of inter- and intra-subject variability.

SimCyp simulator has been developed enormously since it was first introduced. It includes the population mean and inter subject variability of regional luminal pH and bile salt concentrations in the fasted and fed states and enables to assess the solubility and dissolution rate via a bile micelle solubilisation and a diffusion layer (Jamei et al., 2009a). Different populations with different physiology that might affect the drug performance *in vivo* were incorporated as part of the simulator, for example, obesity and renal impairment disease, paediatric, Japanese and Chinese ethnic populations. The interplay of basal luminal fluid, additional fluid taken with dose, biological fluid secretion rates, and fluid absorption rate in the fasted or fed state were modelled in time-dependent fluid volume dynamics model. Gastric emptying, intestinal transit times and their inter subject variability are incorporated for the fasted and fed states. Gut wall passive and active permeability ($P_{eff,\ man}$) can be predicted from *in vitro* permeability measurements (Caco-2, etc.) or using QSAR-type models; regional P_{eff} differences can be specified. SimCyp can also be used to establish physiologically based (PB) *in vitro–in vivo correlations* (PB-IVIVCs).

Thesis overview

The work presented in this thesis explored different methods to estimate f_a and inter-subject variability in humans. It has been proposed to use the well stirred model to estimate absorption using population pharmacokinetics approach (Chapter 2). In addition, different *in vitro* (Chapter 3) and *in vivo* (Chapter 4) techniques were utilised in this research to identify the key causes for high inter-subject variability in oral drug absorption.

In Chapter 2, an attempt is made to establish a data set of f_a and inter-subject variability of different compounds. Calculations of f_a for individuals were carried out based on data from clinical trials with radiolabeled compounds in plasma and urine, clinical trials with urine data alone, as well as calculations based on the well stirred model. Furthermore, correlation analysis of physicochemical properties of the compounds was carried out to identify the key causes of low and erratic absorption. In the second part, phase I clinical trials of four compounds from the AstraZeneca database were utilised to estimate absorption (f_a*f_g) and inter-subject variability. The well stirred model was implemented in the population pharmacokinetic approach (NONMEM software) to identify the rate-limiting step in oral drug absorption, and to estimate the food effect on absorption.

Chapter 3 focuses on identifying the key causes for high inter-subject variability of two model drugs: dipyridamole and furosemide. (AstraZeneca compounds were not available for further investigation *in vitro*, therefore drugs with high reported inter-subject variability were identified.) Compounds were investigated *in vitro* for solubility, dissolution and permeation, the primary processes governing oral drug absorption. In the first section, the regional solubility of the two drugs was tested in pooled gastric and jejunum aspirated fluids from healthy volunteers. In addition, the solubility was measured in ileostomy fluids from 10 individual UC subjects. The ileostomy fluids were characterised with respect to pH, buffer capacity, osmolality and surface tension. Correlation analysis with solubility measurements was then carried out to investigate the underlying causes for variability in solubility. Simulated intestinal fluids were used to investigate the effect of bile salt on the drug solubility. In the second part, the effect of bile salt and pH on the dissolution of these drugs was investigated and finally the dissolution and permeation were investigated simultaneously using the dissolution/permeation system developed by Kataoka *et al.* (2012).

In Chapter 4, different formulation approaches (solid dispersion, self-emulsifying drug delivery system and nano particles), for increasing drug solubility/dissolution and hence absorption, were compared. The formulation performances were evaluated *in vitro* and *in vivo* in rat models to establish '*in vivo in vitro* correlation' (*IVIVC*).

Chapter 2 - Estimation of Oral Drug Absorption and Inter-Subject Variability in Humans

2.1 Chapter Overview

A preliminary account of the extent of oral absorption (f_a) and inter-subject variability instead of the overall bioavailability is required to investigate the absorption process. It is challenging to calculate f_a due to the lack of published pharmacokinetic data from humans in the literature. Therefore, in some studies, manipulations have been made in order to assess f_a. Zhao *et al.* (2001) chose the f_a data for modelling based on one of the following methods: 1) f_a was obtained from bioavailability values after oral administration. If the bioavailability was low, f_a should be equal to or higher than the values of bioavailability 2) f_a was obtained from cumulative urinary excretion of drug-related material following oral administration. If the urinary excretion was low (<80%) and it could not be proved that urinary excretion of the absorbed drug was the main route or nearly all the drug was recovered in urine and faeces, f_a should be equal to or higher than the percentage of urinary excretion of the drug. 3) Intravenous administration showed that nearly the entire drug was excreted in urine or that excretion in bile was small; however, the drug was not completely recovered in urine and faeces. Thus, f_a should be between the percentage of excretion in urine and faeces (100% excreted in faeces).

In another study, Takano *et al*. (2006) used the relative bioavailability of solid dosage form and a solution orally administered in the fasted state. Assuming linear kinetics of drug metabolism in both administrations, lipophilic drugs administered as a solution could be completely absorbed due to their high permeability. Therefore, the relative bioavailability $_{solid/solution}$ is almost equal to $f_{a\ solid}$ in the fasted state. Another manipulation for calculating f_a is the use of relative bioavailability of solid dosage form orally administered in the fasted and fed states. The greater concentration of bile salts and lecithin in the fed state can enhance the solubility of lipophilic drugs. Therefore, the relative bioavailability $_{fasted/fed}$ of these drugs can be regarded roughly as the f_a of a solid dosage form in the fasted state, assuming high permeability to the intestinal wall and linear kinetics metabolism. Moreover, Jamei *et al*. (2009a) have indicated that estimating f_g and f_a from ordinary clinical data is not possible and many reports in the literature erroneously refer to the composite function of f_g*f_a as if it represents only f_a.

After reviewing the literature, with the knowledge gained, up to date, it seems that there is no reliable representative dataset of f_a and its associated inter-subject variability. A dataset that includes information on f_a inter-subject variability will allow scientists to investigate the mechanism behind the variability of f_a by correlating variability to different drug properties. In the first section of this chapter, data for f_a estimation were collected to establish reliable dataset of f_a based on published clinical trials in healthy subjects. Where possible, individual's f_a values were calculated and a correlation analysis was carried out between f_a, inter-subjects variability and the drug physicochemical properties (permeability and solubility properties). In this research, due to the lack of ability to estimate f_g with the available data herein (plasma concentration vs. time), f_g is assumed to be equal to 1. Therefore, whenever the concept absorption is mentioned, it refers to the fraction absorbed and the fraction that escapes gut wall metabolism (f_a*f_g).

When describing inter-subject variability, it is desirable to include as many subjects as possible to identify possible trends and variations in population. Population pharmacokinetics (as implemented in NONMEM) is a useful and readily available tool to analyse pharmacokinetics of datasets. NONMEM is usually used to estimate pharmacokinetics of drugs from clinical data by compartmental analysis. Most of the focus is on drug bioavailability and not f_a. In the second section of this chapter, the well stirred model was incorporated in NONMEM software to estimate f_a*f_g and gain a better understanding on its associated inter-subject variability using the dataset of healthy subjects from phase 1 clinical studies of different formulations.

2.2 Estimation of f_a and Inter-Subject Variability from Published Clinical Trial Data

2.2.1 Introduction

2.2.1.1 Estimation of f_a from *In Vivo* Data in Humans

There are few methods to estimate f_a from *in vivo* plasma concentration vs. time profile in humans. First, a mass balance study of radio-labelled compounds where in this method, intravenous (IV) and oral labelled of a similar dose are given and the f_a is estimated by an accurate determination of the ratio AUC in plasma of the total radio-labelling compound administration. However, radio-labelled studies are not routinely carried out. Another non-invasive method is by determination of the percentage of the parent compound if excreted intact (or metabolites) in urine following oral and IV administration of similar doses (Equation 2.1). This model assumes a significant fraction of the dose must be excreted in urine for this method to be accurate and the metabolism profile in the oral dose should be similar to the IV administration.

$$f_a = \left\{\frac{\sum Excr_{(oral)}}{\sum Excr_{(iv)}}\right\} urine\ data$$

Equation 2.1: Fraction absorbed obtained from urinary data

The well stirred model of hepatic drug clearance was established by Rowland *et al*. (1973) and Wilkinson and Shand (1975). This model allows calculating the hepatic drug clearance based on whole blood drug concentration as a function of hepatic blood flow, the free fraction of drug in the blood and the intrinsic metabolic clearance in the liver based on unbound drug concentration. This model treats the liver as a single well stirred compartment and all the three aqueous spaces within the liver (blood, interstitial space and intracellular space) are well mixed; in addition the distribution equilibrium is achieved so rapidly that the drug in the emergent venous blood is in equilibrium with that in the liver. That means that drug distribution into the liver is perfusion-limited with no diffusion delay and that no active transport systems are involved (Pond and Tozer, 1984).

The total bioavailability is a product of an orally administered drug that is metabolised in the GI tract, the intestinal wall and the liver (Equation 2.2).

$$F_{oral} = (1 - Eh) * f_a * f_g$$

Equation 2.2: Oral bioavailability adapted from Pond and Tozer (1984)

E_h is the hepatic extraction ratio, i.e. the fraction of drug entering the liver that is eliminated by the organ. The hepatic extraction ratio depends on blood flow (Q_h), protein binding (f_u) blood to plasma ration ($C_{b/p}$) and clearance intrinsic. Correction for blood to plasma concentration was made based on the publication from Yang *et al*. (2007). Further on, correction for renal elimination is considered for compounds which were eliminated intact in urine. Whether or not a drug undergoes extensive first pass, elimination can be anticipated from plasma data when the following parameters are known: the ratio of blood to plasma concentration ($C_{b/p}$); plasma clearance (Cl_p); the fraction of the drug in the body that was excreted unchanged in the urine (f_e) and liver blood flow (Q_h).Therefore, by taking all these parameters to one equation, f_a can be estimated based on the Equation 2.3.

$$F = f_a * f_g * (1 - \frac{(1-f_e)*Cl_p}{Q_h * \frac{C_B}{C_P}})$$

Equation 2.3: Calculation of F based on the well stirred model by Yang *et al*. (2007)

2.2.2 Objectives

- To calculate f_a based on human clinical trials published in the literature.
- To establish dataset of f_a and its inter-subject variability from the scientific literature.
- To identify the factors causing high inter-subject variability using correlation analysis to the compounds physicochemical properties.

2.2.3 Methods

2.2.3.1 Estimation of f_a in Humans from Pharmacokinetic Data

In this study, three methods which were described in the introduction were utilised to estimate f_a from human clinical trial studies:

1) Radio-labelled compounds in plasma or urine after IV and oral administration.

2) Urine excretion data following oral and IV administration (Equation 2.1).

3) The well stirred model (Equation 2.3).

Bioavailability data presented in Table 2.1 were calculated based on AUC ratio of IV and oral administration (reported in the publications). Data for all other parameters were collected from different clinical trials; E_h and f_a were calculated for each subject.

2.2.3.2 Critical Review of Clinical Trials

The scientific literature related to clinical trials in humans was examined using the following search engines: Web of knowledge, PubMed, ScienceDirect, Wiley InterScience, SpringerLink and Google Scholar. The key words used were either/or a combination of the following with the drug name: absolute bioavailability, bioavailability, pharmacokinetics parameters, intravenous and oral administration, radiolabelled data, blood to plasma concentration and hepatic extraction ratio.

More than 400 clinical trials were reviewed, and the criteria used for screening:

1. The study was conducted in normal volunteers or in patients with normal kidney and liver function.
2. Mean absolute bioavailability (calculated from AUC) was provided.
3. In the case where few articles were found for the same compounds, the lowest dose given was chosen (to eliminate transporters saturation process).
4. In the absence of IV data in a radiolabel test, the minimum extent of absorption was estimated given that sufficient sampling time was allowed.
5. Where faeces data were not available, the pattern of parent drug and metabolite after IV and oral administration was compared; indicating absorption from the GI tract is complete.
6. Individual's values of plasma clearance, absolute bioavailability based on AUC calculation were presented.

7. Since most of the published clinical trials do not include data regarding the blood to plasma concentration ratio, a separate search for these values was conducted and the mean value was used.
8. The mean value of 1.5 L/h of hepatic flow was included (based on the publication from Bradley *et al*. (1945)).

In addition, the physicochemical properties of the compounds (molecular weight, HBD, PSA, measured logP and intrinsic solubility) were adopted from the publication by Benet *et al*. (2008).

2.2.3.3 Describing Variability

One measure of variability is variance. It is defined as the sum of the squares of the deviations of observations; variance does not allow a ready comparison of variability across sets of observations of different magnitude, or of different dimensions. The coefficient of variation, the square root of variance (the standard deviation) normalized to the mean, overcomes this problem. The terms high and low variability refer to distributions that have high and low coefficients of variation, respectively. Typically, a coefficient of variation of a pharmacokinetic parameter of 10% or less is considered low, 25% is moderate, and above 40% is high (Rowland and Tozer, 2011).

Calculations for the coefficient of variation for bioavailability, hepatic excretion ratio and fraction absorbed were carried out according to Equation 2.4 using Excel:

$$CV = \frac{Standard\ Deviation}{Mean} * 100$$

Equation 2.4: Coefficient of variation.

2.2.4 Results & Discussion

2.2.4.1 f_a Estimations from Published Clinical Trials in Healthy Subjects

In this investigation, more than 400 published clinical trials for different drugs were reviewed. However, only 22 articles contained the required information to estimate f_a based on the well stirred model (Table 2.1-calculations were made based on the well stirred model). Most of the clinical trials which were reviewed did not include IV administration or individual values of plasma clearance or bioavailability. Moreover, parameters such as the blood to plasma concentration ratio or individuals liver blood flow are not routinely measured and reported. Another 20 publications were utilised to estimate f_a based on the measurements of radio-labelled compounds in plasma/urine or based on the assumption that the compound is mainly eliminated in urine (Table 2.2- calculations were made based on methods 1 and 2 described in Section 2.2.3.1).

In this investigation, some of the individuals f_a calculations based on the well stirred model were not realistic (more than 100% or negative values were attained-marked as (*) in Table 2.1). Possible explanation to the discrepancy in the calculations is the use of mean values of hepatic liver blood flow and blood to plasma concentration ratio. As individuals liver blood flow is not measured routinely in clinical trials, the mean value of 1500 mL/min was chosen based on the publication from Bradley *et al*. (1945). Liver blood flow values can be diverse due to population variability (Price *et al*., 2003) and affect liver elimination. Therefore, individuals calculations based on the mean liver blood flow might yield misleading results. Moreover, the use of mean $C_{b/p}$ values might introduce another error in the individuals' f_a estimations. $C_{b/p}$ can vary from 0.5-2 for a drug like molecule, in addition blood to plasma concentration ratio is usually measured *in vitro* by spiking the drug in blood samples and not always blood samples are directly taken from the individuals who participate in the study. The error is expected to be more significant for drugs with high hepatic clearance. A simulation made by Yang *et al*. (2007) revealed that the error in f_h is likely within +0.2 units (for $C_{b/p}$ span from 0.7-2), provided that the hepatic clearance values are not greater than 25% of the liver blood flow.

When omeprazole f_a was calculated based on the well stirred model for each individual, a value of more than 100% was obtained for some individuals. However, when only the mean value was considered in all parameters, it gave 100% absorption which fits the radio-labelled

data reported in the same publication. Similar results were obtained for felodopine (mean f_a=85%). Another example is the labetalol blood/plasma ratio where mean value was reported as 1.36, and ranges between 1.05 to 1.62 (Lalonde *et al.*, 1990). There is no doubt that this range can significantly affect the individual's calculations.

Other explanations for the discrepancy in the calculation; first, an assumption of negligible gut wall metabolism was made to simplify the calculation due to scarcely published f_g data. It might be that the assumption of negligible f_g is not valid for some compounds in this investigation and therefore introduces another error in f_a estimation. Second, Nomier *et al*. (2008) describe an approach utilizing oral/intravenous pharmacokinetic data to estimate oral absorption from animal studies. The author suggested that compounds with E_h higher than 0.5 and low F, might suffer from low success of prediction due to the impact of experimental variability which might be the case herein. Third, another assumption of the well stirred model is that the pharmacokinetics is within the linear range for both PO and IV doses. However, it is likely that some drugs exhibit nonlinear pharmacokinetics. It was found that many of the highly hepatic extracted drugs show dose dependent and time dependent bioavailability. The nonlinearity is most probably due to saturation of metabolism at the higher plasma concentration (Pond and Tozer, 1984).

Table 2.1: Bioavailability, $f_a f_g$ and hepatic excretion ratio (calculated from PK data) and inter-subject variability (CV%). ((*)values exceeding 100% absorption or negative values)

	Compound	Number of participants / Doses(IV/Oral)	F (%)	CV (%)	E_h (%)	CV (%)	$f_a f_g$ (%)	CV (%)
1	Ciclosporine (Hebert *et al*., 1992)	N=6 IV=3mg/kg Oral=10 mg/kg	25.0	38.1	23.2	16.1	32.8	39.8
	Ciclosporine (Gomez *et al.*, 1995)	N=5 IV=2 mg/kg Oral=8 mg/kg	22.4	21.3	33.7	26.1	34.7	28.7
2	Diltiazem (Kolle *et al*., 1983)	N=6 IV=20 mg tablet= 120mg	32.6	30.8	53.1	15.3	72.3	35.3
	Diltiazem (Kolle *et al*., 1983)	N=6 IV= 20mg Solution=120mg	43.8	22.3	53.0	15.3	83.8	19.6
3	Omeprazole (Regårdh *et al*., 1990)	N=9 IV=10 mg/kg Oral=20 mg/kg	53.6	61.5	60.9	54.7	259.3* 105 (mean value)	97.8*
4	Felodipine (Edgar *et al*., 1985)	N=8 IV=2.5 mg/kg Oral=27.5 mg/kg	16.2	37.5	80.7	18.9	57.5* 85 (mean value	200.1*
5	Verapamil (Freedman *et al.*, 1981)	N=6 IV=15 mg Oral=80 mg	23.7	42.8	61.1	13.0	60.6	47.5
6	Flumazenil (Janssen *et al*., 1989)	N=8 IV=2 mg Oral=30 mg	27.8	21.5	74.6	6.7	103.5	1.2
7	Valproic acid (Perucca *et al.*, 1978)	N=8 IV=800 mg Oral=800 mg	99.5	9.8	3.0	11.6	102.6	9.9
8	Labetalol (McNeil *et al*., 1979)	N=6 IV=100 mg Oral=100 mg	38.3	67.9	59.4	35.3	160.5*	124.8*
9	Methadone (Meresaar *et al*., 1981)	N=8 IV=10 mg Oral=10 mg	79.1	26.6	12.9	82.1	92.3	28.6
10	Fluvastatin (Lindahl *et al.*, 1996)	N=9 IV=2 mg Oral=70 mg	32.6	32.9	69.3	2.7	92	25.1
11	Prazosin (Grannen *et al.*, 1981)	N=4 IV=0.5mg/kg Oral=0.5 mg/kg	63.1	20.8	13.3	35.1	73.2	24.4
12	Erythromycin (Somogyi *et al.*, 1995)	N=4 IV=250 mg Oral=300 mg	32.0	23.9	12.9	36.2	36.6	21.5
13	Zidovudine (Klecker *et al.*, 1987)	N=9 IV=120 mg Oral=200 mg	63.4	20.1	100.1	21.3	347.0*	165.4*
14	Nitrofurantoin (Hoener and Patterson, 1981)	N=6 IV=50 mg Oral=50 mg	86.3	14.5	18.8	23.9	106	13.8
15	Haloperidol (Cheng *et al*., 1987)	N=6 IV=1 mg Oral=5 mg	60.0	29.5	35.5	26.2	95.9	36.0

	Compound	Number of participants / Doses(IV/Oral)	F (%)	CV (%)	E_h (%)	CV (%)	$f_a f_g$ (%)	CV (%)
16	Amitriptyline (Schulz *et al.*, 1983)	N=7 IV=40mg Oral=50mg	47.7	22.9	42.9	22.3	83.2	13.1
17	Dipyridamole (Mahony *et al.*, 1982)	N=4 IV=20 mg Oral=50 mg	42.7	30.7	9.3	40.4	47.2	30.8
18	Midazolam (Allonen *et al.*, 1981)	N=6 IV=0.075 mg/kg Oral=15 mg	44.0	38.8	33	15.3	67	42.2
19	Methapyrilene (Calandre *et al*., 1981)	N=6 IV=25 mg Oral=50 mg	14.1	69.4	125.4	43.1	-118.2*	-266*
20	Flunisolide	N=12 IV=1 mg Oral =1 mg	20.1	93.8	68.5	15.6	53.5	49.8
21	Nifedipine (Kleinbloesem *et al.,* 1984)	N=6 IV=0.075 mg/kg Oral=20 mg	51.3	24	29.5	16.2	72.8	24
	Nifedipine (Kleinbloesem *et al.*, 1984)	N=6 IV=0.075 mg/kg Oral=20 mg	51.3	24	29.5	16.2	73.3	35.1
	Nifedipine (Kleinbloesem *et al.*, 1986)	N=7 IV=4.5 mg Oral=20 mg	51.1	33.50	39.12	23.7	66.8	33.5
22	Quinidine Sulphate (Greenblatt *et al*., 1977)	N=7 IV=150 mg Oral=200 mg	79.42	18.80	13.55	31.05	92.53	13.3

Table 2.2: f_a and inter-subject variability (calculated from urine and radio-labelled data)

	Compound	Number of participants / Doses(IV/Oral)	f_a (%)	CV (%)
1	Hydrochlorothiazide (Beermann and Groschinsky-Grind, 1977)	N=7 Oral=25-75 mg	67.73	23.25
2	Venlafaxine (Howell *et al*., 1993)	N=11 Oral=50 mg	92.1	8.79
3	Iothalamate (Prueksaritanont and Chiou, 1987)	N=4 Oral=800 mg	1.09	45.87
4	Ganciclovir (Jacobson *et al.*, 1987)	N=4 Oral=20 mg/kg	3	30.1
5	Practolol (Bodem and Chidsey, 1973)	N=7 Oral=600 mg	95	3.16
6	Phenoxymethylpenicillin (Hellstrom *et al.,* 1974)	N=10 Oral solution=22 mg	49	20.41
7	Vigabatrin (Durham *et al.*, 1993)	N=6 Oral=1500 mg	95.4	19.60
8	Granisetron (Clarke *et al*., 1994)	N=3 Oral=200 mg	97.3	0.92
9	Ketorolac (Mroszczak *et al*., 1987)	N=7 IV=1.7 mg/kg Oral=1.7 mg/kg	99.4	4.12
10	Nitrendipine (Mikus *et al*., 1987)	N=7 IV=2 mg Oral=20 mg	88.38	18.14
11	Felodipine (Edgar *et al*., 1985)	N=8 IV=2.5mg/kg Oral=27.5mg/kg	89.07	4.87
12	Terbutaline (Borgstrom *et al*., 1989)	N=7 IV=0.25 mg Oral=5 mg	73.00	11.10
13	Bretylium (Anderson *et al.*, 1980)	N=10 IV=100 mg Oral=400 mg	25.19	40.39
14	Mercaptoethanesulfonic acid (James *et al*., 1987)	N=8 Oral=800 mg	76.05	21.20
15	Promethazine (Taylor *et al.*, 1983)	N=7 IV=12.5 mg Oral=25 mg	83.00	37.92
16	Salbutamol (Goldstein *et al., 1987)*	N=7 IV=0.4 mg Oral=4 mg	96.67	13.69
17	Mebendazole (Dawson *et al.*, 1985)	N=5 Oral=100 mg	100.93	9.01
18	Metoprolol (Regårdh *et al.*, 1974)	N=5 Oral=5mg	96	1.56
19	Gabapentin (Gidal *et al.*, 2000)	N=50 Oral=600 mg	49.3	27.59
20	Dihydroergotamine (Bobik et al., 1981)	N=6 IV=0.01 mg/kg Oral=0.6 mg/kg	96.38	2.99

2.2.4.2 f_a and Inter-Subject Variability

After excluding negative or exceeding 100% absorption values (marked (*) in Table 2.1), f_a values of 38 drugs were correlated against the inter-subject variability (CV%). The correlation of R^2 =0.41 was obtained (Figure 2.1). The higher correlation (R^2 = 0.63) was obtained when only f_a estimated based on the radiolabel/urine data were included in the analysis (Figure 2.2). Bioavailability was plotted against inter-subject variability in bioavailability for this dataset and a correlation of 0.39 was found (Figure 2.3). For the drugs where f_a was calculated based on the well stirred model, hepatic excretion ratio (E_h) was correlated to the inter-subject variability in bioavailability (Figure 2.4), the R^2 was 0.15. The lack of correlation may indicate that for these compounds the factor which contributes to inter-subject variability in bioavailability is not only the elimination (first pass effect) but also the absorption process. It is important to note that since an individual's plasma clearance was normalised by mean liver blood flow and mean blood to plasma ratio, some aspects of inter-subjects variability in elimination (i.e. changes between individuals liver blood flow) are not considered in this calculation. Therefore, some of the inter-subject variability shown herein might be still related to elimination. In general, it is possible to conclude that there is a general trend where low absorption can be associated with high inter-subjects variability in absorption considering the small dataset sample size and the limitations in this study.

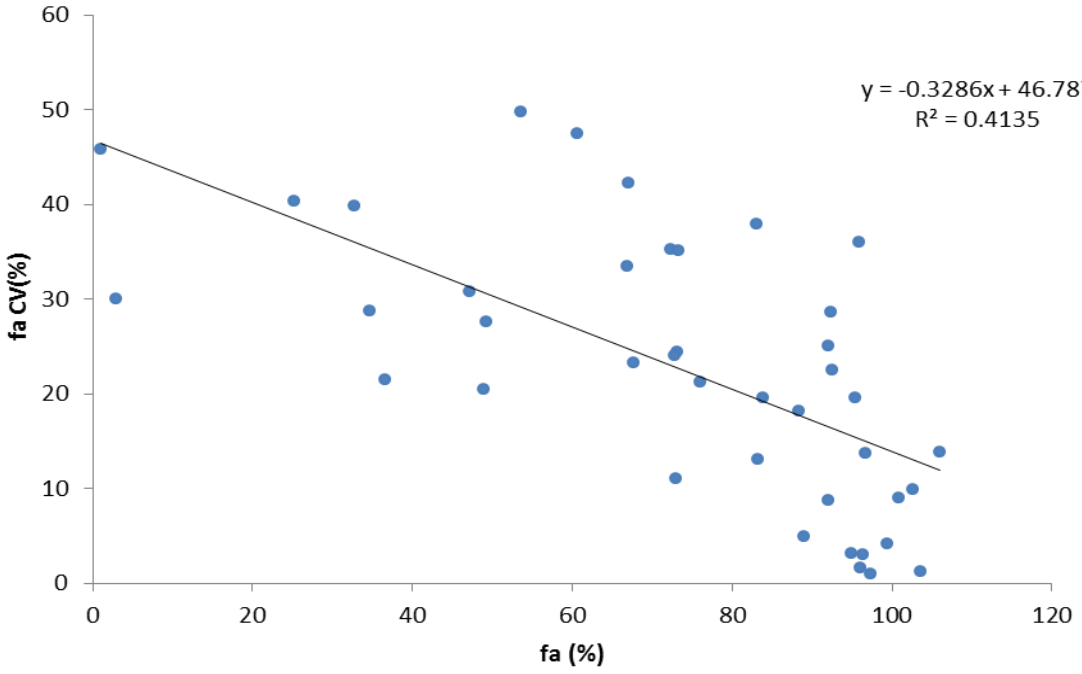

Figure 2.1: f_a vs. inter-subject variability (CV %)

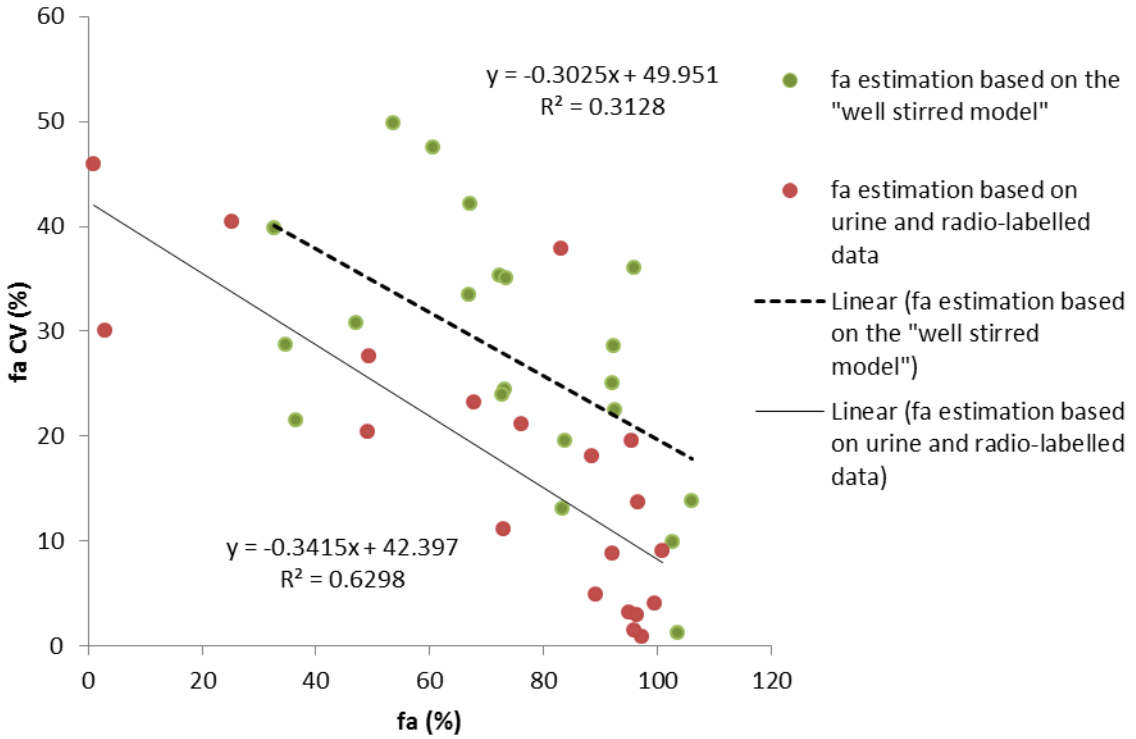

Figure 2.2: f_a vs. inter-subject variability (CV %) showed as two dataset based on different estimations methods of f_a.

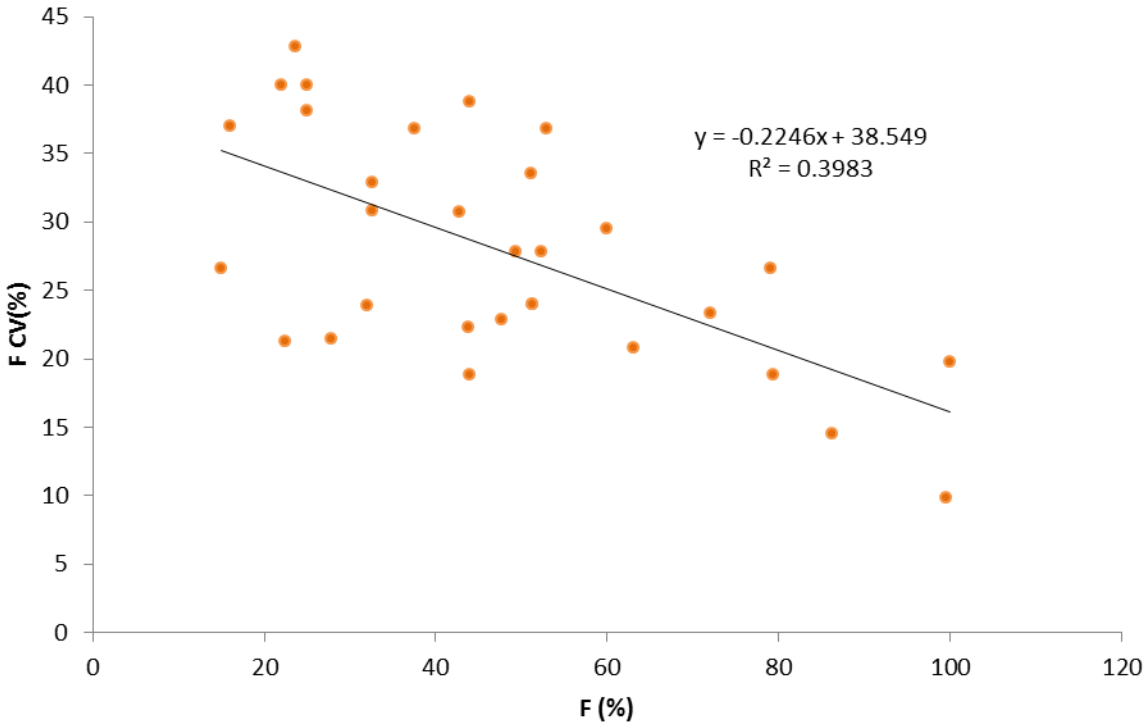

Figure 2.3: Bioavailability vs. inter-subject variability in bioavailability (CV %).

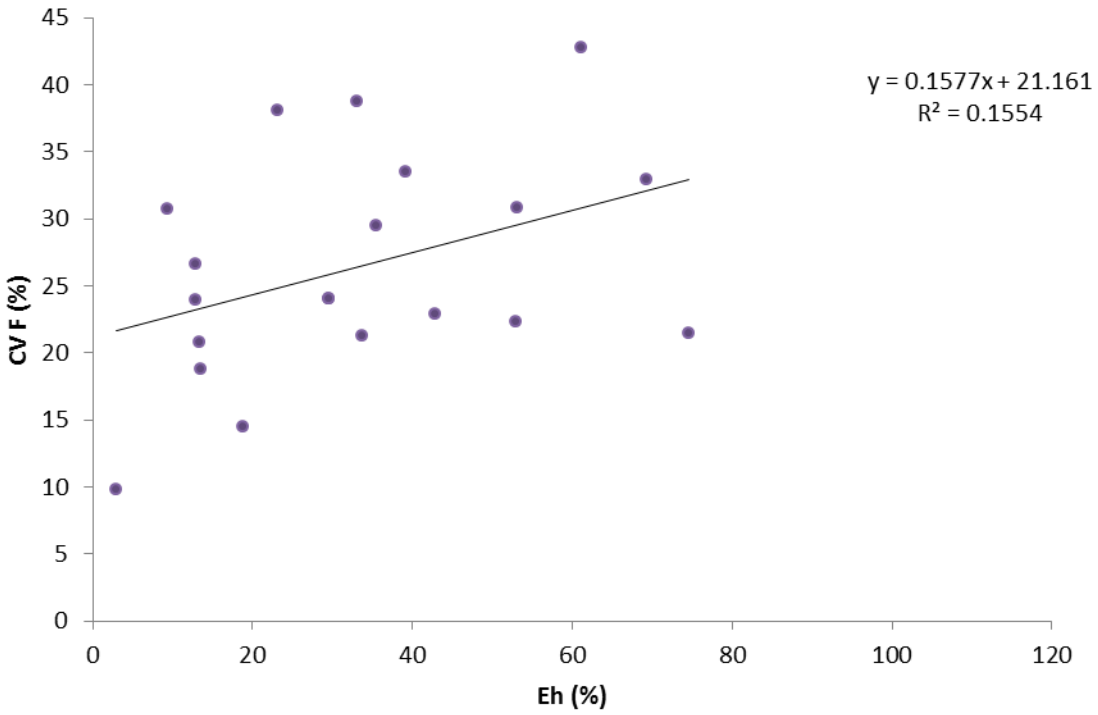

Figure 2.4: Hepatic excretion ratio vs. inter-subject variability in bioavailability (CV%)

2.2.4.3 Correlation of Physicochemical Properties to f_a and Inter-Subject Variability

It has been recognized that drug solubility/dissolution and gastrointestinal permeability are the fundamental parameters controlling the rate and extent of drug absorption (Amidon *et al*., 1995). To identify the factors that might affect absorption and inter-subject variability in this dataset, a correlation analysis was carried out between different physicochemical properties, f_a and inter-subject variability. The intrinsic solubility in water at 37˚C was utilised as a measure for solubility for the convenient comparison of all compounds, since measurements in simulated fluid media are limited and pH might also have an effect on weak acid/base solubility. Polar surface area (PSA- square Angstroms) and a count of the number of hydrogen bond donors (HBD) present in a compound molecule are used to describe passive human P_{eff} (Winiwarter *et al*., 2003). Moreover, when no active transport processes are involved, it is expected that lipid/water partition coefficients will correlate with drug permeability. Further on, Takagi *et al*. (2006) evaluated the correlation of measured LogP and calculated LogP with human jejunal permeability, and showed a correlation in two thirds of the time. Since it is difficult to compare permeability results from different published experiments and *in vivo* human jejunal permeability data is limited, PSA, HBD and measured LogP were chosen as an indication for permeability.

When plotting the intrinsic solubility and the measured LogP against f_a or f_a inter-subject variability, no correlation was observed (Figure 2.5). It might be related to the fact that these physicochemical factors might not reflect loyalty to the situation *in vivo*. As mentioned measured LogP tends to describe better the passive diffusion permeation and the intrinsic solubility is a simplification of the real solubility *in vivo* which depends on pH and bile salts composition among other factors that affect solubility in the GI tract. However, when f_a was plotted against HBD and PSA, a relative trend was observed associated with high absorption and low HBD and PSA properties.

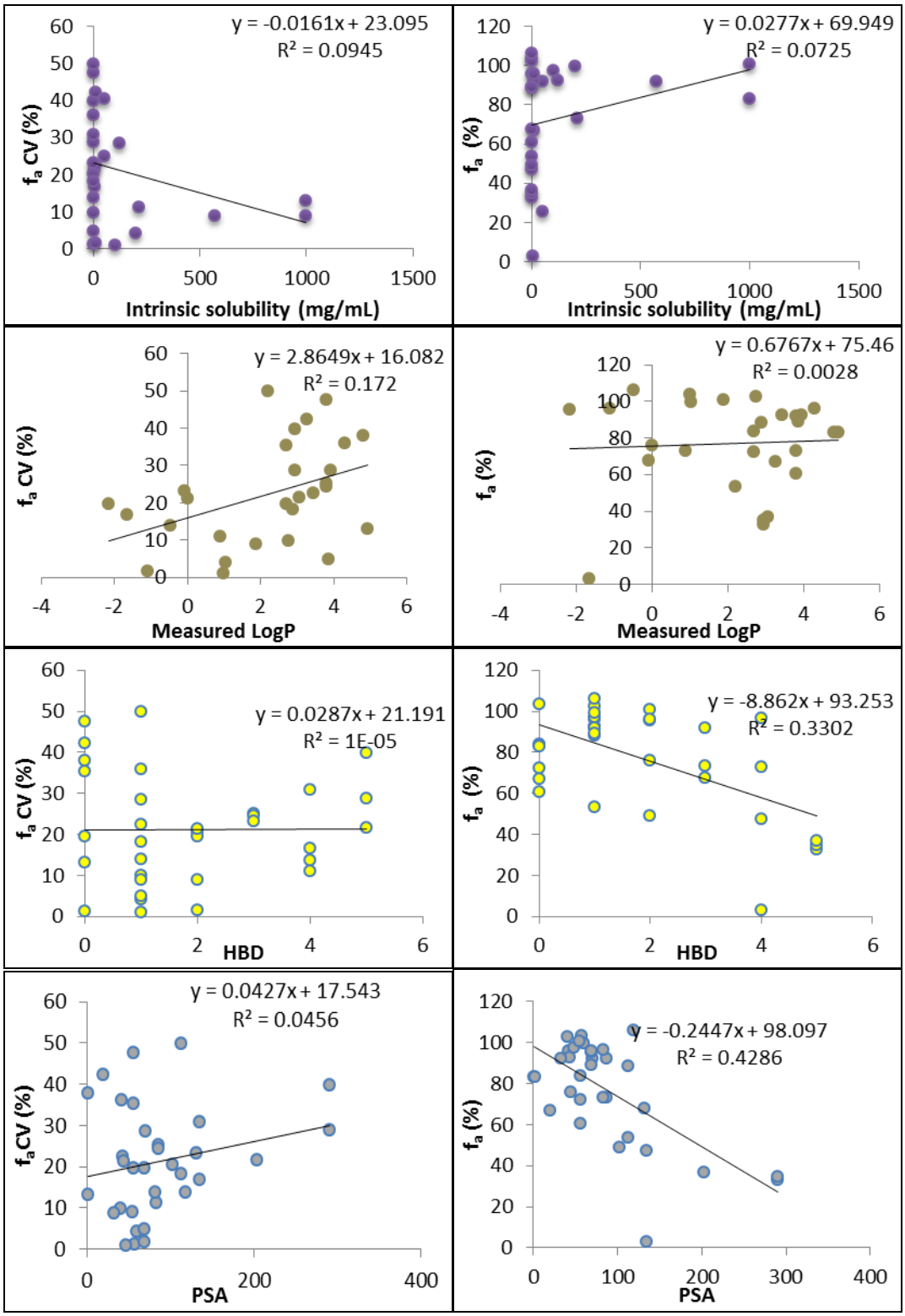

Figure 2.5: Correlations analysis between the inter-subject variability (CV%) of f_a, f_a and different physicochemical properties

f_a inter-subject variability was plotted against the compounds BCS classification (Figure 2.6). Although one would expect that the drugs classified as BCS I will present lower inter-subject variability compared to compounds classified as BCS II, III, or IV, similar inter-subject variability was observed among all classifications. A possible explanation to the high variability found for some BCS I compounds might be related to the fact that the BCS classification definition is based on the extent of permeability and does not take into consideration the rate of permeation through the membrane which might be important even for some highly permeable compounds. A more statistical explanation might be related to the number of subjects that were included in the analysis. In order to understand variability, a large number of subjects are required to identify trends. This study's limitations included relatively low number of subjects in each clinical trial (4-10 subjects) and a limited number of compounds. Therefore, the ability to observe any trend is limited and any conclusion from this research needs to be considered in the light of these limitations.

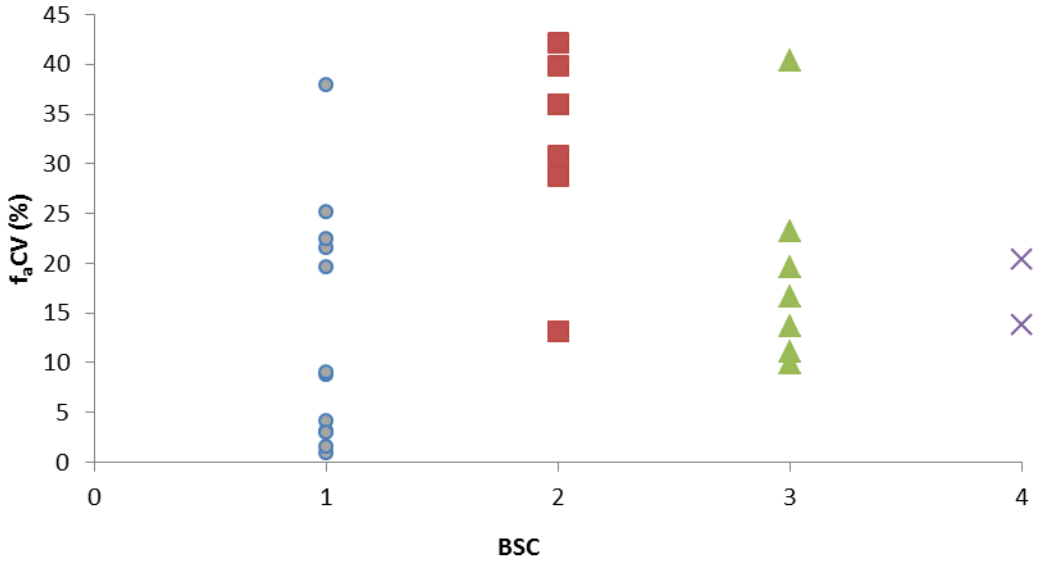

Figure 2.6: BCS classification vs. inter-subject variability in f_a

2.2.5 Summary

Estimation of f_a and inter-subject variability were carried out based on acceptable methods from published clinical trials in healthy subjects. However, it can be concluded that due to scarce published data, it is challenging to estimate f_a from human *in vivo* data alone. Therefore, it is understandable why many scientists made assumptions to simplify f_a estimation. An attempt to identify the factors affecting low and erratic f_a was not successful and might be related to the quality of the chosen parameters to reflect the absorption process *in vivo*. The limitations in this study included; the use of mean values for some parameters to calculate individuals' f_a values, the low number of subjects involved in each clinical trial (4-10 subjects) and the low number of compounds in the analysis (40).

Based on the results found herein, it was decided to utilise larger dataset of subjects (clinical trials from phase 1 studies which included 30-50 subjects) to estimate f_a and its' inter-subject variability. A population pharmacokinetics approach (as implemented in NONMEM) was further employed.

2.3 Estimation of f_a and Inter-Subject Variability Using Population Pharmacokinetics

2.3.1 Introduction

2.3.1.1 Population Pharmacokinetics (popPK)

The idea of popPK models based on preclinical and available clinical data were extensively developed and implemented in the process of drug development. In the early stages of drug development, these models have been used as a significant tool in selecting promising compounds and identifying safe and effective doses and dose regimen. In later clinical phases of full development, mechanistic models have been proposed to characterize drug absorption, taking into account different processes in drug absorption. The description of the variability in drug absorption may become more important, for evaluation of safety and efficacy and differentiation between formulations performance (Miller *et al.*, 2005).

2.3.1.2 NONMEM

When analysing clinical pharmacokinetic data (drug plasma concentrations), it is common to use non-linear mixed-effect modelling, the so-called population approach. This led to the development of software tools, NONMEM (NONlinear Mixed Effects Modelling), with further applications among the pharmaceutical and clinical pharmacology communities (Beal and Sheiner, 1980). The advantage of this modelling approach is the improvement in underlying effects in drug performance which is important in understanding variability in population. The term population pharmacokinetics does not only refer to the mean value but also takes into consideration each individual and his contribution to the mean value. Therefore, this software can handle a large data set of subjects alongside with sparse data. Some of NONMEM applications are: analysis of PK data (either sparse PK data from early stages of drug development or extensively sampled PK data from phase I studies), investigation of pharmacokinetics- pharmacodynamics relationships, use of explanatory model-based analysis, for instance, analysis that estimate the quantitative relationship between inputs (e.g. drug dose and time, patient characteristics, stage of disease) and outcomes (e.g. biomarkers or observable clinical measures) according to some mechanistic views of the relationship (Pillai *et al*., 2005).

The process of finding the optimal model includes four major steps: model definition, model fit, model diagnostics and model evaluation. NONMEM is a tool for building mathematical model of this underlying process using several building blocks. The basic block is the structural model. An example of collected data includes the measurement of the plasma concentration over time. Inferences from the data are drawn and summarized in terms of estimated model parameters, such as drug clearance (CL). Another important component of the model is variability. Therefore, parameters of the model are treated as distributions, rather than single values. This is the second building block called "random effects (the measurements "noise"). In biological data, there are two sources of random variability which are quantified in mixed effect analysis: variability between different individuals – inter-individual variability (IIV) and residual variability (RV). Inter-individual variability is considered at the level of the model parameter and the residual variability is at the level of the observed data point and includes noise due to measurement error, erroneous data records, and changes in individual biology over time, or error due to model misspecification. Often, if the drug was studied on different study occasions, variability between these occasions may also be quantified (inter-occasion variability IOV).

The known, observable properties of individuals (covariates i.e. age, weight and etc...) that cause the descriptors to vary across the population are the fixed effects whereas the random effects cannot be predicted in advance. Modelling population pharmacokinetics as implemented in NONMEM allows scientists to recognize the sources of variability, such as inter-subject, intra-subject, and inter-occasion and it can be used to explain variability by identifying factors of demographic, pathophysiologic, environmental, or drug-related origin that may influence the pharmacokinetic behaviour of a drug. Moreover, it can quantitatively estimate the magnitude of the unexplained part of the variability in the patient population (Ette and Williams, 2004).

One of the more difficult tasks for a modeller is to find an appropriate structural description of drug absorption, as the population pharmacokinetic modelling approach should be executed while taking into account the physicochemical properties of a drug, the physiology of the subject and the variability of all the different mechanisms of absorption. The traditional models used to describe the absorption process are simple and include a parameter describing the absorption rate (first or zero order absorption rate constant), bioavailability and usually a lag time parameter characterizing any potential absorption delay. Some of the limitations in

developing an absorption model are the design and execution of studies that will allow precise characterization of drug absorption. Given the importance of characterizing absorption, more effort should be expended on developing these models.

Due to the limitations in estimating f_a and its inter-subject variability in the previous section, it has been decided to explore AstraZeneca (AZ) compounds database to identify different compounds with several phase 1 clinical studies to calculate f_a utilising the population pharmacokinetics approach. The population approach allows the determination of the magnitude of inter-subject (individuals) variability and can handle large numbers of subjects than presented in one single phase 1 study. The well-stirred model was implemented in NONMEM to estimate f_a*f_g (absorption) instead of bioavailability. The population pharmacokinetics of 4 AZ compounds with different reported bio-availabilities was tested by the simultaneous fitting of data from different drug formulations, including oral solution, immediate-release (IR) formulations, extended-release (ER) and prolonged release (PR) formulations.

The purpose of this investigation was to obtain an accurate estimation of f_a*f_g from plasma concentration vs. time data and therefore only parameters that characterise first pass and renal elimination were included in the model building (i.e. liver blood flow, blood to plasma ratio and renal clearance). Moreover, within this study, f_a*f_g was estimated without incorporating *in vitro* permeability data of gut wall metabolism; therefore, whenever the concept absorption is mentioned, it refers to the fraction absorbed and the fraction that escaped gut wall metabolism.

2.3.2 Objectives

- To develop a population pharmacokinetic model to estimate f_a*f_g and inter-subject variability (NONMEM).
- To estimate f_a*f_g of different compounds to understand formulation effect on absorption and inter-subject variability.
- To investigate the rate limiting step in absorption by comparison f_a*f_g from oral solution and solid dosage forms administrations.
- To estimate f_a*f_g to investigate food effect on absorption and inter-subject variability.

2.3.3 Methods

2.3.3.1 Data

Four AZ compounds with low (AZD7009), intermediate (AZD1305 and AZD0865) and high (AZD242) bioavailability were identified from the AstraZeneca compounds database. All datasets were phase 1 studies performed in healthy volunteers, conducted in accordance with the Declaration of Helsinki, which were compliant with the International Conference on Harmonisation (ICH)/Good Clinical Practice (GCP) and regulatory requirements, and also the AstraZeneca policy on Bioethics. Compound selection was based on availability of intravenous data and differing physicochemical and pharmacokinetic properties. Compound physicochemical properties and pharmacokinetic parameters based on non-compartmental analysis are specified in Table 2.3 and Table 2.4 (Data on files from AstraZeneca).

In addition to the intravenous data, clinical trials which investigated different formulations under different conditions were incorporated in the analysis and f_a*f_g was estimated for the oral solution and different formulation. The clinical trials, administered doses and formulations' description are detailed in Appendix 1.

Table 2.3: Compounds pharmacokinetics parameters based on the non-compartmental analysis (Data on files from AstraZeneca).

Properties	AZD0865	AZD242	AZD7009	AZD1305
Bioavailability (%) ± SD (CV%)	57.1±12.2 (22.2%)	103±4 (4.4%)	15.5±4 (25.8%)	32±10 (30%)
Plasma Clearance (L/h)	3.8	0.16	213	34-57
Renal clearance (L/h)	0.11	0.03	12	5.4
Distribution volume (L)	27	9	19.7	152-344
Blood to plasma ratio	0.625	0.7	1	0.93

Table 2.4: Compounds physicochemical properties Data on files from AstraZeneca).

	AZD0865	AZD242	AZD7009	AZD1305
MW (g/mol)	366.4	408.5	446.5	434.5
Solubility	Solubility in water (pH=8), FaSSIF (pH=6.5), SGF (pH=1.7) and human intestinal fluids (pH=6.9), are 0.003, 0.006, 3.05 and 0.005 mg/mL respectively. Salt solubility in water is approx. 0.35 mg/mL.	Ranged from 0.02 mg/ml at pH 2.5 to 86 mg/ml at pH 7.2.	0.2, 9 and 50 mg/mL in water (pH=9.8), phosphate buffer (pH= 7.7) and 0.1M HCl (pH=1.2) respectively.	3.1, 3.1 and 0.56 mg/mL at pH 9, 7 and 5, respectively
pka	6.1	3.7	9.7	9.9
Partition coefficient log K_D	4.2	2.8	3	2.5

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2.3.3.2 Model Building

Population pharmacokinetic model building was undertaken using NONMEM VII (V-12, Icon plc). Individuals' plasma concentration vs. time profiles, from different clinical trials of the same formulation were pooled to form a single dataset, with mass units expressed in nanomoles. The covariates available for all datasets were age, height, weight and body mass index (BMI). Raw plots of plasma drug concentration vs. time were generated using R software (Version R-3.1.1, available on http://www.r-project.org/) and inspected for possible trends in the structural models. Disposition of each compound was determined by modelling the intravenous data alone. One-, two-, three- and four-compartment models with linear intrinsic hepatic clearance (CLi) were tested. The IV data were analysed with first-order conditional estimation (FOCE) plus interaction (between inter-individual and residual variability). For the IV data analysis, the ADVAN7 TRANS1 subroutine in NONMEM was used. Once an adequate structural model was identified, the disposition parameters were then fixed, based on the assumption that the disposition parameters would remain unchanged when the oral dose is administered. Additional (e.g. oral) data from each formulation were pooled with the intravenous data, and the absorption model was developed.

For the f_a*f_g estimation the following equations were included in NONMEM coding (presented in Appendix 1).Within NONMEM hepatic clearance was calculated based on the intrinsic clearance. The following equations were included in NONMEM coding. Liver volume (LV) is associated to the subject weight as indicated by Price *et al*. (2003)):

$$LV = 0.05012 * WT^{0.78}$$

Equation 2.5: Liver volume based on publication from Price *et al*. (2003) normalised by weight

Liver blood flow (Q_h) in males in was reported as 50.4 L/h the blood/plasma ratio was used to take into account the total blood to total plasma drug concentration ratio (BPR) (Equation 2.6):

$$Qh = 50.4 * LV * BPR$$

Equation 2.6: Liver blood flow

Calculation of clearance hepatic was based on the intrinsic clearance (CLi), and liver blood flow (Equation 2.7)

$$Clh = \frac{Qh * Cli}{(Qh + Cli)}$$

Equation 2.7: Clearance hepatic calculation based on intrinsic clearance

Allometric weight scaling was added to renal clearance fixed effects *a priori*, standardized to a body weight of 70 kg according to the following relationships Equation 2.8 (Holford, 1996).

$$CL = CLh + CLr$$

$$Clr = Clr * (WT\backslash 70)^{0.75}$$

Equation 2.8: Renal clearance normalised by weight

The entrohepatic circulation (ER) was calculated based on the intrinsic hepatic clearance (CLi) and liver blood flow (FQ) (Equation 2.9) and was further utilised to estimate f_a*f_g based on bioavailability (F1) NONMEM estimation (Equation 2.10):

$$ER = \frac{CLi}{(FQ + CLi)}$$

Equation 2.9: Entrohepatic circulation

$$F1 = fa * fg * (1 - ER)$$

Equation 2.10: Calculation of f_a*f_g based on the well stirred model

For this NONMEM analysis, the ADVAN5 TRANS1 subroutine was used. Inter individual variability for different PK parameters was estimated using an exponential model (log normal model), except for the f_a*f_g, where a Logit transformation was used to ensure the individual estimate remained between 0 and 1 and to reduce potential numerical instability during computation. Inter individual variability was added in a step-wise fashion, firstly to clearance and volume parameters, and then to absorption parameters. Proportional and mixed additive models were tested for residual error.

A Lag-time or a discrete number of transit compartments were utilised in the absorption model, to mimic more closely the *in vivo* absorption process. Transit compartments for administration of the solid dosage formulations were used to mimic a delay in absorption onset and a gradual increase in absorption rate in a more physiological manner while lag time offered a good fit in the case of oral solution administration. Drug transfer from the final transit compartment to the central compartment occurred through an absorption compartment, from which the drug was absorbed according to the first-order rate constant k_a. The optimal number of transit compartments (n) was estimated and chosen based on the lowest OFV (objective function value) (Figure 2.7).

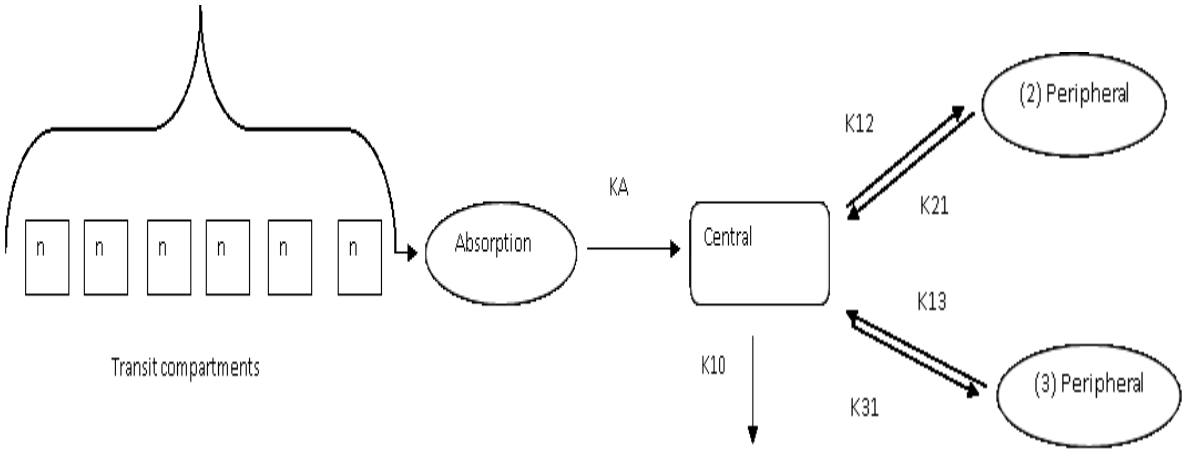


Figure 2.7: Structural model used in NONMEM.

2.3.3.3 Pharmacokinetic Model Evaluation

Model selection was achieved by observing successful convergence, use of the objective function value (OFV- an objective function value is the sum of squared deviations between the predictions and the observations. In NONMEM, the objective function is -2 times the log of the likelihood. A difference in objective function value of 3.84 is considered to be significant at $p<0.05$ with one degree of freedom, based on chi squared distribution); successful covariance step (estimations of RSE values), by examination of relative standard error values and goodness-of-fit plots (prediction vs. observations, and plots of residuals vs. population prediction and time). Xpose (Version 4.0) and R based model building aid (Version 3.1.1) were used for the graphical goodness-of-fit-analysis. A visual predictive check (VPC's) was employed to characterize the model's simulation properties. The final model was used to simulate 1000 new datasets, based on the design of the original dataset. For each of the original data points, a 95% prediction interval was obtained by extracting the 2.5% and 97.5% percentiles of their simulated distributions. These were then plotted against the observations using PsN (Version 3.5.3) and Xpose (version 4.0). The software tool Perl-speaks-NONMEM was used to run a nonparametric bootstrap of 200 iterations to provide unbiased estimates of the standard errors and the 95% confidence intervals of the estimated parameters.

2.3.3.4 CV Calculations

Simulations of the model using R software in 1000 subjects were carried out to estimate variance from the model, the square root of the variance being the standard deviation. The coefficient of variation (CV%) was then calculated by dividing the standard deviation in the mean value. Moreover, the nonparametric test, bootstrap, was used to confirm the 5-95% CI (confident interval). The terms high and low variability refer to distributions that have high and low coefficients of variation, respectively. Typically, a coefficient of variation of a pharmacokinetic parameter of 10% or less is considered low, 25% is moderate, and above 40% is high (Rowland and Tozer, 2011).

2.3.4 Results & Discussion

In this investigation, the well stirred model was successfully implemented in NONMEM to focus on the drug absorption, and not the overall bioavailability. The advantage of using pooled data in NONMEM enabled the estimation of population variability in absorption. The disposition parameters (The intrinsic clearance (CLi), volume of distribution (V) of the central compartments and similar parameters for the peripheral compartments are presented in Table 2.5). f_a*f_g, the absorption rate constant (k_a), lag time or transit time were also estimated (Table 2.6, Table 2.7, Table 2.8 and Table 2.9).

The best fit for the disposition model for all four compounds was achieved with three compartment disposition model. For AZD0865, AZD242, AZD1305 and AZD7009, the OFV decreased by 51, 35, 79 and 167 units respectively, when moving from a two compartment model analysis to a three compartment fit. A successful covariance step was obtained for all structural models. When a four compartment model was assessed for all compounds, either the covariance step was unsuccessful, or the OFV increased, which can both indicate model misspecification. Reasonable goodness-to-fit plots were achieved where the lack of trend indicates that the structural model adequately described the data at all-time points. The goodness-to-fit plots and VPC's plots are presented in Appendix 1.

Table 2.5: Disposition parameter based on IV administration

	Model Parameter	AZD0865	AZD242	AZD1305	AZD7009
Fixed effects (θ) (% RSE)	Intrinsic CL (L/h)	3.99 (7%)	0.16 (10%)	55.7 (9%)	282 (41%)
	Volume (L)	8.59 (10%)	4.09 (9%)	45.3 (26%)	19.7 (27%)
	Q1(L/h)	28.9 (18%)	0.436 (36%)	9.89 (26%)	132 (14%)
	V1 (L)	9.35 (10%)	5.09 (13%)	99.9 (11%)	123 (9%)
	Q2(L/h)	0.437 (18%)	0.543 (25%)	204 (11%)	19.3 (7%)
	V2 (L)	9.65 (35%)	1.54 (27%)	128 (5%)	236 (5%)
Inter-individual variability (Ω) (% RSE)	Intrinsic CL (L/h)	21.4% (24%)	26.2% (27%)	29.3% (24%)	102% (41%)
	Volume (L)	27.3% (27%)	21.4% (22%)	77.7% (33%)	56.4% (19%)
	Q1(L/h)	37.7% (39%)	13.4% (33%)	54.9% (33%)	37.7% (22%)
	V1 (L)	26.2% (29%)	8.9% (25%)	25% (33%)	22.3% (25%)
	Q2(L/h)	45.1% (23%)	29.4% (26%)	11% (55%)	0 FIXED
	V2 (L)	71.1% (16%)	42.3% (6%)	0 FIXED	0 FIXED
Residual error (Σ) (% RSE)	Variance	0.0066 (8%)	0.00244 (24%)	0.0226 (6%)	0.0185 (8%)
	Additive	105 (18%)	1.6 (134%)	0	0

Table 2.6 : f_a*f_g and k_a estimations of AZD0865 formulations

	Model Parameter	**Oral solution**	**IR tablet in the base form**	**IR tablet in the salt form**	**IR tablet in the base form**	**IR tablet in the salt form**
Fixed effects (θ) (% RSE)			Normal gastric pH		Elevated gastric pH	
	k_a (h^{-1})	2.59 (7%)	3.24 (21%)	2.49 (9%)	0.624 (14%)	2.53 (18%)
	f_a*f_g (%)	60.8 (15%)	58.2 (40%)	68 (10%)	16 (8%)	29 (12%)
	Lag time/ KTR (min)	0.198 (2%)	38.3 (10%)	23.1 (7%)	14.7 (12%)	42.4 (12%)
Inter-individual variability (Ω) (% RSE)	k_a (%)	21.2% (13%)	49.1% (33%)	85.7% (11%)	36.5% (32%)	65.8% (23%)
	f_a*f_g (%)	40.2% (14%)	34.8% (30%)	42.8% (17%)	45.7% (23%)	36.6% (23%)
	Lag time/ KTR (%)	13.9% (15%)	35.6% (18%)	58.1% (13%)	0 FIX	39% (30%)
Residual error (Σ) (% RSE)	Variance	0.018 (2%)	0.001 (15%)	0.062 (8%)	0.01 FIX	0.001 (48%)
	Additive	60.4 (9%)	88.6 (26%)	949 (24%)	25000 (7%)	28200 (9%)

Table 2.7: f_a*f_g and k_a estimations of AZD242 formulations

	Parameter	Oral solution
Fixed effects (θ) (% RSE)	k_a (h^{-1})	10 (34%)
	f_a*f_g (%)	98.5 (29%)
	Lag time/ KTR (min)	0.179 (12%)
Inter-individual variability (Ω) (% RSE)	k_a (%)	59.7% (35%)
	f_a*f_g (%)	30.8% (45%)
	Lag time/ KTR (%)	11.4% (78%)
Residual error (Σ) (% RSE)	Variance	0.0112 (5%)
	Additive	2.69 (25%)

Table 2.8: f_a*f_g and k_a estimations of AZD1305 formulations

	Model Parameter	Oral solution fast	Oral solution fast	Oral solution fed	ER formulation fast	ER formulation fed
Fixed effects (θ) (% RSE)	k_a (h^{-1})	1.42(12%)	1.6 (8%)	0.965 (6%)	0.165 (10%)	0.278 (15%)
	f_a*f_g (%)	63 (35%)	60 (43%)	77 (24%)	71.3(30%)	71.1 (28%)
	Lag time/ KTR (min)	0.15 (1%)	0.24 (4%)	0.22 (4%)	0.46 (10%)	0.43 (12%)
Inter-individual variability (Ω) (% RSE)	k_a (%)	60% (13%)	51.2% (9%)	38.1% (18%)	41.3% (23%)	60.5% (20%)
	f_a*f_g (%)	102% (16%)	90.8% (15%)	122.5% (19%)	101.5% (23%)	100.5% (24%)
	Lag time/ KTR (%)	0 FIX	26.2% (17%)	28.5% (20%)	51.9% (15%)	53.8% (17%)
Residual error (Σ) (% RSE)	Variance	0.062 (2%)	0.0174 (5%)	0.0179 (5%)	0.093 (4%)	0.123 (4%)

Table 2.9: f_a*f_g and k_a estimations of AZD7009 formulations

	Model Parameter	Oral solution	PR tablet
Fixed effects (θ) (% RSE)	k_a (h^{-1})	1.6 (10%)	0.0452 (7%)
	f_a*f_g (%)	33 (20%)	60 (92%)
	Lag time/ KTR (min)	0.157 (1%)	4.95 (15%)
Inter-individual variability (Ω) (% RSE)	k_a (%)	60.2% (15%)	60.7% (9%)
	f_a*f_g (%)	64.8% (21%)	0 FIXED
	Lag time/ KTR (%)	0 FIX	157.5% (12%)
Residual error (Σ) (% RSE)	Variance	0.101 (3%)	0.118 (2%)
	k_a (h^{-1})	46.3 (8%)	0

Lag-time estimation was carried out for the oral solution data, and appeared to improve the fit for all oral solution formulations (decrease of 911, 702, 462 and 407 units in the OFV for AZD0865, AZD242, AZD1305, AZD7009, respectively). For AZD0865, two different IR tablets were given as the base form and the salt form of the drug. For the IR tablet in the base form and in the salt form, it was found that the 13 and 8 transit compartments improved the model fit. The same formulations were administered with elevated gastric pH after IV administration of 80mg omeprazole. For these data sets, 4 and 10 transit compartments for the base and the salt form in elevated gastric pH gave the best model fit. The food effect for AZD1305 pharmacokinetics was investigated when an oral solution and an ER formulation were given. 2 compartments and 1 compartment transit increased the model fit by a decrease in OFV for ER tablet under fasted and fed conditions. The addition of one transit compartment for AZD7009 PR formulation yielded a decrease in OFV compared to the lag time.

The goodness of fit plots all solid dosage formulation presented in Appendix 1. It can be seen that for some compounds (AZD242 and AZD0865) there is a trend in the CWERS plots vs. time in late time points (after 24h). This might be attributed to the long elimination phase of the drug due to the very low clearance of the drug. However, since the focus of this research is in the absorption phase alone, the model fit was reasonably accepted (when data points after 24h were ignored a reasonable goodness of fit was accepted).

The visual predictive check for all compounds presented in Appendix 1, indicates that the final model was able to simulate data with a similar distribution to the observed data. The VPC is showing the median, 5^{th}, 50^{th} and 95^{th} s of the observations lie within the 95% CI of model simulation.

The software tool Perl-speaks-NONMEM was used to run a nonparametric bootstrap of 200 iterations to provide unbiased estimates of the standard errors and the 95% confidence intervals of the estimated parameters. The median and 5-95% CI values are presented in Table 2.10.

Table 2.10: Bootstraps results for f_a*f_g

Compound		fa*fg (%)	Bootstrap - fa*fg (%)median	Bootstrap 5-95% CI
AZD0865	Oral solution	60.8%	61%	57.9-65.2%
Normal gastric pH	IR tablet in the base form	58%	57.5%	51.4-65%
	IR tablet in the salt form	73%	68%	65-70%
Elevated gastric pH	IR tablet in the base form	15.7%	15.7%	12.5-20.8%
	IR tablet in the salt form	28.5%	28.1%	23.7-32.4%
AZD242	Oral solution	99%	100%	92-100%
AZD1305	Oral solution fasted state	60%	60.9%	52-67%
	Oral solution fed state	77%	77%	69-85%
	ER fasted state	71%	73%	60-84%
	ER fed state	71%	68%	61-76%
AZD7009	Oral solution	33%	33.5%	27.3-39.7%
	PR tablet	57%	53%	48-60%

Table 2.11: CV% estimations for f_a*f_g based on simulations for AZD compounds

Compound	Formulation	CV% Based on simulations
AZD0865	Oral solution	15%
Normal gastric pH	IR tablet in the base form	14.2%
	IR tablet in the salt form	13.5%
AZD0865	IR tablet in the base form	38%
Elevated gastric pH		
	IR tablet in the salt form	25%
AZD242	Oral solution	9%
AZD1305	Oral solution fasted state	33%
	Oral solution fed state	28%
	ER fasted state	30%
	ER fed state	27%
AZD7009	Oral solution	39.3%
	PR tablet	N/A

AZD242

AZD242 was chosen as a control drug based on the assumption of 100% absorption and low inter-subject variability, to confirm that the f_a*f_g values generated by NONMEM with the fitted "well-stirred" model equations are valid. The absorption rate constant was high for oral solution, indicating fast absorption. 100% absorption was estimated for the oral solution with short lag time. With regards to variability, NONMEM assessed relatively low inter-subject variability (9%). This emphasises the fact that high absorption is associated with low variability.

AZD0865

AZD0865 is a weak base with a pka of 6.5. Based on the *in vitro* studies, it is expected that AZD0865 solubility would be highly dependent on the gastrointestinal pH, and therefore drug precipitation might occur as a consequence of the pH increase from acidic in the stomach (especially in the fasted state) to near-neutral in the small intestine (Carlert *et al*., 2010).

The oral solution bioavailability was reported to be 55%. Based on the results of the f_a*f_g estimation for the oral solution, f_h is around 90%, indicating low hepatic extraction; therefore, the relatively low bioavailability can be attributed to absorption (60%). A relatively low inter-subject variability for the oral solution of 15% was estimated (CV %). Absorption decreased slightly when the drug was administered as the base form, and increased as the salt form. For all formulations the f_a*f_g inter-subject variability was similar. When the drug was given after administration of omeprazole for both IR formulations, a significant decrease in the absorption was observed (15% and 30% for the base and the salt form, respectively- Figure 2.8). In addition, an increase in inter-subject variability was observed.

To identify the rate limiting step in absorption there is a need to compare the oral solution to the solid dosage form formulation. In the case of AZD0865, f_a*f_g from the base form did not differ from the oral solution. At first, it would appear that permeability is the rate-limiting step, given that the solution and solid dosage form in the base form gave similar f_a*f_g values. However, an increase in f_a*f_g for the salt tablet was observed. Therefore, it can be assumed that the absorption is solubility/dissolution rate-limiting. Salt formation is the first and the most common approach to increase drug solubility in the pharmaceutical industry (Kawabata

et al., 2011; Korn and Balbach, 2014)). In the case of AZD0865, mesylate as a crystalline powder was developed to overcome the solubility issue. In addition, the permeability data from *in vitro* studies in Caco-2 cells (AstraZeneca data on file) indicate that AZD0865 is a highly permeable drug.

Considering the dose and the high solubility of the drug in the gastric fluids, it is reasonable to assume that for all dosage forms, a complete dissolution in the stomach occurred. However, with stomach emptying, the drug from either solution or tablets can precipitate, and low dissolution occurs in the small intestine. It seems that the salt IR tablet managed to minimize this effect, and yielded a super-saturated state for a longer period of time to allow higher absorption. To emphasise that, f_a*f_g of the IR tablet in the base form decreased by more than half under elevated gastric pH. A lower decrease was attained for the salt form, with twice the extent of f_a*f_g compared to the base form. This indicates that gastric pH plays an important role in drug absorption. A median gastric pH of 5.8 was expected based on a study with the same omeprazole regimen (Röhss *et al*., 2007). At elevated gastric pH, the AZD0865 solubility in the gastric fluid is low, and almost all the drug would be emptied into the duodenum from the stomach in the undissolved form. Both the rate and extent of absorption are therefore limited by intestinal drug dissolution.

A separation of the fraction that escapes gut wall metabolism (f_g) from the fraction absorbed (f_a) was not made in this investigation. However, based on clinical studies where AZD0865 was administered with grapefruit juice, and which did not seem to affect the pharmacokinetics of AZD0865, this indicates that metabolism by CYP 3A4/3A5 in the gut is of minor importance for the pharmacokinetics of AZD0865.

The inter-subject variability estimated herein was similar for all formulations around 15%, and increased under elevated gastric pH conditions. The increase in solubility of the drug using the salt formation did not affect the inter-subject variability. It might be attributed to the slight increase in absorption (only 10%) when the drug was administered in the salt form. In the case of elevated gastric pH, the differences in gastric pH due to omeprazole administration can explain the increase in variability.

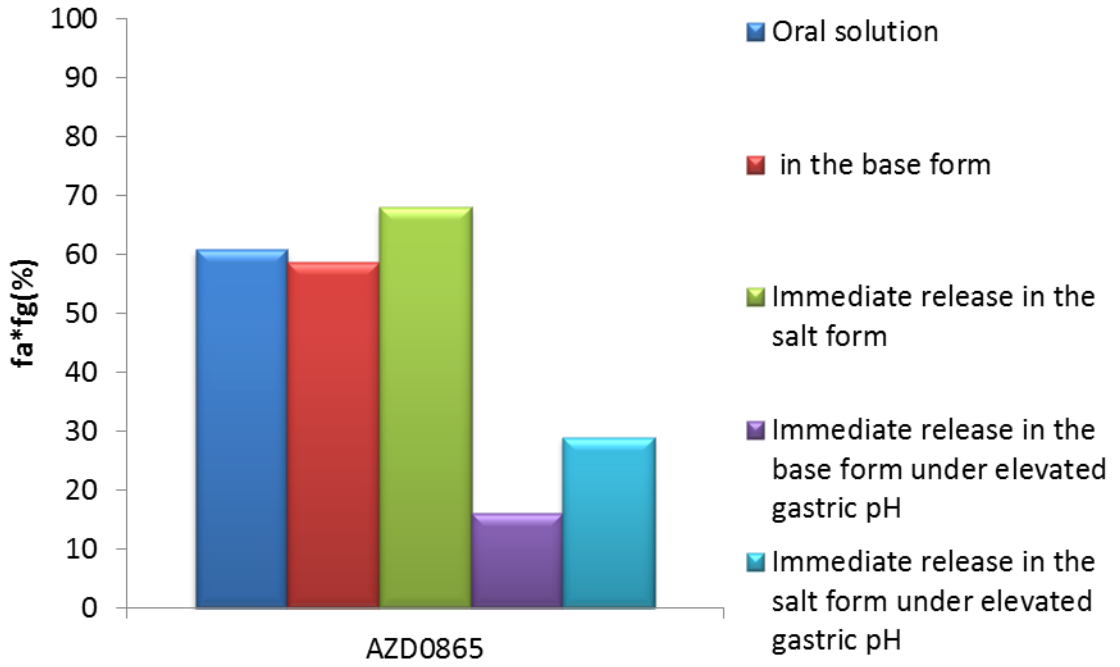


Figure 2.8: f_a*f_g values of AZD0865

AZD7009

AZD7009 is a weak base (pka=9.7), and as such its solubility in aqueous solutions is pH dependent, and increases with a decrease in pH. The oral bioavailability is 16%; C_{max} varied between 16-62% in the single dose- escalating study.

The absorption rate was faster in the case of the solution compared to the PR formulation (3.2 and 0.04 h^{-1}) respectively, with higher inter-subject variability in the solution absorption rate than in the solid dosage form. The solution lag time was shorter compared to the transit time for the PR tablet with high inter-subject variability in transit time for the PR formulation. Low f_a*f_g was estimated for the oral solution with high inter-subject variability whereas the f_a*f_g for the PR tablet increased to 60% (Figure 2.9). The low f_a*f_g after oral solution administration (30%) indicates f_h is around 50%. Therefore, the low bioavailability (16%) in the case of the solution dosage form can be attributed to both absorption and hepatic elimination. Solubility or dissolution should not be the rate-limiting step for this compound, as its pka is higher than the gastrointestinal pH range. The increase in f_a*f_g for the PR formulation might indicate a possible stability issue for the drug in the upper part of the gastrointestinal tract. Allowing for a low dissolution rate in the upper part of the gut will

enable more of the drug to reach the lower parts of the gut, thus prolonging absorption. However, no *in vitro* stability data were found to support this hypothesis.

The fact that in the upper part of the gut the drug might be more susceptible to gut wall metabolism can be ruled out based on clinical trial data that showed that no effect on drug pharmacokinetics when co-administered with the P-gp inhibitor verapamil (AstraZeneca data files).

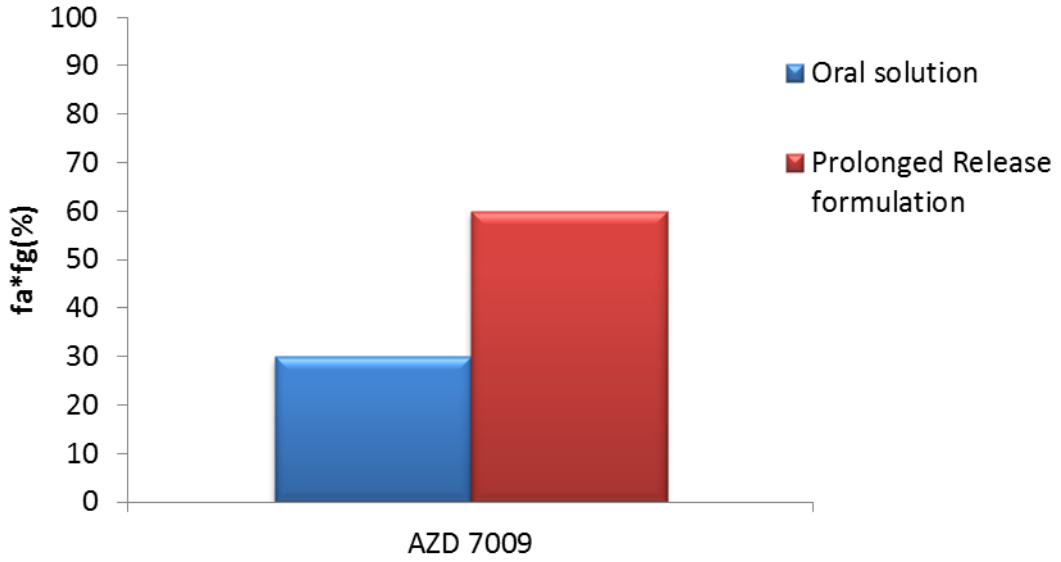


Figure 2.9: f_a*f_g values for AZD7009

AZD1305

AZD1305 is a base with pka of 9.9. The solubility is pH-dependent, and increases at pH below 9.9. The permeability data from *in vitro* studies in Caco-2 cells (AstraZeneca data on file) indicate that AZD1305 is a highly-permeable drug. The absolute bioavailability for the oral solution and the ER formulation ranged from 31 to 50% and 22 to 61%, respectively.

The k_a value for the oral solution was relatively high compared to the ER formulation, indicating a slow release of the drug from the tablet matrix in the GI tract, and slow absorption. It can be seen that the inter-subject variability in the rate of absorption was higher in the case of the solid dosage forms as compared to the solution, which might be attributed to the differences in the disintegration and dissolution of the drug resulting from the difference between individual GI physiology. The oral solution f_a*f_g in the fasted state was estimated as 60% and increased in the fed state (77%). Comparing the ER formulation and the solution in the fasted state, it can be seen that absorption increased by 10%. In addition, no food effect was observed for the ER formulation (71% vs. 68% under fast and fed states respectively) (Figure 2.10). Interestingly, inter-subject variability was high for both the oral solution and the ER tablet under the fasted and fed states (greater than 30%).

The physiology of the gastrointestinal tract changes in the fed state, and may consequently affect drug absorption. The remarkable changes in the stomach under the fed state notably include a rise in gastric pH thanks to buffering and dilution effects, along with an increase in the gastric fluid volume and a decrease in gastric emptying time. In the small intestine, an increase in bile salt concentration, decrease in fluid volumes and in some cases inhibition of CYP enzymes and efflux transporters are expected (Varum *et al*., 2013). Since AZD1305 is a free base with pka of 9.9, it would be expected to have high solubility in the gastric fluids, and its solubility should not decrease significantly in the administered clinical dose in the intestine in the fed or the fasted state. However, degradation of the drug in low pH conditions might explain the increased absorption under fed state. *In vitro* studies have shown support for this hypothesis (Sigfridsson *et al*., 2012). In the fed state, both the elevated gastric pH and the low retention time in the stomach might contribute to the drug stability, and therefore more drug arriving to the small intestine that is available for absorption. In addition, it might be that an increase in bile salt concentration and gastric fluid volumes might have a positive food effect on the drug absorption under fed conditions. The food effect vanished when the

extended release formulation was given under both fed and fasted states. Thanks to a slower dissolution in the stomach, less of the drug is deemed susceptible to degradation in the acidic conditions of the stomach, and more available to be absorbed in the small intestine.

High inter-subject variability can be attributed to the absorption process for all formulations (CV=35%). Although a positive food effect caused an increase in f_a*f_g in the fed state, formulating the drug as an extended-release tablet did not improve the inter-subject variability either in the fasted or fed states. It might be that the drug stability differs significantly between individuals, even in the lower parts of the GI tract, due to differences in the pH in the small intestine.

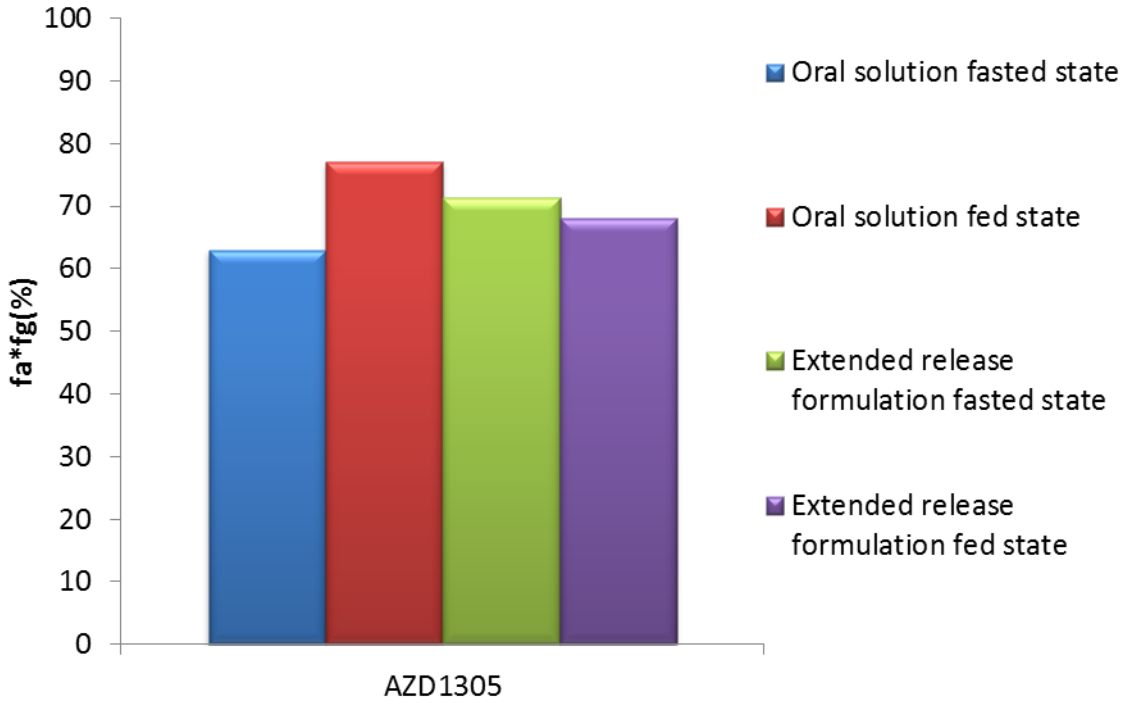


Figure 2.10: f_a*f_g values of AZD1305

2.3.5 Summary

The well stirred model was successfully implemented in NONMEM to estimate population absorption. Estimations of f_a*f_g and inter- subject variability were obtained for 4 AZ compounds with different formulations. The salt form of AZD0865 increased the drug absorption indicating absorption is solubility/dissolution limited, as the drug possibly precipitated to a significant extent in the small intestine. Due to the drug's basic nature, low absorption was estimated under elevated gastric pH. Inter-subjects variability in absorption was relatively low for AZD0865. The PR tablet of AZD7009 increased the absorption *in vivo*. The food effect on AZD1305 absorption disappeared when the extended-release formulation was administered, indicating that in the absence of food the oral solution is less stable in the lower stomach pH. Obtaining an accurate estimation of absorption and variability in absorption from phase 1 clinical trial will enable scientist to understand better the absorption process and evaluate formulation performance with combination of *in vitro* data. In addition, understanding inter-subject variability in early stages of drug development will help scientists to plan and interpret phase 2 and 3 clinical trials.

As has been described, PBPK models are readily available to estimate f_a, and most of them rely on *in vitro* data. Therefore, a prerequisite for the absorption estimation is reliable *in vitro* methods. These PBPK models can be incorporated in population approaches (as has been done in the SimCyp simulator and GastroPlus). For the purpose of this investigation and for simplification reasons, only parameters that characterise elimination were included in the model building (i.e. liver blood flow, blood to plasma ratio and renal clearance). Further work will include adding physicochemical (i.e. measurements of solubility, dissolution and permeability) and physiological parameters (i.e. gastric emptying time and transit time) to the model to mimic closely the situation *in vivo*.

2.4 Chapter Conclusions

Focusing on f_a*f_g instead of the overall bioavailability enabled a better understanding of possible loss of drug in the GI tract and accordingly developing suitable formulations to increase absorption in early stages of drug development. Therefore, an accurate estimation of f_a*f_g is an essential key in this investigation. In the first section, different methods to estimate f_a*f_g from human clinical trials were utilized. However, due to the lack of published individuals' pharmacokinetics values, in addition to the small number of subjects who participated in the published clinical trials, it was not possible to yield a reliable large dataset of f_a and inter-subject variability. Considering these limitations, f_a*f_g and inter-subject variability for 38 compounds were estimated. A moderate correlation was obtained between f_a*f_g and inter-subject variability. In addition, the physiochemical parameters chosen in this study did not yield a significant correlation to the low and erratic absorption. Based on these conclusions, phase 1 clinical trials for 4 compounds with different formulations were utilized to estimate f_a*f_g in the second section. In addition, utilizing the population pharmacokinetics approach (NONMEM) enabled to estimate the inter-subject variability in a relatively large set of data (30-50 subjects). In this section, the population pharmacokinetic model was successfully developed to estimate f_a*f_g and inter-subject variability. Due to the complexity and multiple factors affecting drug absorption in the GI tract, there is a need to further verify these results *in vitro*. In the next chapter, different *in vitro* experiments were utilized to investigate the important factors in causing high inter-subject variability in absorption. Since the compounds investigated in this chapter were not available for further experiments, two model drugs (dipyridamole and furosemide) with high inter-subject variability in bioavailability (attributed to absorption) were investigated to understand the mechanism causing variability.

Chapter 3 - Inter-subject Variability in Solubility, Dissolution and Permeability *"In Vitro"* of Two Model Drugs

3.1 Chapter Overview

The biopharmaceutics drug classification scheme (BCS) for correlating *in vitro* drug product dissolution and *in vivo* bioavailability was developed based on the recognition that drug solubility/dissolution and gastrointestinal permeability are fundamental parameters controlling the rate and extent of drug absorption (Amidon *et al*., 1995). Based on this assumption, it is important to consider the inter-subject variability in solubility, dissolution and permeability and which factors contribute to inter-subject variability in absorption.

In situ measurements of drug concentration in the gastrointestinal fluids are hindered by numerous ethical and practical concerns regarding a healthy subject's safety. Therefore, a more practical approach to estimating drug solubility, dissolution and permeability in the GI tract is to use different *in vitro* methodologies (Dressman *et al.*, 2007). In the previous chapter, absorption and inter-subject variability were estimated in humans from drug concentration in plasma and hypothesis based on *in vitro* data was drawn to underline the reasons for low and erratic absorption. To our knowledge, there is no single *in vitro* system that composes the complexity of the GI tract to investigate all variable parameters that can influence absorption variability in the GI tract. Understanding the mechanism for absorption variability in the early stage of drug development by using *in vitro* systems will assist in designing formulations to increase absorption and decrease inter-subject variability.

In this chapter, due to the complexity of the GI tract environment and lack of one *in vitro* model to estimate all GI parameters simultaneously, solubility, dissolution and permeability were assessed separately as for which parameters in the GI tract might affect drug absorption. In the first section, solubility was tested in real human fluids and simulated fluids. Characterisation of these fluids enables the underlining of the parameters in the GI tract that contribute to changes between individuals. Parameters that showed significant correlation to variability (i.e. bile salt and pH) in the drug's solubility were further investigated with regards to dissolution and permeability. In the second part, the extent and dissolution rate of these drugs were investigated using simulated intestinal fluids, in particular, under different conditions of pH and bile salt concentration. Based on the understanding that it is often difficult to extrapolate from *in vitro* dissolution data alone on the *in vivo* absorption, adding permeability data will add a deeper understanding of the absorption process and inter-subject variability.

Due to difficulties obtaining the API's and the formulations of the compounds investigated in chapter 2, a literature research for well formulated compounds with highly variable bioavailability in humans was carried out. Two compounds that were commercially marketed were chosen. Dipyridamole (BCS II) is a poorly soluble weak base with pKa =6.4 (Williams *et al.*, 1981). Its bioavailability is 52%, and varies significantly between subjects in the range of 20- 70% (Rajah *et al.*, 1977; Tyce *et al*., 1979). Furosemide, a weak acid with pKa =3.8, is reported to be a poorly soluble and permeable drug and the FDA classified it as a BCS IV drug (Granero *et al*., 2010). Furosemide mean bioavailability was reported to be about 60%; however, its bioavailability is highly variable and erratic, with values ranging from 12% up to 100% (Hammarlund *et al*., 1984; Kelly *et al.*, 1974; Ponto and Schoenwald, 1990). Since neither drug is extensively metabolized in humans, it is reasonable to assume that bioavailability is a good indicator to absorption. Therefore, the inter-subject variability in bioavailability might be attributed to the absorption process rather than the elimination process.

3.2 Inter – subject Variability in Gastrointestinal Drug Solubility

3.2.1 Introduction

As described in the previous chapter, gastrointestinal fluid is complex, dynamic and fluctuating. Solubility in the GI contents is determined by its crystalline/amorphous form, drug lipophilicity, solubilisation by native surfactants and co-ingested food stuffs, pH, buffer capacity and viscosity. In addition to the composition of the fluids, the total fluid volume is an important factor which influences the solubility of drugs.

Many publications have characterised chemically the fluids available for drug dissolution in the upper GI tract in humans, and examined variations regarding the physicochemical properties of these fluids. Important considerations like, osmolality, surface tension (Dressman *et al*., 1998), buffer species and ionic composition (Fadda and Basit, 2005), and gastric, pancreatic and microbial enzymes (Sousa *et al*., 2008), and bile salts (Holm *et al.*, 2013) in the luminal fluid may significantly influence drug solubility/dissolution and hence absorption. The composition and characteristic of the fluid from the human GI tract is summarised in Table 3.1.

Table 3.1: Characteristic of fluids aspirated from the human GI tract

		Gastric fluids	Jejunal fluids	Ileal fluids	Colonic fluids
pH	fast state	1.23-7.36[a]	6.8	7.4	6.8
	fed state	6.4-7[a]			
Buffer capacity (mmol*L^{-1} *Δ pH^{-1})	fast state	7-18[a]	2-13[c]	6.4	37.7
	fed state	14-28[a]	13-30[c]		
Pepsin (mg/mL)	fast state	0.11-0.22[a]			
	fed state	0.26-0.58[a]			
Osmolality (mOsm*kg^{-1})	fast state	98-140[a]	271		224
	fed state	217-559[a]			
Surface tension (mN*m^{-1})	fast state	41.9-45.7[a]	28 -26[d]		33
	fed state	30-31[a]	27-37[d]		
Bile salts (mM)	fast state	0.2-0.8[b]	2.9		0.6
	fed state	5-18[a]			

(Kalantzi *et al*. 2006) [a]; (Holm *et al*., 2013)[b];(Moreno *et al*., 2006; Persson *et al.*, 2005)[c] (Clarysse *et al*., 2009a; Persson *et al.*, 2005)[d]; (Fadda *et al.*, 2010a)[e];(Diakidou *et al.*, 2009) [f]; (Lindahl *et al.*, 1997)[g];

The solubility of weak acids and bases depends on their ionization constants, k_a and the pH of the dissolution medium (Bhattachar *et al*., 2006), and as such, the pH of the GI fluids remains one of the most important influences on the saturation solubility of ionisable drugs. This pH also varies widely with location in the gastrointestinal tract, and even there are complex variations in pH between the fed and fasted state (Hörter and Dressman, 2001). Yet another parameter that affects the solubility and dissolution rate is the buffer capacity of the GI fluid, particularly for ionisable drugs. The buffer capacity of human gastric aspirates was reported to be 14 mmol/L/△pH 30 minutes post-ingestion of a liquid meal, increasing to 28 mmol/L/△pH 210 minutes post-meal ingestion. The buffer capacity of the duodenal aspirates varied between 18 and 30 mmol/L/△pH during 30 to 210 minutes post-meal ingestion (Kalantzi *et al*., 2006).

Bile salts in the intestine also have a considerable impact on the solubility of lipophilic drugs. The solubility is further influenced by inter-subject variability in bile salt concentration; such variability is also magnified in the fed state. Holm *et al.* (2013) reported that under fasted conditions, bile salt concentration ranges from 2 to 6.4mM and in the fed state, the concentration varies significantly with reported values ranging from 0.5 to 37mM. In the fasted state, bile salt concentration is mainly affected by the basal secretion of each individual, whereas in the fed state, food intake and its composition can markedly affect bile salt levels in the gut. Surface tension also affects drug dissolution through its influence on wetting, with higher surface tension values leading to decreased wetting. Furthermore, variations in gut osmolality can affect drug release from different formulations and excipient performance, with osmolality values range from 124-278mOsm/kg and 250-367mOsm/kg in the fed state in the upper small intestine.

In this work, ileostomy fluids from 10 subjects with ulcerative colitis (UC) of the colon were characterized in terms of pH, buffer capacity, osmolality and surface tension, and solubility measurements were also made. For comparison, and to understand the regional differences in the solubility of dipyridamole and furosemide along the GI tract, solubility experiments were carried out in pooled gastric and intestinal fluids from healthy volunteers, and in simulated GI fluids

Studies are consistently published on attempts to mimic the GI fluids *in vitro* in order to characterise drug solubility, dissolution and permeability. However, most of these approaches aim to mimic the average person. To extend our knowledge of possible factors that might cause variability in drug solubility, the effects of bile salt concentration and pH were studied by use of simulated intestinal fluids. Different solutions of FaSSIF buffers were prepared to predict how the intestinal solubility of furosemide and dipyridamole varies within the normal range of bile salt and pH in the human small intestine.

3.2.2 Objectives

- To determine the solubility of dipyridamole and furosemide along the GI tract in gastric, intestinal pooled fluids and from ileostomy fluids.
- To characterise 10 individual ileostomy ulcerative colitis (UC) patients' fluids with regards to osmolality, buffer capacity, surface tension and pH.
- To investigate the effect of bile salt concentration and pH on drug solubility using simulated intestinal fluids.
- To identify and mechanistically understand the key causes of inter-subject variability in solubility of dipyridamole and furosemide.

3.2.3 Materials

Dipyridamole (D9766) and furosemide (F4381) were obtained from Sigma Aldrich Chemicals (Poole, UK). SIF powder was purchased from Biorelevent.com. All salts to prepare the buffers were of analytical grade and purchased from VWR Chemicals Ltd. (Poole, UK). Solvents used in HPLC were: HPLC water, acetonitrile and phosphoric acid. All were of HPLC grade and purchased from Fisher Scientific (Loughborough, UK).

3.2.4 Methods

3.2.4.1 Human Fluids

Healthy pooled gastric and intestinal fluids were supplied by AstraZeneca, Sweden. Gastric and intestinal fluids were aspirated from healthy volunteers via an oral intubation tube (Loc-I-Gut, synectics Medical, Sweden (Bønløkke *et al*., 1997). The fluids were collected, pooled and stored in -80˚C until analysis.

Ileostomy fluids were supplied from the Singleton Hospital in Swansea. Fluids were collected from stoma bags in patients who were undergoing a routine change of their stoma bag, or who were undergoing surgery for stoma reversal. Ileostomy fluids were collected from 10 different patients. End ileostomy is usually constructed as a permanent stoma for patients with ulcerative colitis or Crohn's disease. The terminal ileum is brought through the abdominal wall in the right iliac fossa area. This is usually the outcome of proctocolectomy (Keighley and Williams, 1999). The samples were not pooled, and therefore each sample corresponds to one patient. Patients were not fasting and their diet was not controlled.

3.2.4.2 Sample Preparation

The GI fluids were centrifuged with Centrifuge 5415D (Eppendorf AG, 22331 Hamburg, Germany) at 16,110xg RCF (relative centrifugal force, equivalent to 13200rpm) for 20 minutes. The supernatant obtained were kept in a freezer (-80˚C) until analysed.

3.2.4.3 Osmolality Measurement

Osmolality was determined with a Digital Micro-Osmometer (Type 5R), (Hermann Roebling Messtechnik, Berlin, Germany). The operating principle of this instrument is based on freezing point depression. Samples were thawed to room temperature before measurements were taken, and a volume of 100µL was used for each measurement.

3.2.4.4 Surface Tension Measurement

Surface tension was measured with a Delta 8 Tensiometer (Kibron Inc) controlled by Delta-8 manager software (version 3.8). The measurement was performed using DynePlates (96-well plate designed for tensiometer) with a 50µL sample in each well.

3.2.4.5 Buffer Capacity and pH Measurement

Buffer capacity was measured by using a pH meter (HI99161) equipped with an FC202 electrode, designed for viscous and semi-solid materials (Hannah Instruments, Bedfordshire, UK). Buffer capacity was measured at a pH change of 0.5 and 1.0 units. This was performed by the aliquots addition of accurate amounts of HCl (intestinal fluids) to a 300µL sample to achieve the desired pH change. Buffer capacity was then calculated using Equation 3.1. Buffer capacity measurement was only performed in one direction due to the limited availability of gastrointestinal fluids and time.

$$\beta = \frac{\Delta AB}{\Delta pH}$$

Equation 3.1: Buffer capacity calculation

In equation 1, Δ AB is the small increment in mol/L of the amount of acid or base added to produce a pH change of Δ pH in the buffer. Equation 1 was further modified (Equation 3.2) to account for smaller volumes (300µL) of the sample in contrast to 100mL in conventional measurements, which was then used for the calculation of the buffer capacity.

$$\beta = \left[\frac{M_{\frac{acid}{base}} \times V_{\frac{acid}{base}(mL)}}{\Delta pH}\right] \times \frac{1000}{V_{sample\,(mL)}}$$

Equation 3.2: Modified equation for buffer capacity calculations

$M_{acid/base}$ and $V_{acid/base}$ are the molarity and volume of the acid/base added to the V (mL) of the sample to produce a pH change of Δ pH in the sample.

3.2.4.6 Simulated intestinal fluids Preparation

Two important variables in the composition of intestinal fluids that might affect drug solubility were simulated *in vitro*, bile salt concentration and pH. Total bile salt concentration in aspirated fluids from the fasted duodenum and jejunum ranged from 0.6 to 5.5mM (Kalantzi *et al*., 2006; Lindahl *et al*., 1997; Moreno *et al.*, 2006). With respect to pH, the range in the duodenum and jejunum was found to be around 5.5-7.4 (Evans *et al.*, 1988; Fallingborg *et al.*, 1989; Kalantzi *et al.*, 2006; Lindahl *et al.*, 1997; Pedersen *et al*., 2000b). These conditions were simulated *in vitro* using FaSSIF-V1. FaSSIF-V1 was prepared according to the recipe from Dressman *et al*. (1998) (Table 3.5). pH was adjusted in the range of 5.5-7, and the bile salt concentration ranged from 1 to 6mM.

3.2.4.7 Solubility Measurements

Solubility measurements were performed in simulated intestinal fluids, healthy GI fluids and ileostomy human fluids. An excess of the drug was added to micro centrifuge tubes (Eppendorf AG, Hamburg, Germany) containing 200µL of fluid. The samples were placed in a shaking bath, maintained at 170 shakes per minute at 37°C for 24 hours. After 24 hours, the samples were centrifuged (4472 rcf /5000rpm) at a temperature of 40 °C using a Centrifuge 5804R (Eppendorf AG, 22331 Hamburg, Germany) for 10 minutes at 37°C (a temperature set point of 40°C was used to maintain the required temperature in the micro tubes during centrifugation). The supernatant was transferred to a centrifuge filter tube (cellulose acetate membrane, pore size 0.22µm, sterile Corning® Costar® Spin-X®) and then centrifuged at 5000rcf (Eppendorf AG, Hamburg, Germany) for 20 minutes at 37°C (40°C set point). Aliquots of the filtrate (50µL) were removed and diluted with the mobile phase. The amount of drug dissolved in the sample was assayed and determined by using HPLC-UV /fluorescence, as described in the following section. Calibration curves and blank samples for

the drugs were prepared. Spiking the different media with known concentrations of drugs showed recovery between 92-100%. The same samples were also analysed after only 5 hours of incubation and similar concentrations were measured compared to 24 hours' incubation. Thus, it is possible to exclude degradation in biological fluids or binding to the filters.

3.2.4.8 High Performance Liquid Chromatography (HPLC) for Assaying Drug Solubility

Drug concentrations were determined by reverse-phase HPLC analysis with UV and fluorescence detection. The equipment consisted of an integrated HP 1200 Series HPLC system comprising an HP1200 autosampler, an HP 1200 pump and an HP 1200 multiple wavelength detector system, a Vis-UV spectrophotometric detector and fluorescence detector (Agilent Technologies, West Lothian, UK). The detector was interfaced with a pv with PC/Chrom + software (H&A Scientific Inc, Greenville,NC, USA). Separation of furosemide was achieved with C-18 column, Hypersil Gold 150*4.6 mm 3 µm (Fisher Scientific) at 40˚C. The mobile phase used for analysis consisted of 25:75% (V/V), acetonitrile and 0.05M phosphate buffer adjusted pH to 2.5. The flow rate was 1mL/min, the injection volume was 20µL, and the detection wavelength for vis-UV was 238nm. For fluorescence excitation and emission, the wavelengths were set to 233 and 389nm respectively. The drug retention time was 12.5 min. Separation of dipyridamole was achieved with c-18 column, Atalntis, 150*4.6 mm, 5µm (Waters) at 40˚C. The mobile phase used for analysis consisted of 40:60 % (V/V), acetonitrile and 0.1% TFA in water. The flow rate was 1ml/min and the injection volume was 20µL; the detection wavelength for vis-UV was 285nm, and for fluorescence detection, excitation and emission the wavelengths were set to 295 and 485 respectively. The drug retention time was 6.4 min. Calibration curves were prepared in the corresponding mobile phase as saturated drug solutions were subjected to 20 fold dilution.

3.2.4.9 Statistical Analysis

All solubility data presented and fluid measurements herein are the mean value of triplicate experiments. The coefficient of variation (CV%) was calculated by dividing the standard deviation in the mean value. The terms high and low variability refer to distributions that have high and low coefficients of variation, respectively. Typically, a coefficient of variation of a pharmacokinetic parameter of 10% or less is considered low, 25% is moderate, and above 40% is high (Rowland and Tozer, 2011).

Correlation analysis was carried out using Excel. Standard multiple regression using SPSS statistics software 22.0.0 (SPSS Inc., Chicago, IL) was conducted to assess the feasibility of predicting the solubility of furosemide in ileostomy fluids from the following variables: buffer capacity and pH.

3.2.5 Results & Discussion

In this study, dipyridamole solubility in pooled gastric and intestinal fluids was 9.3mg/mL and 0.016mg/mL respectively. Furosemide solubility in pooled gastric fluids was 0.003mg/mL, while in pooled intestinal fluids increased to 2.9mg/mL. Solubility data for dipyridamole and furosemide in different individuals are graphically presented in Figure 3.1 and Figure 3.2 respectively. The mean solubility of dipyridamole was 0.043mg/mL (range 4-69µg/mL). The furosemide solubility mean value in the ileostomy fluids was 1.7mg/mL (range 0.2-5.3mg/mL). High variability between subjects in the solubility of both drugs was observed with CV values of 88% and 63% for furosemide and dipyridamole accordingly. It can be seen that solubility of both compounds in FaSSif were slightly different compared to the solubility in human intestinal fluids and ileostomy fluids (Table 3.2).

Table 3.2: Solubility in human intestinal fluids and ileostomy fluids (mean ± SD)

	Dipyridamole (mg/mL)	**Furosemide (mg/mL)**
Gastric pooled fluids	9.3 ± 0.04	0.003±0.0006
Intestinal pooled fluids	0.016 ± 0.0001	2.9±0.05
Ileostomy fluids	0.043 ± 0.023	1.8±1.6
Simulated intestinal fluids FaSSIF	0.028 ± 0.001	1±0.01

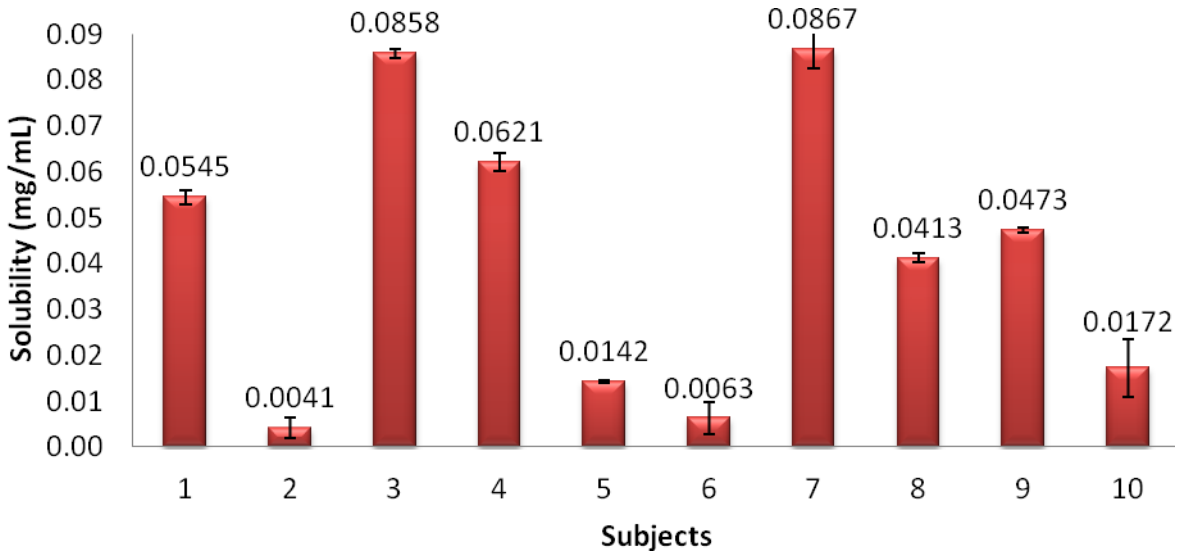


Figure 3.1: Dipyridamole solubility in ileostomy fluids from 10 individual

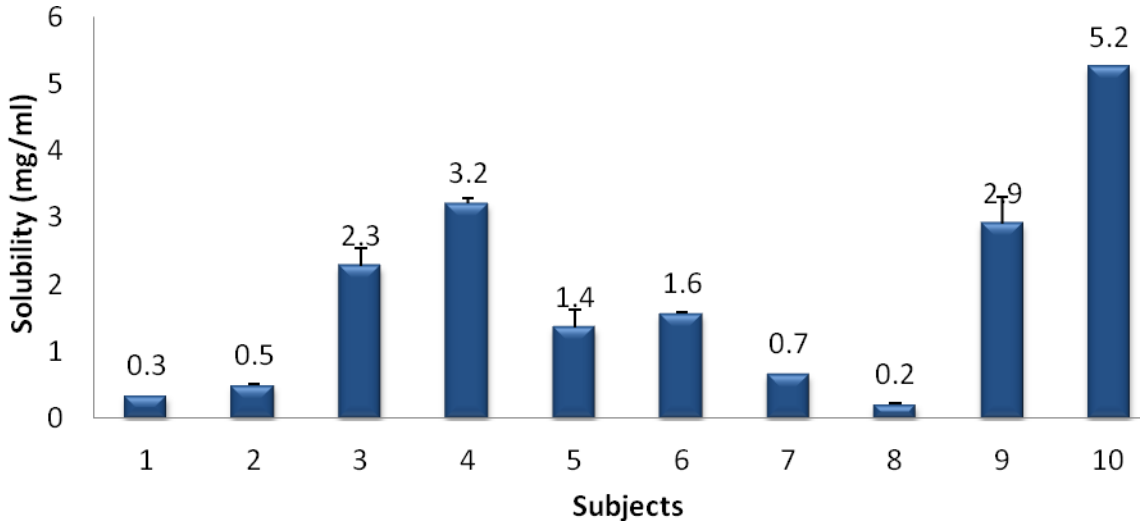


Figure 3.2: Furosemide solubility in ileostomy fluids from 10 individuals

pH, buffer capacity, surface tension and osmolality were later measured in 10 individual ileostomy fluids (individuals, mean, SD and CV% values presented in Table 3.3). In this study, the mean value of surface tension in ileum fluids was 42.9±6mN/m (range 34-49 mN/m, CV=14%) Osmolality mean value was found to be 398 ±81mOsmol/kg (range 328-619mOsmol/kg CV=20%). The mean value of buffer capacity and pH was 18.3± 11mM/L/ΔpH (range 5.6-45mM/L/ΔpH CV=60%) and 6.7±0.91 (range 6.16-7.88, CV=14%), respectively. Buffer capacity in ileostomy fluids was reported to range between 11-54mM/L/ΔpH with a mean value of 22.4mM/L/ΔpH. pH in ileostomy fluids ranged from 5.8-8 with a mean value of 7.1 which is in agreement with the data presented herein (Fadda *et al.,* 2010a).

Table 3.3: Characterisation of ileostomy fluids (mean ±SD and CV as measure to variability)

Subject no.	Surface Tension (mN/m)	Osmolality (mOsmol/kg)	Buffer Capacity (mM/L/ΔpH)	pH
1	36.5	368.0	6.62	6.16
2	43.3	433.7	12.54	5.65
3	49.5	357.3	17.16	7.54
4	48.3	349.3	14.98	7.88
5	48.3	434.7	14.15	6.85
6	49.2	328.0	5.62	7.88
7	39.6	370.0	13.71	6.08
8	37.4	363.3	17.85	5.37
9	49	351.7	20.57	7.68
10	35	619.0	32.44	7.65
Mean ± SD	43±6	398.5±81	15.9±7.2	6.7±0.9
CV (%)	14	20.3	45.5	13.5

To identify the factors which contribute to the inter- subject variability in solubility of the two drugs, poorly soluble, weak acid and base, correlation analysis was carried out and the results presented as R square values in Table 3.4. Dipyridamole solubility in ileostomy fluids was significantly correlated with the pH changes within individuals giving R squares value of 0.79. No correlation was found between dipyridamole solubility to the buffer capacity, osmolality and surface tension in ileostomy fluids. Furosemide solubility in ileostomy fluids was relatively well correlated with pH changes of individuals and buffer capacity (R^2 = 0.58 and 0.56 respectively).

Table 3.4 : Correlation analysis (R^2 values)

	Dipyridamole solubility	**Furosemide solubility**
pH	0.79	0.58
Buffer Capacity	0.079	0.56
Osmolality	0.034	0.23
Surface Tension	0.2	0.03

pH correlations to dipyridamole and furosemide solubility in ileostomy fluids were confirmed using simulated intestinal fluids, where the correlation of R^2=0.82 and 0.63 between solubility and pH changes for dipyridamole and furosemide respectively were found (Figure 3.3 and). To further understand how bile salts concentration affects dipyridamole and furosemide solubility, solubility measurements were made under different conditions of bile salts concentration in simulated intestinal fluids. For dipyridamole, correlation of R^2= 0.94 in the range of 1-6mM bile salt was obtained (Figure 3.5). Furosemide solubility in simulated intestinal fluids increased slightly when bile salt concentration increased with solubility ranging from 1 to 1.5mg/ml (Figure 3.6). The buffer capacity of these fluids was measured, and a correlation of 0.64 was attained to furosemide solubility.

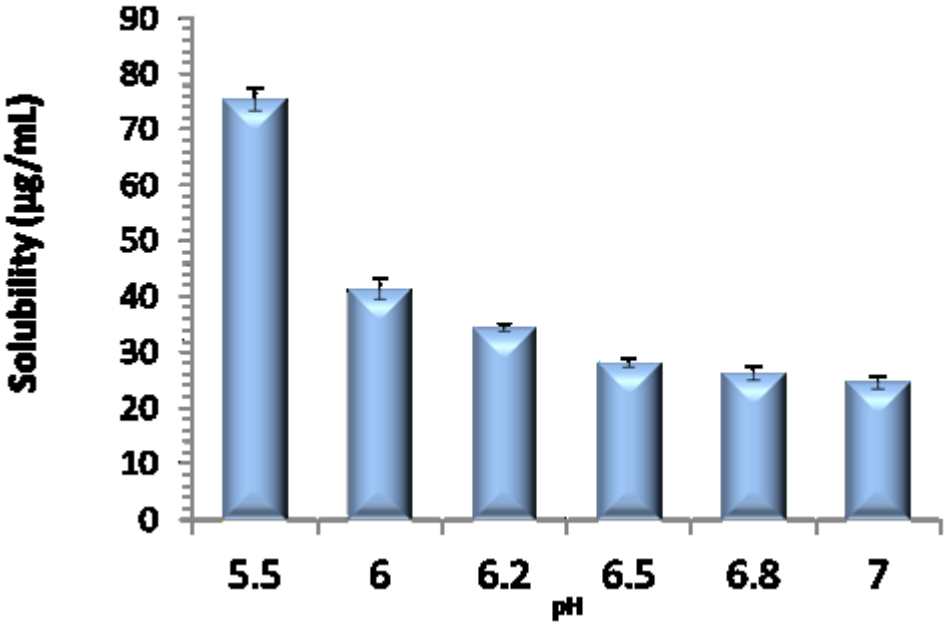


Figure 3.3: Dipyridamole solubility as function of pH in simulated intestinal fluids

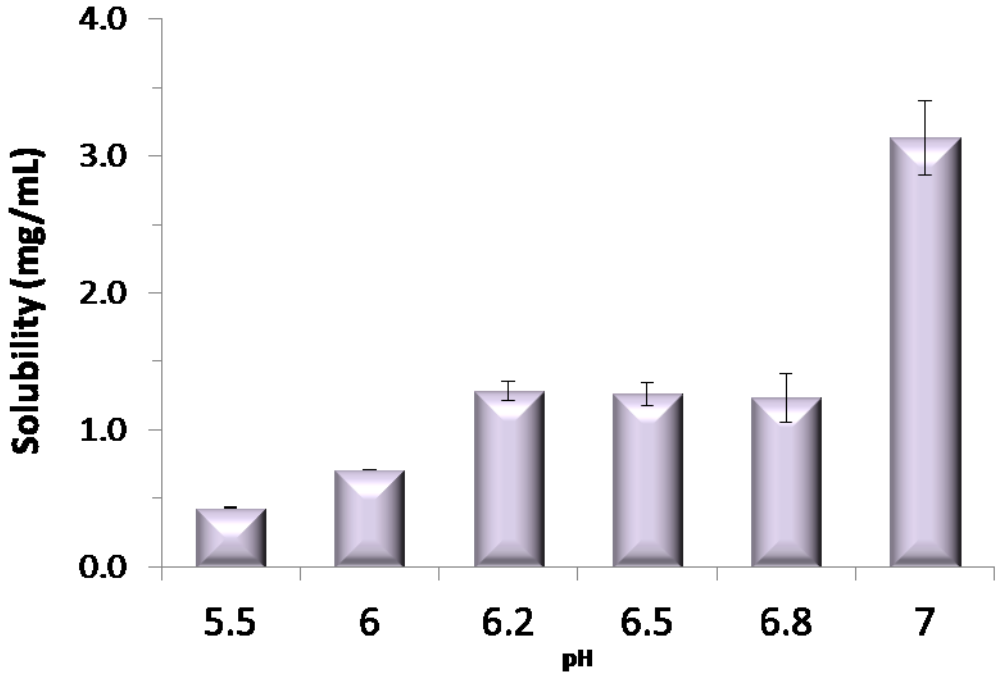


Figure 3.4: Furosemide solubility as function of pH in simulated intestinal fluids

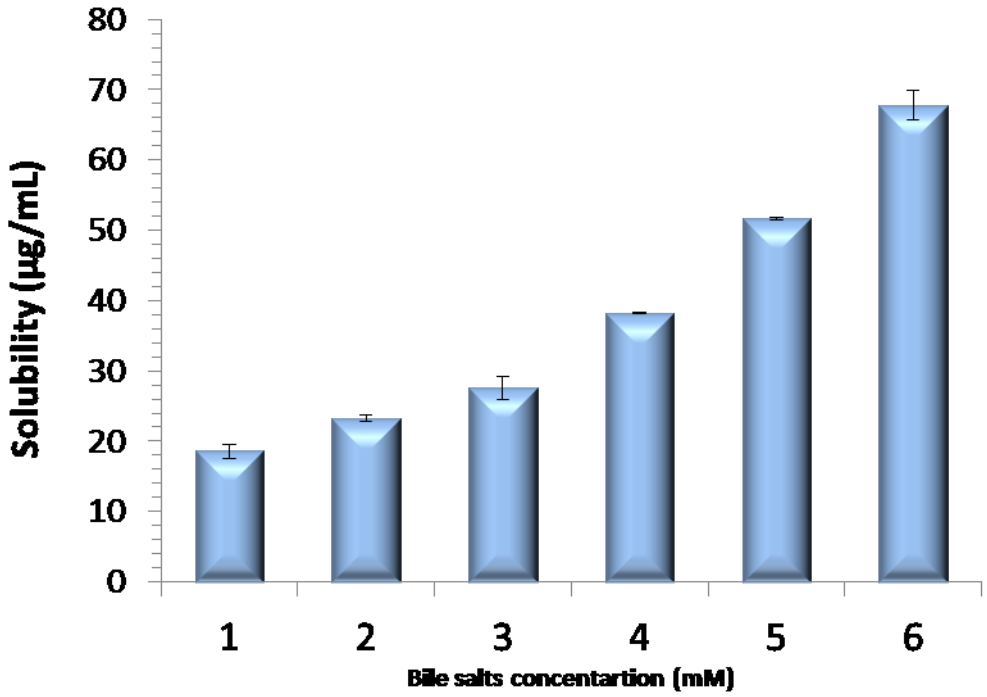


Figure 3.5 : Dipyridamole solubility as function of bile salt concentartion in simulated intestinal fluids

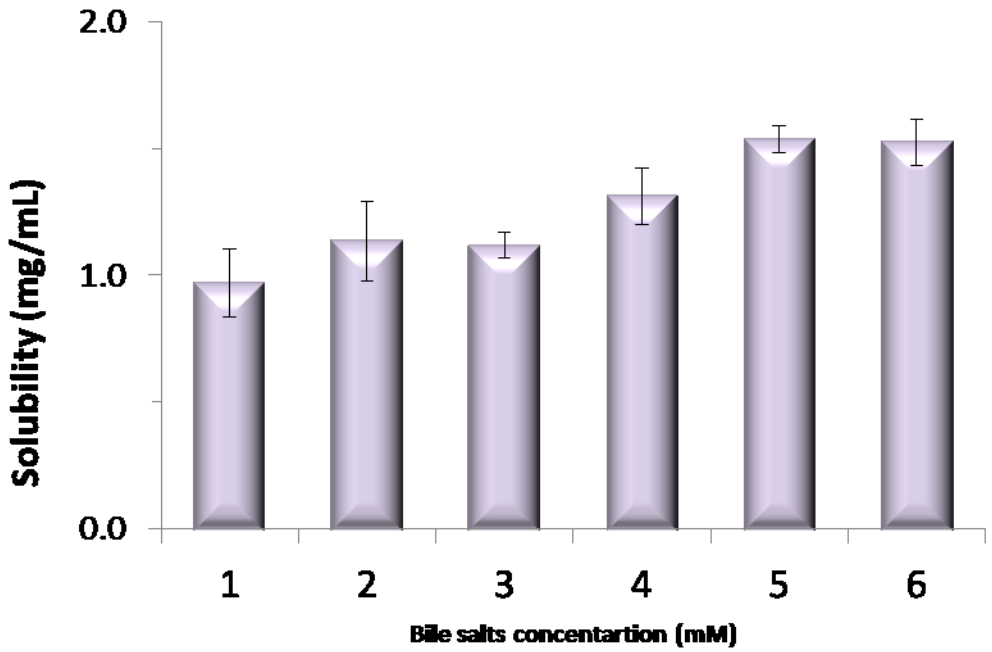


Figure 3.6: Furosemide solubility as function of bile salt concentartion in simulated intestinal fluids

A practical approach to estimating drug solubility in the GI tract is to aspirate fluids from the human GI tract and measure the solubility in these fluids '*ex vivo',* albeit one hindered by numerous ethical and practical concerns regarding the safety of healthy subjects. Moreover, the amount of fluid which can be aspirated is small, and individual samples are usually pooled to produce a large volume of samples for solubility measurements. In this study, the solubility experiment and the measured parameters in individual subjects were carried out in ileostomy fluids taken from UC subjects. These fluids are readily available, easy to collect and the volume obtained from ileostomy bags is sufficiently large to perform individual solubility measurements. Therefore, it was possible to analyse the complexity of intestinal fluids to further underline the factors that might affect variability between individuals in drug solubility.

Although many researchers have investigated the inter-subject variability of GI fluids (Augustijns *et al*., 2013), few have correlated GI fluid variability to that of variability in drugs solubility (Annaert *et al*., 2010; Clarysse *et al.*, 2011; Clarysse *et al.*, 2009b; Pedersen *et al.*, 2000a; Pedersen *et al.*, 2000c). Therefore, it is desirable to obtain correlations and an account of the possible factors that are important for drug solubility. Most of the published solubility measurements and correlations were conducted in aspirated duodenal and jejunal fluids. Despite the fact that dipyridamole and furosemide are commonly given in immediate release forms and their main site of absorption is the duodenum and jejunum, as poorly soluble drugs they are highly likely to reach the lower parts of the gut, namely the ileum and colon. Therefore, the solubility of these poorly soluble drugs in lower parts of the gut fluids is of particular relevance and interest, in particular in the light of low volumes of fluids availability in the lower parts of the gut.

For weak bases and acids, the pH changes along the GI tract influence drug solubility. The solubility of furosemide (weak acid, pka 3.9) in gastric fluids is very low due to the acidic condition of the stomach fluids. The solubility was shown to increase in intestinal fluids as expected mainly due to the increase in the pH of the fluids. Furosemide solubility in pooled duodenum fluids was reported as 1.9 and 3.8 mg/mL by Clarysse et al. (2011) and Heikkilä et al. (2011). Similarly, due to the pH increase, dipyridamole solubility (weak base pka 6.4) decreased from the stomach to the jejunum. In previous reports, the dipyridamole solubility in pooled duodenum and jejunum fluids was 0.022 and 0.029 mg/mL, respectively, (Kalantzi et al., 2006; Söderlind et al., 2010) higher than the value reported herein. This can be

explained by pH differences as pH was measured at 6.7 in these reports and herein the pooled jejunum fluids pH was 7.4.

Using simulated fluids as a predictor for drug solubility *in vivo* is a simplified method of the more complex situation *in vivo*. In a recent review publication, Augustijns *et al*. (2013) investigated the correlation between the solubilising capacity of FaSSIF versus fasted human intestinal fluids. A relatively strong correlation (R^2=0.85) was observed. A better correlation for neutral molecules was obtained compared to ionisable molecules. This indicates that interplay of a few parameters of the intraluminal fluids affects the solubility of these ionized, lipophilic compounds. Nevertheless, it was concluded that for an initial estimation of drug solubility, these findings show that FaSSIF can be used for drug solubility screening to predict solubility in human intestinal fluids. In this study, furosemide solubility in FaSSIF underestimated furosemide solubility in the upper parts of the gut, whereas dipyridamole solubility in FaSSIF overestimated dipyridamole solubility in the upper parts of the intestine. FaSSIF pH was 6.5 while pH of the pooled jejunum fluid was 7.4, and this increase in pH might explain the increase in dipyridamole solubility and the decrease in furosemide solubility in FaSSIF.

With regard to characterisation of the ileostomy fluids, osmolality measurements reported herein were higher than the normal values published in healthy subjects (124-278mOsmol/kg in fasted state and 250-367mOsmol/kg in the fed state). Ileal fluids from healthy subjects and ileostomy fluids differ mainly in their volume, with approximately 1.5L of fluid passing through the ileo- caecal valve per day. However, the average ileostomy contents are less than a third of this volume (Fadda, 2007), and so we would expect ileostomy fluids to therefore be more concentrated. Furthermore, transit through the final part of the ileum is slower in ileostomates compared to normal subjects. High retention time in the ileum generates greater exposure to the bacterial flora, indirectly facilitating the generation of metabolites such as short chain fatty acids, thereby increasing the osmolality in ileostomy fluids compared to ileal fluid (Ladas *et al*., 1986). Vertzoni *et al*. (2010) characterised the ascending colon fluids from subjects with ulcerative colitis with regard to surface tension and osmolality. Surface tension was found to be around 41mN/m, with no difference between subjects in remission or in relapse. Osmolality was 290 and 199mOsmol/kg in subjects in remission and in relapse states respectively, with no significant difference, implying high variability in the fluids of these subjects. pH in ileum fluids from patients was found to be around 7.3 -7.7, and no significant

decrease was observed compared to healthy subjects (Ewe *et al*., 1999). In view of the inter-subject variability found in ileostomy fluids, it can be seen that for most of the intraluminal measured parameters, the variability is intermediate (CV<20%). However, for the buffer capacity parameter, great variability was found between the individuals (CV=60%). As described, some of the factors that can cause inter-subject variability in healthy subjects include food intake, gut secretions, age and gender. The severity of the disease and the inflammation location along the gut can also vary markedly between patients, likely affecting the absorption and secretion of ions, bile salts and fatty acids, and all of which will influence the solubility of ionizable drugs. As the diet of the individuals in this study was not controlled, however, the data presented should also reflect the variability among individuals with varying diets, reflecting circumstances in everyday life. The combination of all these factors can explain the high variability in the solubility of both drugs.

The positive correlation to pH in the case of furosemide in both simulated fluids and ileostomy fluids is quite surprising. As discussed, furosemide is a weak acid with pka of 3.8, and its solubility- dependent pH would not be expected at this pH range. A possible explanation of its pH dependent solubility in that range might be related to the buffer capacity of these fluids. In our *in vitro* measurement at equilibrium drug solubility, pH values in the human and simulated media were decreased for furosemide experiments due to an excess of weak acid (attributable to the low buffer capacity of the fluids). For example, the final pH values decreased by 1 to 0.5 units in the simulated media relative to the starting pH. The changes in pH may affect the saturation solubility of the drug, as previously described by Avdeef (2007). *In vivo*, if the fluids buffer capacity is low and the administered dose is high, this may likely cause changes in the intraluminal pH. A further decrease in the gut fluids pH might also be observed in the case of a weak acid, hence decreasing drug solubility.

Further regression analysis was performed to understand the effect of both pH and buffer capacity on the solubility of furosemide in ileostomy fluids. Standard multiple linear regression (SPSS statistics software release 22.0.0, SPSS Inc., Chicago, IL) was used to explore the influence of pH and buffer capacity of intestinal fluids on the solubility of furosemide. Solubility was found to be dependent on both buffer capacity and pH (R^2 =0.983, F=57.98 p<0.01), and it was found that the solubility of furosemide increases with higher buffer capacity and higher pH values (Figure 3.7). Equation 3.3, obtained by multiple

regressions, enables the prediction of solubility of furosemide in the human intestinal fluids based on the knowledge of pH and buffer capacity in the range measured in this publication.

$$S = -3.569 + 0.523(pH) + 0.41(pH * \beta) - 0.174(\beta)$$

Equation 3.3: The effect of buffer capacity and pH on furosemide solubility

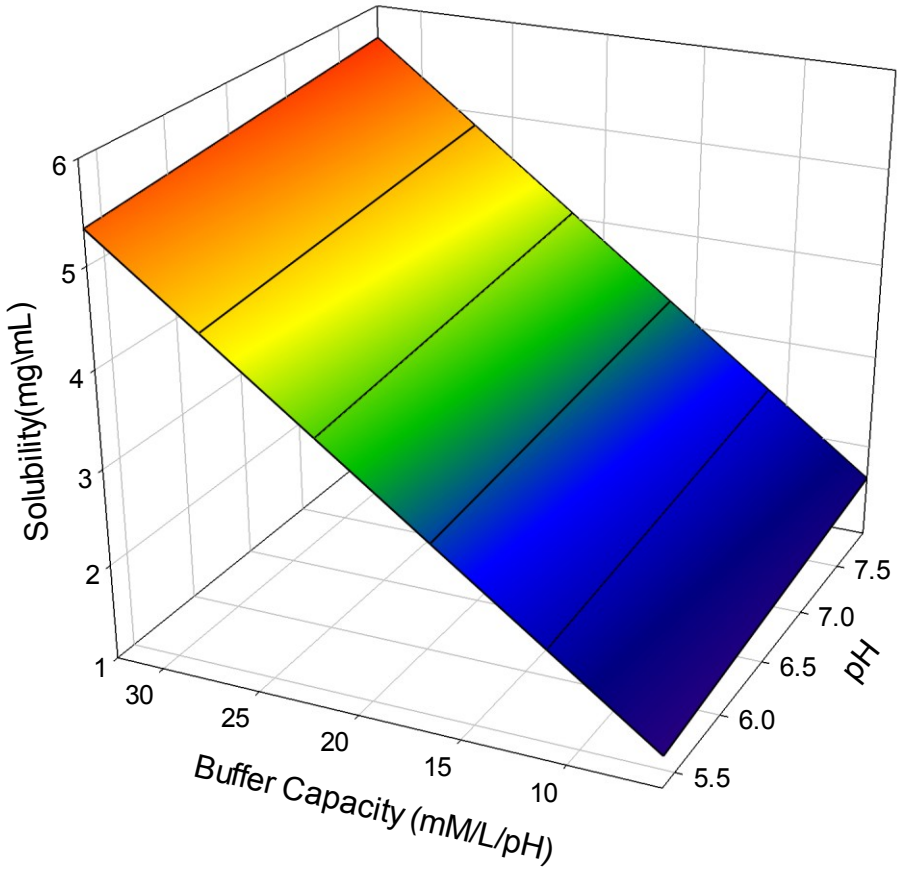


Figure 3.7: The Effect of Buffer capacity and pH on furosemide solubility

Aside from pH and buffer capacity, furosemide solubility also increased with bile salt concentration in simulated fluids. Therefore, it is reasonable to assume that due to its low solubility in gastric fluids and considering its lipophilic nature, solubilization of furosemide in intestinal fluids may be dependent on the presence of bile salts in the intestine. It can be seen that for furosemide, pH changes in simulated fluids yield a 90% increase in solubility, whereas bile salt concentration changes from the lowest to the highest in simulated intestinal fluids yield an increase of only 30%. Buffer capacity changes furthermore cause an increase of 45% in solubility, indicating that both pH and buffer capacity play important roles in influencing the solubility of furosemide *in vivo*.

Dipyridamole is a weak base with pka of 6.4. As expected for weak bases, the solubility is significantly influenced by changes in pH in both ileostomy fluids and simulated fluids. Therefore, significant differences can be anticipated between individuals with different pH values along the GI tract. However, with weak basic drugs, there is a need to take into consideration possible precipitation when transferring from low gastric pH to the higher pH in the duodenum. It is reasonable to assume that changes in factors such as gastric pH and gastric emptying time will add to the complexity of GI fluids composition, and augment an increased variability in drug solubility. Furthermore, dipyridamole showed an increase in solubility as bile salts concentration increased in simulated fluids. It can be assumed that the effect of bile salts will be more significant in the intestine, owing to their lower solubility in a region of higher pH.

3.2.6 Summary

In this study, the regional gastrointestinal solubility of furosemide and dipyridamole – two model drugs with reported high inter-subject variability in bioavailability in man - was investigated. Characterization of ileostomy fluids from individuals revealed high variability for buffer capacity and to a lesser degree for pH. Solubility measurements in ileostomy fluids for both furosemide and dipyridamole showed high inter-variability. The correlation analysis to solubility measurement showed that dipyridamole solubility in these fluids is pH-dependent, whereas furosemide solubility was highly correlated to buffer capacity and pH. Simulated intestinal fluids were used to investigate possible effects of bile salt concentration on drug solubility; dipyridamole solubility correlated with bile salt concentration in the fasted state, while slight variation in furosemide solubility was observed in the same bile salt concentration range. Based on these results, it was decided to further investigate the rate and extent dissolution of dipyridamole under different conditions of pH and bile salt concentration in bio-relevant media.

3.3 Evaluation of the Effects of Bile Salt and pH on the Dissolution of Dipyridamole and Furosemide

3.3.1 Introduction

3.3.1.1 *In Vitro* Dissolution Tests

There is an increasing interest in the development of dissolution tests to establish *in vitro in vivo* correlations (*IVIVC*). A number of GI factors should be considered when developing *in vitro* dissolution test models, such as pH, ions, surfactants, lipid, enzymes, volumes, flow rate, viscosity, and mechanical stress. In addition, the variation of these factors may also be included in the *in vitro* model.

Many attempts have been made to simulate the dissolution rate and extent of different formulations *in vitro*, the most conventional and widely used systems being the USP I/II apparatus, in which dissolution is performed in litre round-bottom vessels usually containing 900mL of dissolution media and either a rotating basket or rotating paddle to mimic GI hydrodynamics. More recent developments based on these models have included the USP apparatuses III/IV to TIM-TNO's intestinal model, which incorporates different GI compartments (Kostewicz *et al.*, 2013). Further attempts have also been made to develop bio-relevant dissolution media and so improve the *IVIVC*. The stomach is the main region where IR drug products disintegrate after oral administration, and the acidic pH environment here can be crucial for the dissolution of poorly soluble weakly basic compounds: Indeed, in the fasted state, weakly basic drugs dissolve primarily in the stomach, whereas the weak acid will remain largely undissolved. In order to assess dosage form performance in the stomach, simulated gastric media are often employed - the most simple dissolution media simulating gastric fluids being that media used in the USP method. Vertzoni *et al*. (2005), for instance, developed a FaSSGF media with more relevant pH, surface tension and pepsin level values, and low levels of taurocholate, along with other attempts by other researchers to make dissolution media utilised more clinically relevant (Pedersen *et al*., 2013; Vertzoni *et al*., 2005). For the lower compartments of the gut, Dressman *et al*. (1998) proposed and evaluated the fasted stated simulated intestinal fluids (FaSSIF) and the fed stated simulated intestinal fluids (FeSSIF) as bio-relevant media to test the dissolution of poorly soluble drugs (Vertzoni *et al*., 2004). Second-generation simulated media in turn are largely based on better understandings of gastrointestinal conditions and contents (Jantratid *et al*., 2008), and Fotaki

and Vertzoni (2010) published a comparative account of different media in relation of IVIVC. The content and properties of these media are described in Table 3.5 and Table 3.6.

Table 3.5: The content and properties of FaSSIF media (Jantratid et al., 2008)

	FaSSIF	**FaSSIF-V2**
Sodium taurocholate (mM)	3	3
Lecithin (mM)	0.75	0.2
Dibasic sodium phosphate (mM)	28.65	
Maleic acid (mM)		19.12
Sodium hydroxide (mM)	8.7	34.8
Sodium chloride (mM)	105.85	68.62
pH	6.5	6.5
Osmolality (mOsmol/kg)	270±10	180±10
Buffer capacity (mmol/L/pH)	12	10

Table 3.6: The content and properties of FeSSIF media (Jantratid et al., 2008)

	FeSSIF- V1	**FeSSIF-V2**
Sodium taurocholate (mM)	15	10
Lecithin (mM)	3.75	2
Glyceryl monooleate (mM)	--	5
Sodium oleate (mM)	--	0.8
Acetic Acid	144	--
Maleic acid (mM)	--	55.02
Sodium hydroxide (mM)	101	81.65
Sodium chloride (mM)	173	125.5
pH	5	5.8
Osmolality (mOsmol/kg)	635±10	390 ± 10
Buffer capacity (mmol/L/pH)	76	25

It can be seen, however, that the buffer capacity of 10mM/L/ΔpH (fasted) and 25mM/L/ΔpH (fed), used in the updated version is still higher then what has been measured in luminal fluids (Fadda *et al*., 2010a). In addition, the ion composition in these media is not representative of physiological fluids in humans; small intestinal luminal fluids are buffered primarily by bicarbonate, which is secreted by the pancreas and epithelial cells in the gut. Aside from pH, the constituent buffer salts, ionic strength and buffer capacity of the dissolution media can influence the drug release from ionisable polymers and compounds (Boni *et al.*, 2007; McNamara *et al.*, 2003; Sheng *et al.*, 2009).

Sheng *et al.* (2009) investigated the significance of physiological buffer species and concentration without inclusion of any bile salts in dissolution testing of BCS II acidic drugs: It was found that the higher the concentration of bicarbonate, the faster the drug flux. However, the intrinsic dissolution rates in phosphate buffers were higher than in all bicarbonate buffers, even when pH was maintained and the same buffer concentration was used. It was concluded that not only the pH but also the buffer species and concentrations should be considered in composing the *in vitro* dissolution media to closely reflect the *in vivo* dissolution fluids. That would be highly important to consider for weak compounds with pka values close to or higher than the intestinal pH range. For weak bases with pka lower than the pH range and very low solubility, the intrinsic flux is independent of the buffer species or concentration. Hence, bicarbonate buffers can be said to closely resemble the intestinal environment, and provide a more physiological medium for the *in vitro* assessment of drug release.

Based on the results from the previous section where pH and bile salt were found to have an effect on the drugs solubility, further investigations into these conditions impact on the extent of drug release and the dissolution rate were carried out. Bicarbonate buffers which were further modified by Fadda *et al*. (2009b) and Liu *et al.* (2011) were utilised in this research to better evaluate ionisable drug release, under different conditions of pH and bile salt concentration simulating different individuals. As has been described, the bicarbonate buffer mimics the intestinal fluids closely in regards to buffer capacity and ion composition, but lack bile salts otherwise present in human intestinal fluids. In this investigation, the bicarbonate buffer used was modified by adding crude bile salts. In addition, since the buffer conditions resemble the intestinal fluids from the average person, a number of further changes were made to the buffer pH and bile salts concentration in order to mimic 9 different individuals in the fasted state, and to understand how these differences affect the dissolution rate and extent. Moreover, to understand how different conditions in the gastric fluids with respect to pH and bile salt concentration affect the dissolution of weak acid, FassGF was utilised in this research. pH and bile salt concentration ranged from 1.2 to 2.5 and 0.03 to 0.6mM respectively, based on a recent publication from Pedersen *et al*. (2013).

3.3.2 Objectives

- To test dipyridamole and furosemide dissolution in the form of the active pharmaceutical ingredient and the commercial tablet in Hanks buffer under different conditions of pH and bile salt concentration to simulate possible changes between individuals in the proximal small intestine in the fasted state, and investigate possible effects on drug dissolution.
- To test the dissolution of furosemide in FaSSGF under different conditions of pH and bile salt concentration to simulate possible changes between individuals in the stomach in the fasted state, and investigate possible effects on drug dissolution.

3.3.3 Materials

3.3.3.1 Chemicals and Supplies

Magnesium sulphate heptahydrate, sodium hydroxide, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, potassium chloride, calcium chloride were purchased from VWR (Dorset,Uk). Carbon dioxide gas and medical oxygen gas were purchased from BOC (UK). Bile salt (B8756) and pepsin from porcine gastric mucosa (77161) were purchased from Sigma. SIF powder containing taurocholate and lectin was purchased from biorelevant.com. Dipyridamole (D9766) and furosemide (F4381) of pure pharmaceutical grade, in crystalline powder form, were obtained from Sigma-Aldrich (St Louis, USA). Dipyridamole Tablets 100 mg and Furosemide 40 mg BP Tablets, from Generics UK Ltd (Potters Bar, England) and Teva UK Ltd (Eastbourne), respectively, were selected as immediate release commercial solid dosage forms.

3.3.3.2 Instruments

USP-II Dissolution Apparatus (PTWS, Pharma Test, Hamburg, Germany) controlled by software IDIS EE2.11.16 (Icalis Data System Ltd., Berkshire, UK) equipped with in –line UV spectrophotometer (Cecil 2020, UK), pH meter (pH 211 Microprocessor) equipped with H11131 probes (Hannah Instruments, Bedfordshire, UK), Sho-Rate gas flow meter (Brooks, Veenendaal, Netherlands) calibrated for carbon dioxide. The pH stabilization was confirmed through pH measurements about every ten minutes with a pH electrode (H11131, Hanna Instruments Ltd.) attached to a pH 211 Microprocessor (Hanna Instruments).

3.3.4 Methods

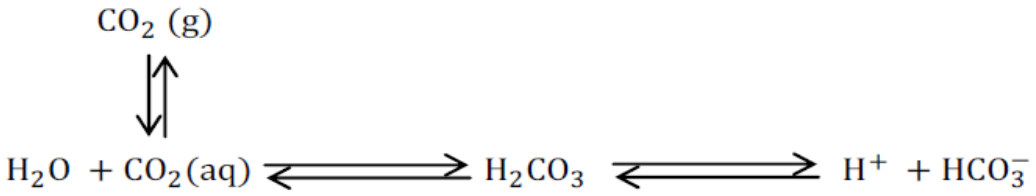
3.3.4.1 Buffers Preparation

- ***m*Hanks**

Hanks balanced salt solution closely resembles the ionic composition of the small intestinal fluids, however, it has a pH of 7.4, which is too high, and a buffer capacity of 1 mmol/L/ΔpH, which is too low, as compared to that of human jejunal fluids (Table 3.7). Consequently, this buffer was further modified to achieve a pH of 6.8 and a higher and more relevant buffer capacity by Liu *et al.* (2011). Hanks solution is primarily a bicarbonate buffer, in which bicarbonate (HCO_3^-) and carbonic acid (H_2CO_3) co-exist, along with CO_2 (aq) resultant from the dissociation of the latter (Equation 3.4).

Table 3.7: Comparison of the ionic composition (mM) and buffer capacity of the small intestinal fluids, phosphate buffer and *m*Hanks adopted from Liu *et al.* (2011)

Composition	Human Jejunal fluids	Phosphate bufeer (0.05M)	*m*Hanks buffer
Bicarbonate	7.1	Not present	4.17
Phosphate	0.8	50	0.8
Potassium	5.1	50	5.8
Sodium	142	29	142
Chloride	131	Not present	143
Calcium	0.5	Not present	1.3
Magnesium			0.8
pH	6.8	6.8	6.8
Buffer Capacity (mmol\L\ΔpH)	3.2	23	3.1



Equation 3.4: Bicarbonate disassociation

Where bicarbonate (HCO_3^-) and carbonic acid (H_2CO_3) coexist, along with $CO_{2(aq)}$ resultant from the dissociation of the latter. The pH of the buffer system can be altered by adjusting the concentration of the acid (H_2C0_3) and its conjugate base (HCO_3^-). Therefore, purging $CO_{2(g)}$ into Hanks buffer in excess increases the concentration of $CO_{2(aq)}$, promoting the formation of carbonic acid and thus resulting in a decrease in the pH of the buffer system.

The modified Hanks bicarbonate buffer was prepared by mixing 136.9mM NaCl, 5.37mM KCl, 0.812mM $MgSO_4*7H_2O$, 1.26mM $CaCl_2$, 0.337mM $Na_2HPO_4*2H_2O$, 0.441mM KH_2PO_4 and 4.17mM $NaCO_3$ (Liu *et al.* (2011)) The preparation of the media included the addition of bile salts (mixture of sodium cholate and sodium deoxycholate) in the following concentration 1, 3, 6mM. pH was measured after the addition of bile salts and was around 7.4: To further adjust the pH, $CO_{2(g)}$ was purged into the buffer solution using polyurethane flow gas tubes (Freshford Ltd., Manchester, UK) to obtain pH 6.8, and for a longer time to obtain a pH of 6.4. The flow of the gas was monitored using the gas flow meter. Each tube was positioned 2cm below the liquid surface of its corresponding vessel at a very low flow rate (at pressure of 2mbar) compared to what had been used previously, so as to avoid significant influences in the hydrodynamics of the dissolution apparatus. Buffer capacity measurements for all buffers were carried out according to the method described in section 3.2.4.5.

Cross-over study design was used to simulate the conditions of different individuals' proximal small intestine with respect to pH and bile salt concentration. The following pH condition and bile salt concentrations were chosen as 6.4, 6.8, 7.4 and 1, 3, 6mM (Table 3.8). Increasing the concentration of bile salt further was not possible, however, given that the continuous purging of CO_2 with higher bile salt concentration produced foam in the vessel. Lower pH could also not be attained by purging CO_2 alone without changing the initial bicarbonate concentration in the media.

Table 3.8: Cross over study to simulate different individuals' proximal small intestine with changes in pH and bile salts concentration

pH \ Bile salt concentrations	**1mM**	**3mM**	**6mM**
6.4	X	X	X
6.8	X	X	X
7.2	X	X	X

.

- **FaSSGF**

Simulated gastric fluids, FaSSGF, were prepared according to Vertzoni *et al*. (2005). The concentrations of the components are given in Table 3.9. Some modifications were made with respect to f pH and bile salt concentration so as to mimic individual's variability in gastric fluids, based on publication from Pedersen *et al*. (2013), where aspirated gastric fluids were characterised. The pH was found to range from 1.16 to 5.96. The high measurement above pH 5, however, was probably due to the dilution of the gastric contents by saliva and/or nasal secretion, or due to high reflux from the intestine. In terms of bile salt concentration, a mean value of 0.3mM was found, ranging from 0 up to 0.6mM. Therefore, the following pH conditions were chosen to simulate differences between individuals as 1.2, 1.8 and 2.5 and 0.08, 0.3 and 0.6mM bile salts. Cross over study design was again used to simulate different individuals' stomach conditions with respect to pH and bile salt concentration (Table 3.10)

Table 3.9: Simulated gastric fluids composition Vertzoni et al. (2005)

Composition	
Sodium taurocholate(µM)	80
Lecithin (µM)	20
Pepsin (mg/mL)	0.1
Sodium chloride (mM)	34.2
Hydrocholic acid	QS pH 1.6
pH	1.6

Table 3.10: Cross over study to simulate different individuals' gastric fluids with changes in pH and bile salts concentration.

pH\Bile salt concentration	**0.08**	**0.3**	**0.6**
1.2	X	X	X
1.8	X	X	X
2.5	X	X	X

3.3.4.2 Drugs

Furosemide (40mg) and dipyridamole (100mg) were weighted into gelatine capsules, and the drug release under different conditions was evaluated using USP-II apparatus. In addition, the commercial immediate release tablets of furosemide and dipyridamole were tested under the same conditions. The amount release from capsules was determined using an in line UV spectrophotometer with 1 or 10mm flow cells at 285nm and 238nm for dipyridamole and furosemide, respectively. Data were processed using Icalis software and the tests were conducted in triplicate, in 900mL dissolution medium maintained at 37±0.5˚C. A paddle speed of 50 rpm was employed. The tests were lasted for 2 hours and pH was measured periodically along the experiment and was maintained at the desired pH ±0.5 by sparging CO_2 into the media.

3.3.4.3 Calculations and Statistical Analysis

The dissolution profiles were analysed by one way ANOVA repeated measurements using general linear model followed by a Tukey post- hoc analysis in PASW statistics 22 (SPSS Inc., Illinois, USA).

Dissolution Efficacy (AUC_{2h}/ $AUC_{Theoretical}$ *100) was calculated at 120 min and the effect of both bile salt and pH was evaluated by a mixed effect model. The experimental assay was adapted to the structure of a two factorial experimental design -pH and bile salts concentration. A multiple standard regression was used to quantify the effects of all variables under study on the dissolution of both drugs and to construct the corresponding response surfaces graph (SPSS, v.22).

Dissolution rate was calculated based on the following equation for all dissolution profiles (Equation 3.5).

$$\text{Dissolution rate (\% release/ min)} = \frac{(\text{Extent release T30min} - \text{Extent release T0})}{(\text{T30min} - \text{T0})}$$

Equation 3.5: Calculation for dissolution rate

CV % for the dissolution efficacy and dissolution rate was calculated by dividing the standard deviations to the mean.

3.3.5 Results & Discussion

Further modifications of *m*Hanks were implemented successfully in this research. Addition of bile salt up to concentration of 6mM did not change the initial pH and decrease in pH to 6.4 was achievable without changing the recipe composition. The continuous purging of CO_2 along the experiments did not create any foam during two hours of experiments. Buffer capacity was measured for all solutions. Buffer capacity increased with bile salts concentration (5mmol/L/ΔpH for media with 6mM bile salt concentration) and decreased with pH changes (1.4mmol/L/ΔpH for media with pH 7.4).

It can be seen that dipyridamole was not dissolved completely after 2 hours under all conditions (Figure 3.8). In addition, the dissolution rate and extent of dipyridamole release were influenced primarily by bile salt concentration. At higher bile salt concentration, the extent of release after 2 hours was around 60%. pH did not influence the drug release to a similar extent, as under different pH conditions at the same bile salts concentrations the percentage of release was not significantly different. It was also found that release from the commercial dipyridamole tablet followed the same dissolution pattern as for the active ingredient alone (Figure 3.9). The solubility/dissolution increase of poorly soluble drugs in the presence of bile salts can be attributed to two mechanisms: When the bile salts are present at a level below their CMC, adsorption of bile salts on the surface of dipyridamole particles may reduce their free surface energy and facilitate wetting and removal of molecules, with a parallel increase in saturation solubility in the bulk solution. However, at concentrations higher than the critical micelle concentration (CMC), bile salts should theoretically enhance drug dissolution by forming submicron-mixed micelles in which the lipophilic dipyridamole molecules are solubilised (Holm *et al*., 2013).

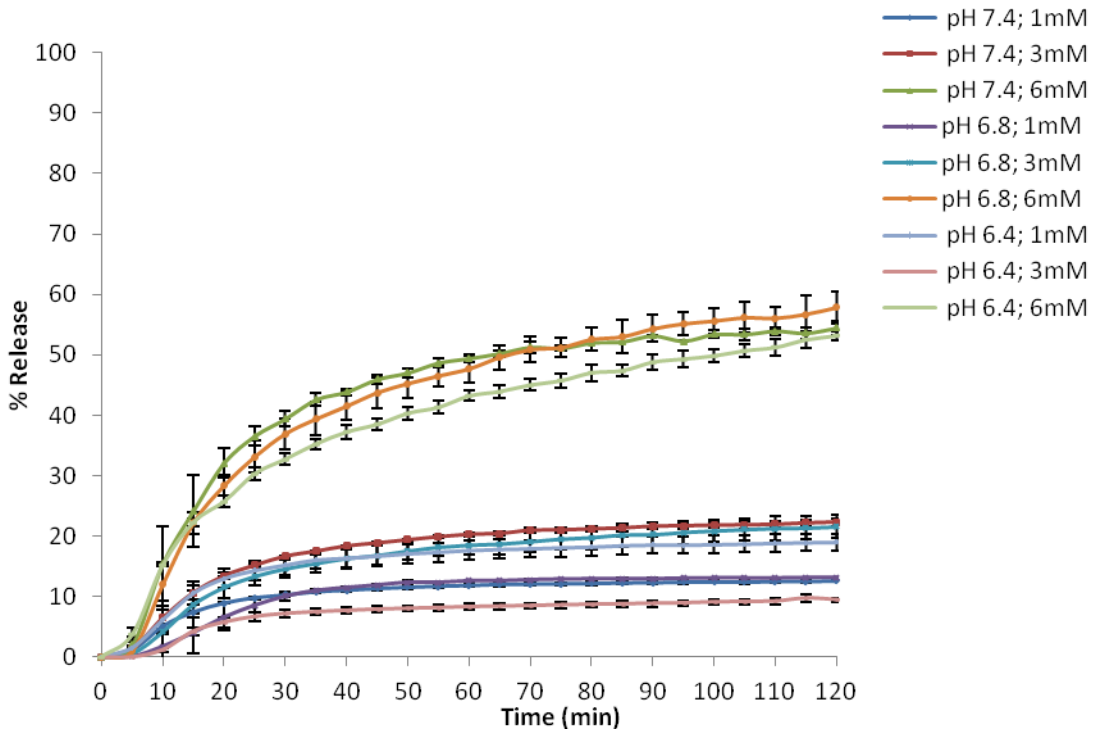


Figure 3.8: Dissolution of dipyridamole in *m*Hanks buffer under different conditions of bile salt and pH.

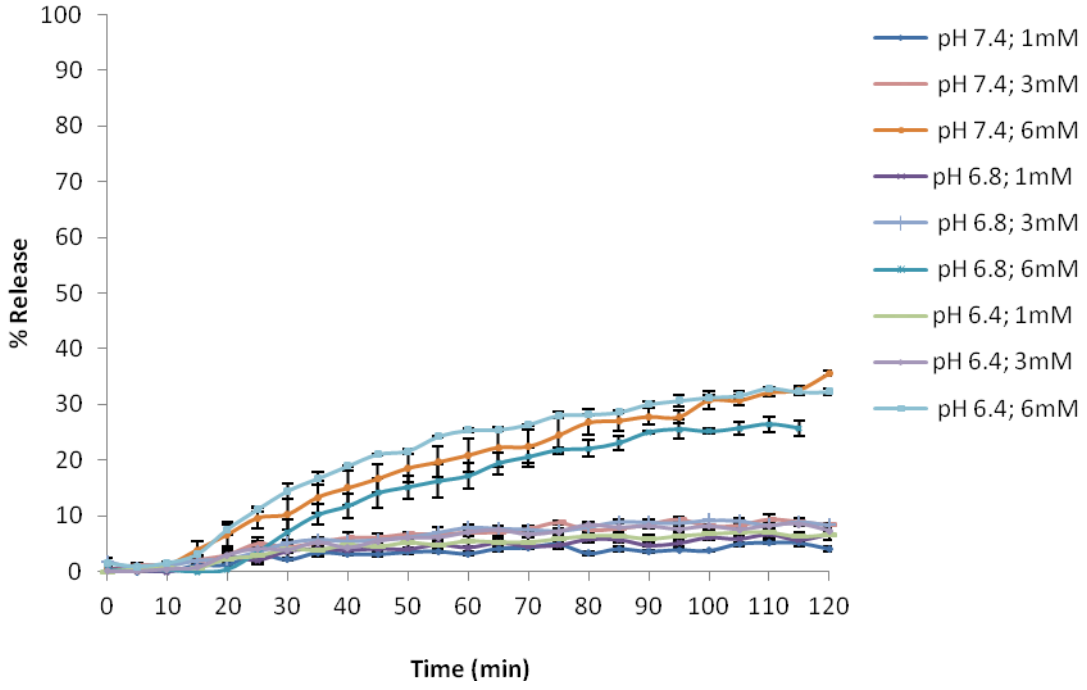


Figure 3.9: Dissolution of dipyridamole commercial tablet in *m*Hanks buffer under different conditions of bile salt and pH.

In comparing dissolution profiles of commercial tablets of dipyridamole and the API, it is safe to conclude that the excipients in the commercial tablet did not affect the drug dissolution and were not able to minimize bile salts effect.

Further regression analysis was performed to understand the effect of pH and bile salts concentration on the extent of dipyridamole release ($AUC_{0\text{-}120}$) of in *m*Hanks. Standard multiple linear regression (SPSS statistics software release 22.0.0, SPSS Inc., Chicago, IL) was used to evaluate the influence of pH and bile salts concentration in *m*Hanks on the drug release. The drug release was found to be primarily dependent on bile salt concentration and marginally on pH (R^2 =0.923, F=19.97 $p<0.01$). Equation 3.6, obtained by multiple regressions, enables the prediction of drug release in the *m*Hanks media based on the knowledge of pH and bile salts concentration in the range measured in this research.

$$AUC\ (0-120) = -36.24 - 4.873(pH) + 1.188(pH * \beta) + 0.143(\beta)$$

Equation 3.6: The effect of bile salts and pH on dipyridamole release in *m*Hanks.

Interestingly, at that clinical dose, the furosemide mean extent of release was around 80%. Furosemide dissolution did not show any particular trend as for pH and bile salt concentration (Figure 3.10) However, great variability in the percentage of release was observed under different conditions, ranging from 70 to 100% at 120 min (10% CV in dissolution efficacy under all conditions). In addition, high variability in the dissolution rate (between 0-30 min) was observed with calculated CV of 80%. Surprisingly, the dissolution of the commercial tablet gave around 70-80% release with low variation in the extent and dissolution rate (2% and 30% CV in dissolution efficacy and dissolution rate, accordingly) under all conditions (Figure 3.11).

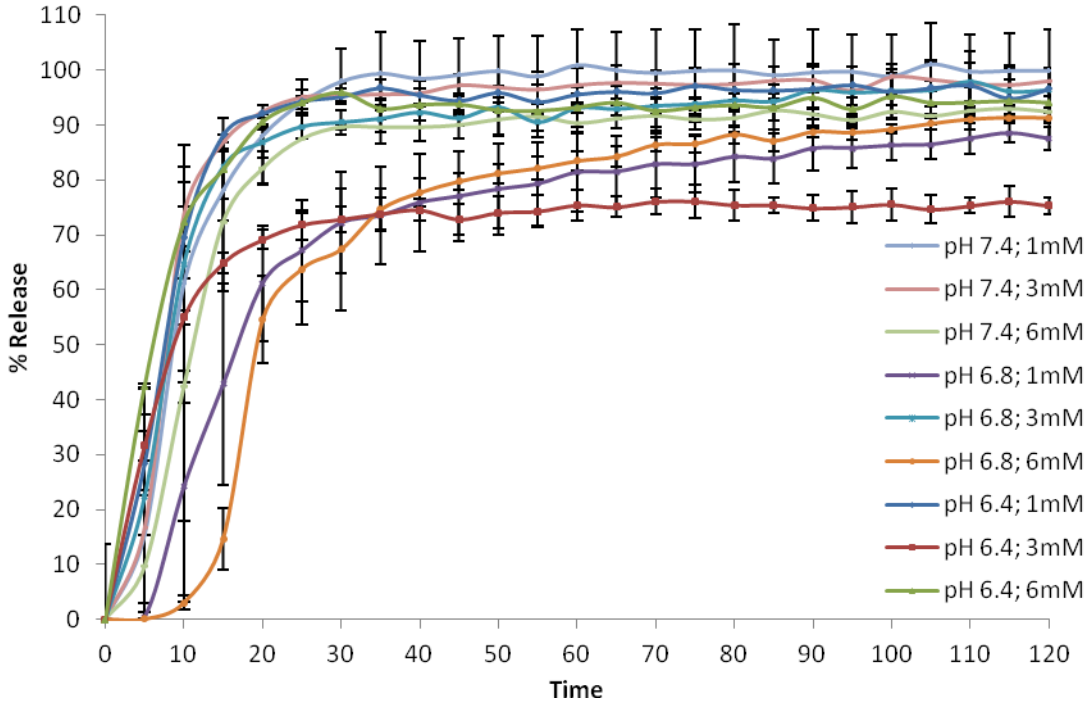


Figure 3.10: Dissolution of furosemide in *m*Hanks buffer under different conditions of bile salt and pH.

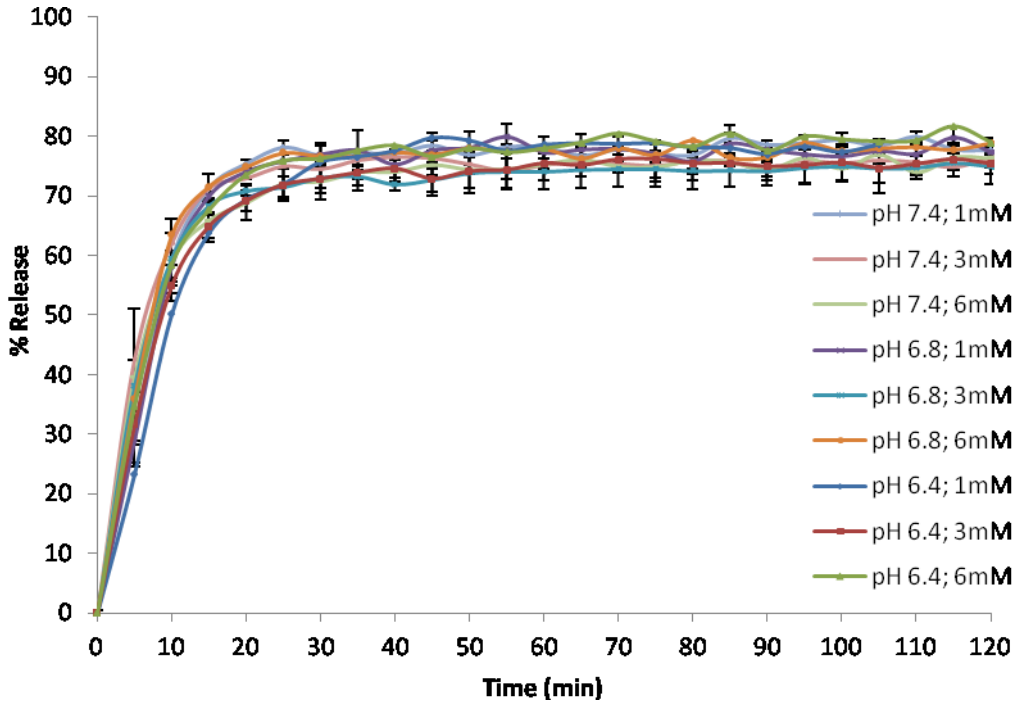


Figure 3.11: Dissolution of furosemide commercial tablet in *m*Hanks buffer under different condition of bile salt and pH.

As has been described previously, furosemide is a weak acid. As its disintegration and dissolution first occur in the stomach for immediate release forms, changes in the stomach fluid need to be considered in terms of their effects on furosemide. The dissolution of furosemide under different conditions of both API and the commercial tablet in the stomach gave a low percentage release from 8 to 16%. Again, no trend was observed as for pH or bile salt concentration changes, with great variability around dissolution rate and percentage of release at 120 minutes (Figure 3.12 and Figure 3.13).

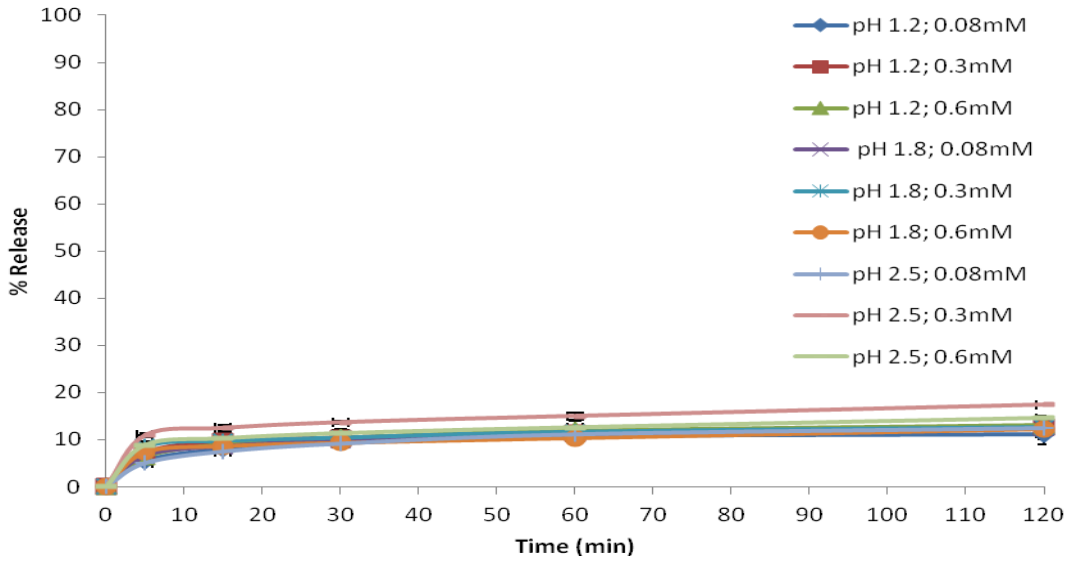


Figure 3.12: Dissolution of furosemide in FaSSGF buffer under different conditions of bile salt and pH.

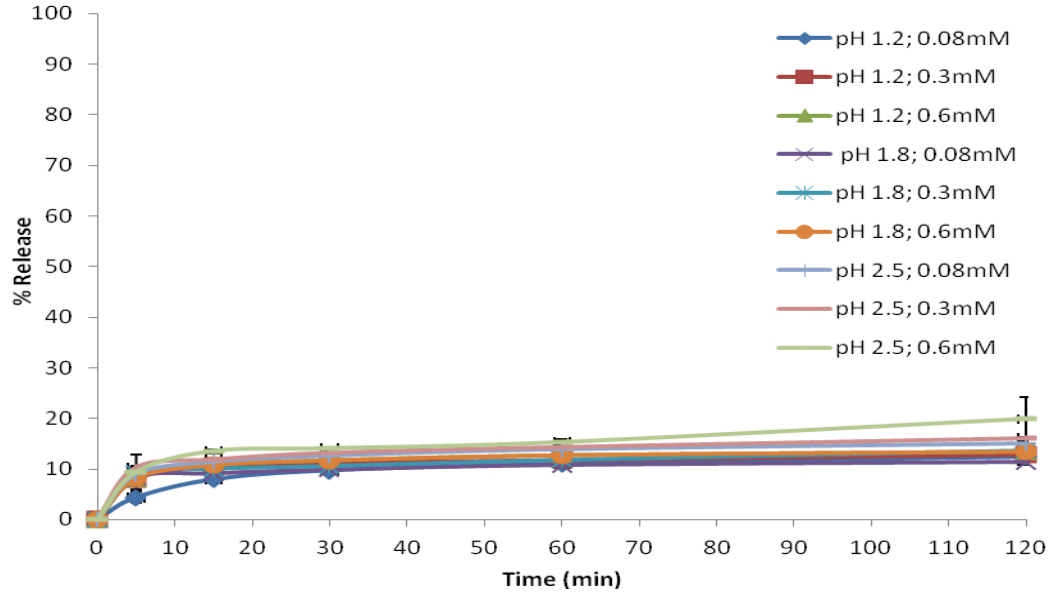


Figure 3.13: Dissolution of furosemide commercial tablet in FaSSGF buffer under different conditions of bile salt and pH.

The great variability under different conditions of furosemide API can be attributed to the wetting capacity of the powder after the gelatine capsules dissolved. Based on the lab work experience with furosemide, furosemide powder was very difficult to handle (fluffy and had static properties). It was observed that in a simple buffer solution, furosemide was not dispersed equally and instead agglomerates to bigger particles. This was not observed in the commercial tablet dissolution, and it may well be that the excipients or compressing the powder in the form of tablet contributed to the wetting and dispersion of the tablet. In section 3.1, it was found that saturated solubility of furosemide is both pH and bile salt dependent. However, when examining the dissolution in bicarbonate buffer and FaSSGF, no dependency on pH or bile salts was observed. This can be attributed to the low ratio of dose to the dissolution volume. However, the high variability in the rate of furosemide dissolution observed *in vitro* due to the powder characterisation might explain some of the variability *in vivo*. Moreover, based on the low dissolution of furosemide in the stomach, it might be that erratic gastric emptying time between individuals will markedly influence the drug dissolution rate and eventually on the extent of absorption in combination of variation in intestinal transit time.

3.3.6 Summary

As for solubility studies, use of simulated intestinal fluids in dissolution test can provide valuable information about the different factors affecting changes in drug release between individuals. It is always preferable to simplify the situation in order to understand variability mechanism; therefore, based on the results from the solubility study in section3.2, bile salt and pH were investigated in relation to dissolution. It was found that dipyridamole dissolution was relatively low and varied significantly with increasing bile salt concentration and to a lesser extent with pH. The dissolution of furosemide was complete although fairly variable under all conditions and did not vary with increasing bile salt concentration or in the investigated pH range. These results are relatively surprising considering the results from the previous study. Moreover, as furosemide is classified as a BSC IV drug in the administered clinical dose, one would not expect complete drug release. Based on these results, it will be interesting to investigate if these conditions affect the drug permeability and how it affects the overall process of drug absorption.

3.4 Evaluation of the Dissolution and Permeability of Dipyridamole and Furosemide under Different Conditions of Bile Salt Concentration and pH

3.4.1 Introduction

3.4.1.1 Prediction of Absorption in the Dissolution Permeation System

In vitro evaluation of dissolution or solubility-limited absorption focuses on the estimation of intraluminal concentrations by dissolution and precipitation assessment in setups that ignore intestinal permeation. To predict drug flux and hence the fraction absorbed, estimated dissolved concentrations need to be combined with drug permeability. The advantages in modelling a set up that includes both dissolution and permeation assessments are that it better mimics sink conditions for highly permeable drugs. Secondly, it was reported that in some cases, developing new formulations in order to increase solubility, might compromise its permeability (Beig *et al*., 2012; Kostewicz *et al.*, 2013; Miller and Dahan, 2012; Miller *et al.*, 2011). As such, an account of both dissolution/solubility and permeability is highly valuable to simulate the dissolution and permeation process *in vitro*. The first published attempt to develop an integrated dissolution Caco-2 system to predict dissolution–absorption relationships describes a system where first dissolution occurs in bio-relevant media. Due to incompatibility of the Caco-2 monolayer to the conditions of the bio-relevant media, samples were treated to adjust its composition similar to Hanks balance buffer, and their permeability tested through the monolayer (Ginski and Polli, 1999) (Figure 3.14). It was found that the fast and slow formulations of piroxicam, metoprolol, and ranitidine, predicted dissolution-absorption relationships from a continuous dissolution/Caco-2 system qualitatively matching the *in vivo* data. However, it is important to note that this system requires that drug solubility and permeability are not adversely affected by conversion from the dissolution medium to the final Caco-2 donor solution. A decrease in solubility may precipitate the drug and reduce the donor concentration in the Caco-2 permeation studies. In addition, the pump flow rate might also affect the permeation assessment.

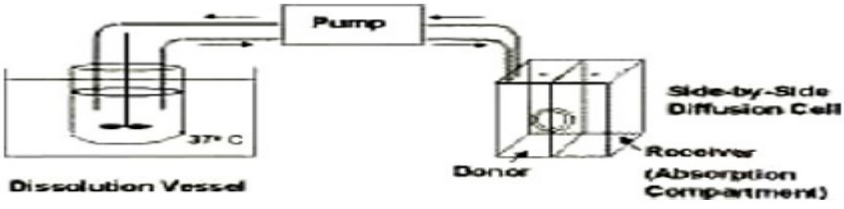


Figure 3.14: Dissolution/Caco-2 system developed by Ginski and Polli (1999)

Vertzoni *et al*. (2012) assessed danazol permeation through Caco-2 monolayer using real human intestinal fluids (Figure 3.15). Duodenal aspirates were collected after administration of the lipophilic drug danazol together with a meal, and increasing luminal lipid concentration reduced danazol permeability. However, increasing the solubility of danazol in the aspirates thanks to the lipids more than compensate for the reduced permeation flux obtained with the aspirates, leading to a higher overall rate of transport.

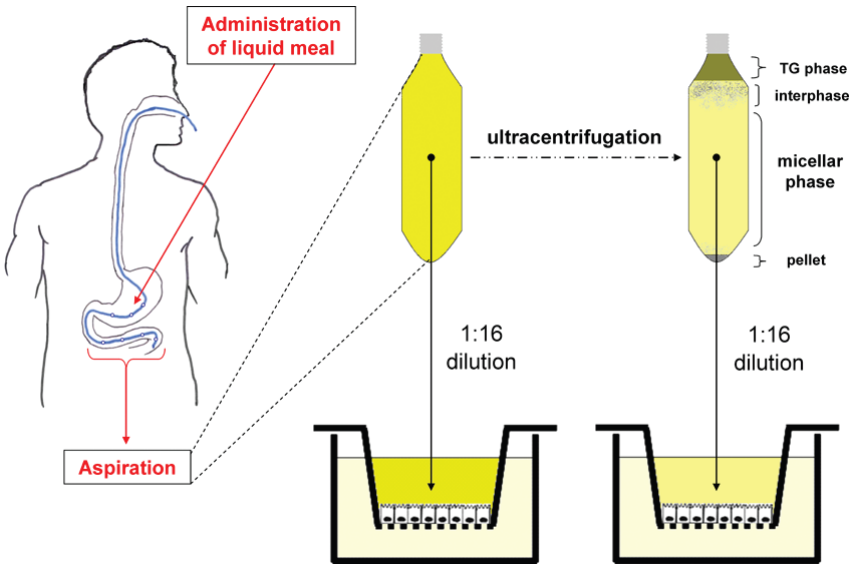


Figure 3.15: Assessment drug solubility by real human fluids following permeation through Caco-2 cells (Vertzoni *et al.*, 2012).

A further development of this system by addition of the gastric compartment was done by Kobayashi *et al*. (2001). This system was designed to enable prediction of the absorption rate of not only water-soluble drugs, but also drugs that have poor water solubility (Figure 3.16). For instance, dissolution and permeation of albendazole and dipyridamole from different formulations were tested in this system (Sugawara *et al.*, 2005).

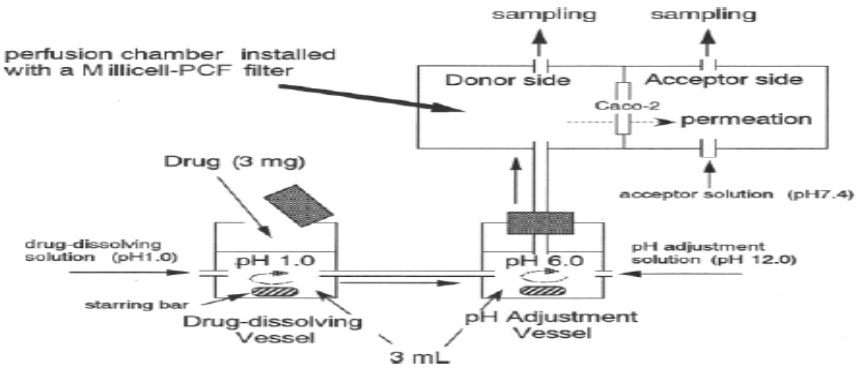


Figure 3.16: Adding gastric compartment to assess dissolution/permeation (Kobayashi *et al*., 2001)

Motz *et al*. (2007) utilized USP apparatus 4 combined with Caco-2 permeation flow cell using a stream splitter. The apparatus has been validated using several formulations of propranolol HCl, but its added value for more challenging drugs has not been reported (Figure 3.17).

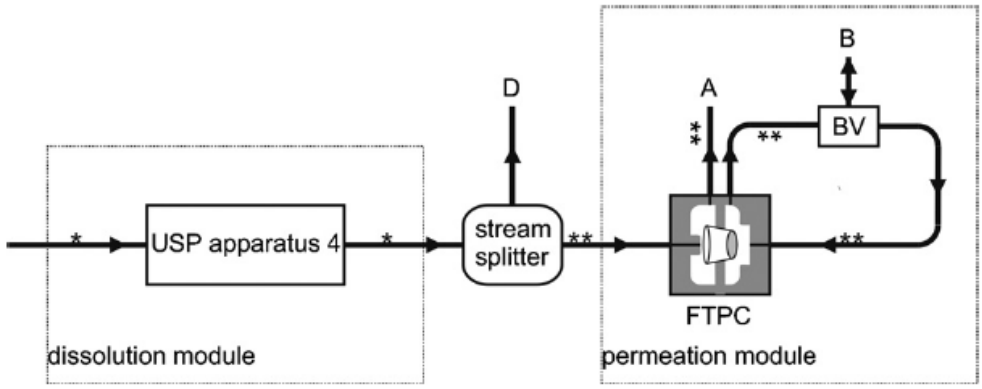


Figure 3.17: USP apparatus 4 combined with Caco-2 permeation flow modifications developed by Motz *et al*. (2007)

All the systems mentioned above integrated separate absorption compartments, and the transfer of the dissolved samples was dependent either on flow rate detected by pumps or sample treatment prior permeation assessment. Moreover, sink conditions were not maintained. Kataoka *et al.* (2012) optimized a side-by-side dual chamber system to allow for dissolution of solid dosage forms at the apical side of a Caco-2 cell monolayer, known as the dissolution –permeability system (D/P system- Figure 3.18).

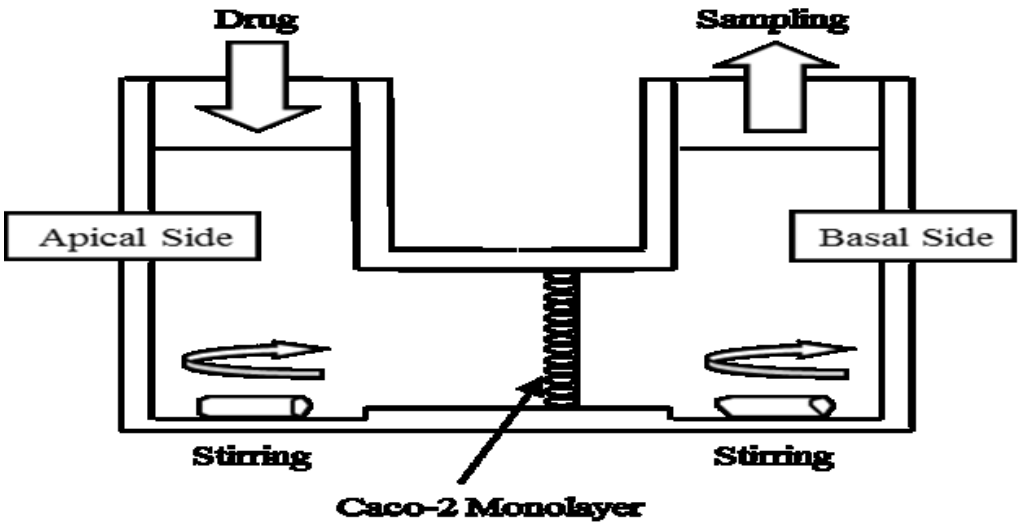


Figure 3.18: A side-by-side dual chamber system called the dissolution –permeability system developed by Kataoka *et al.* (2012)

In this setup, buffer media were optimized to simulated intestinal fluid. Since FaSSIF media based on phosphate ions due to its high osmolality caused a rapid decrease in the monolayer TEER (indicating damage to the monolayer integrity), a modified FaSSIF based on Hanks balanced buffer with low osmolality at pH 6.5 was used as the apical medium (8mL)(Kataoka *et al.*, 2006). Isotonic buffer (pH 7.4) with serum albumin was used as basal medium (5.5mL) to ensure sink conditions. Both compartments were stirred at 200rpm (Kataoka *et al*., 2003). The advantage of this system is that permeation and dissolution are determined simultaneously at the same time point, ensuring that dissolved drug can permeate the intestinal membrane. *In vitro–in vivo* correlations *(IVIVC)* using the D/P system was demonstrated by using clinically relevant doses. The group showed that an increased amount of the applied dose especially for poorly soluble drugs increased the permeated amount. Therefore, in order to evaluate *in vivo* absorption by using *in vitro* experiments with the D/P system, the applied amount should be decided based on the *in vivo* clinical dose of each drug. 1% of clinical dose should be applied to the D/P system when correlating the results to the *in vivo* absorption data. The explanation for that is the GI fluid volume was reported as approximately 500mL and 900–1000mL at fasted and fed state, respectively and considering the apical volume (8mL) in the D/P system, a proportion of 1/100 corresponds to about the *in vivo* volume. As such, a correlation between the human fraction absorbed and the permeated amount in the D/P system has been established for poorly water-soluble reference drugs (Kataoka *et al.*, 2003) (Figure 3.19).

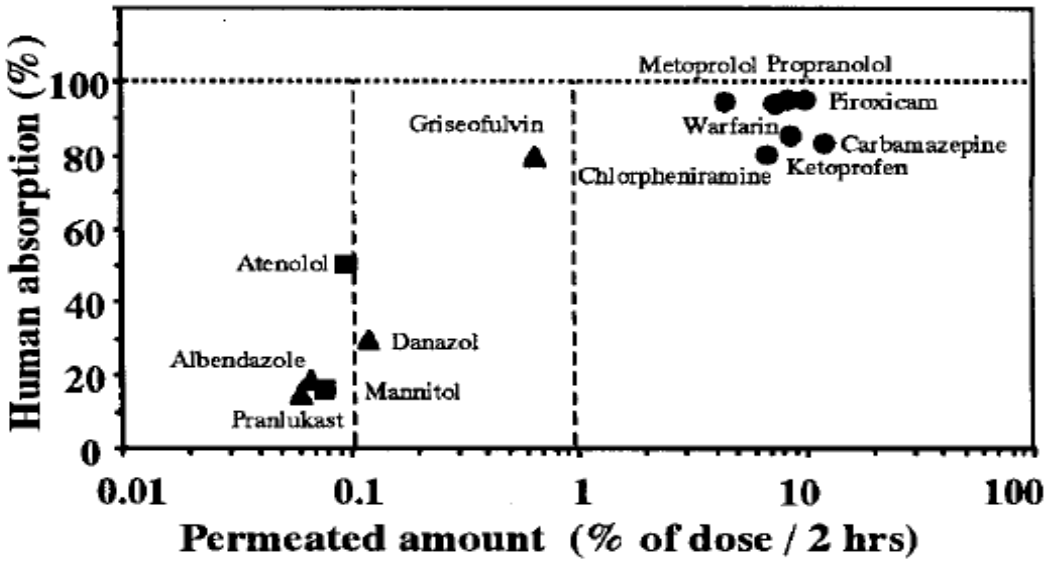


Figure 3.19: Correlation between *in vivo* human absorption and *in vitro* permeated amount in the D/P system (Kataoka *et al*., 2003) .

The D/P system has been further utilised to predict the net food effect on the absorption of poorly soluble drugs. 13 drugs were tested in the fed and fasted states, and good correlation was obtained to the fraction absorbed reported in the literature. In addition, the D/P system has also been used to rank the effectiveness of drug formulations to improve the oral absorption of poorly soluble drugs. Buch *et al*. (2009) tested different formulations (solid dispersion, nano- and microsized) of fenofibrate in the D/P system, and successfully predicted the formulation performance in rats. Similarly, Katoka *et al*. (2012) investigated the effect of different formulations of danazol (BCS II) and pranlukast (BCS IV) using the D/P system for evaluation of solubilizing and supersaturating effects. On the apical side, an increased extent of release was observed with both drugs in the D/P system; however, the permeated amount was only improved for danazol. Good *IVIVC* was obtained for danazol with increasing bioavailability in the rat, but was not observed for pranlukast. These results demonstrate the importance of simultaneously assessing dissolution and permeation when evaluating absorption-enhancing strategies. Another application of the D/P system demonstrated the use of the system to learn about transporter-related drug-drug, drug-food, and drug-excipient interactions. When erythromycin - an inhibitor of the efflux carrier P-gp – was applied to the apical medium, permeations of fexofenadine and talinolol P-gp substrates were significantly enhanced without change in their dissolution. Moreover, the effect of the surfactant Cremophor EL as a P-gp inhibitor and surfactant was tested on the dissolution and permeation of saquinavir, and it was found that both solubility/dissolution and permeation as a result of P-gp inhibition were increased (Kataoka *et al.*, 2011). Recently, the group demonstrated that the D/P system enables evaluation of the limiting steps in the oral absorption of poorly water-soluble drugs, and prediction of dose-dependent pharmacokinetics. For instance, increasing applied doses of zafirlukast slightly decreased the dissolution and permeation in the D/P. Although the difference in the dose of zafirlukast was eightfold (10–80mg), the obtained f_a value was predicted to be around 35%, irrespective of the dose. It was suggested that the limiting step of zafirlukast absorption could thus be dissolution rate, and not solubility.

An understanding of both dissolution and permeation simultaneously is crucial to understand drug absorption. Dissolution tests practically show the free concentration in the fluids which is available to be absorbed, however it does not mean that the entire drug in solution will be able to permeate through the gut wall such in the case of BCS IV drugs. In the previous section, different conditions of pH and bile salt concentration of GI fluids resembling individuals with different composition of GI fluids have been shown to affect the dissolution profile of dipyridamole. Therefore, it will be highly desirable to understand how these changes might be reflected in the permeation profiles and affectively its absorption. In addition, no significant differences in the dissolution of furosemide were observed previously under different conditions of bile salt concentrations and pH. However, as furosemide is a poorly permeable drug, it will be useful to determine whether these different conditions affect its permeation. In this research, the D/P system was utilised to investigate the effect of different bile salt concentrations and pH conditions, relevant to the fasted state, on the dissolution and the permeability of dipyridamole and furosemide in order to predict possible variable absorption in human.

3.4.2 Objective

- To investigate the effect of bile salt concentrations and pH, relevant to the GI fasted state, on the dissolution and the permeation of dipyridamole and furosemide to predict possible variable absorption in humans

3.4.3 Materials

Caco-2 cells human adenocarcinoma cell line (86010202), Dulbecco's modified Eagle's medium (DMEM)(D5671), fetal bovine serum (F7524) , non essential amino acids (M7145), L glutamine (G7513), 0.25% trypsin-EDTA (T4049), gentamicin (G1264) (50mg/ml), HBSS 10X with no calcium and magnesium (55037C), sodium bicarbonate (S5761) , dextrose anhydrous (158968), HEPES (hydroxyethyl piperazineethanesulfonic acid) (H3375), bovine albumin serum (A9418), NaOH and HCl 5M standard solution, acetonitrile and water HPLC/LCMS grade were purchased from sigma Aldrich (Dorset, UK). SIF powder was obtained from Biorelvent.com (London, UK). Transwells Cornining Costar Corporation (6 wells, 2.4cm^2 surface area, 3µm pore size, PET clear membrane, 3452), syringe filters (Millex, SLLHH04NL, 4mm, 0.45µm, Millipore) and 162 cm^2 flasks were obtained from Fisher (Leicestershire , UK).

3.4.4 Methods

3.4.4.1 Cell Culture

The following protocol was adapted from Ashiru D, PhD thesis (2009)

- Cell maintenance

Caco-2 cells were grown and maintained in culture as previously described (Hidalgo et al., 1989). Briefly, cells were grown in 162cm^2 cell culture flasks and subcultured weekly on achieving 80-90% confluency. Cell culture growth medium was DMEM, supplemented with 10% v/v fetal bovine serum, 1% v/v non-essential amino acids, 1% v/v L- glutamine, 0.1% v/v gentamicin (50 mg/mL). Cells were stored in an incubator at 37˚C with a humidified environment of 95% and 5% CO2. The medium was changed every 2-3 days. All processes were carried out using trained techniques and precautions relative to cell culture in class II flow cabinet.

- Subculture

On reaching 80-90% confluency, the cells were visualised under an inverted microscope to verify the general appearance of the culture and look for signs of microbial contamination. The culture was also observed with an unaided eye to look for fungal colonies that could be floating at the medium- air interface.

The Caco-2 monolayers in the flasks were detached from the surface with 0.25% trypsin-EDTA. The trypsin was inactivated by the addition of medium containing FBS. The exact procedure is thus:

1. Using a sterile pipette, old culture medium was removed and discarded.
2. The monolayer was rinsed with 5mL of calcium and magnesium-free phosphate buffer (CMF-PBS) to remove all traces of FBS.
3. 3mL pre-warmed trypsin solution was then added to the flask, cells incubated for at least 1 minute on the bench and then the flasks were transferred to a shaking incubator for detachment of cells.

In order to avoid subpopulation selection, 100% of cells were detached at each passaging procedure. The process of detaching the cells took between 5 to 10 minutes; the cells were regularly inspected under an inverted microscope to determine the point at which all cells had been detached. The cells from the flask were transferred to a centrifuge tube, and the cell suspension centrifuged at 200xg for 5 minutes with the pellet then re-suspended in medium. A cell count was performed by taking a 100µL sample from the cell suspension and combined with 100µL trypsin blue; this was mixed vigorously, and the suspended cell density determined using a Neybauer Hamocytometer. The number of cells/mL was calculated, and the required cell concentrations generated by appropriate dilution.

- Cell freezing

At regular intervals, cells that had reached confluency in 162cm^2 flasks were prepared for cryopreservation. Prior to freezing, as with subculturing, the cells were visualised under an inverted microscope to verify the general appearance of the culture looking for signs of microbial contamination. The culture was also observed with an unaided eye to look for fungal colonies that could be floating at the medium- air interface. The cells were harvested in the same manner as described above for subculture. However prior to centrifugation, a sample was taken for counting. Whilst the cells were spinning, a viable cell count was carried out as previously described, and the number of cells/mL was calculated as well as the total cell number. After centrifugation, the supernatant was removed from the centrifuged cells and the cell pellets re-suspended in enough cryprotective medium (freezing medium in DMSO) to give a final cell concentration of 1 to 2*106 cells/mL.

Cryogenic vials were labelled with the cell line, passage number and date, and 1.8 mL of the freezing media containing cell suspension was added to each of the vials and sealed. The cells

were frozen in a -80˚C freezer using a 'Mr Frosty' cell freezer containing room temperature isopropanol in the bottom compartment to ensure a gradual freezing of the cells. After 24 hours, the vials were transferred to a liquid nitrogen storage vessel until required.

- Cell revival

The required vial was transferred from the liquid nitrogen storage and rapidly thawed in a 37˚C water bath within 60 to 90s. The contents of the vial were transferred to a flask containing 15mL of cell culture medium. The tube was centrifuged for 5 min at 200 x g. After centrifugation, the supernatant was removed from the centrifuged cells, and the cell pellets were re-suspended in fresh growth media. The cell suspension was transferred to 25cm^2 flask and was incubated for at least 3 days until a monolayer was formed, after which the media was replaced by fresh media until 90% confluency was achieved.

- Seeding cells on transwells

For D/P studies, cells were seeded at a density of 60,000 cells/cm^2 (1.2 x 10^5 cells/well) onto 6 Transwell polyesther membranes with 24 mm diameter, poor size of 3µm and surface area of 2.4 cm^2. Cells growing on transwells membranes were provided with fresh complete medium three times a week until the time of use. To feed the cells, 1.5mL of complete medium was added to the top of the cell layer, and 2.6mL was added to the bottom of each transwell. All cells used in this study were between passages 47 to 56. The filters were used between the 17th and 21st day of culture.

3.4.4.2 Buffer Preparation

- Preparation of transport medium

100mL DI water were transferred in a 300mL volumetric flask and HBSS solution was added. Sodium bicarbonate, glucose and HEPES were added and dissolved stepwise. The volume was completed to 300mL with purified water, and pH was adjusted to 6.5 using NaOH/HCl solution (Table 3.10).

Table 3.11: Transport medium, pH 6.5

Composition	Amount per 300mL
HBSS Solution	30mL
Sodium bicarbonate	105mg
Dextrose anhydrous (D+/-glucose)	750mg
HEPES*	675mg
DI Water	QS 300mL

- Preparation of fasted state simulated intestinal fluid (FaSSIF)

SIF powder was weighted to create the following concentrations of 1,3 and 6mM of sodium taurocholate, and dissolved in about 50 mL of transport medium. The volume was completed to 50mL, and the pH was adjusted to 6, 6.5, 7.4 using NaOH/HCl solution (Table 3.12).

Table 3.12: FaSSIF, pH 6.5

Composition	Amount per 150mL
SIF powder *	328mg
Transport medium	QS to 150mL

- Preparation of Basal Solution

BSA (bovine serum albumin) was weighted in a beaker, and 100mL of the transport medium were added. Gentle stirring was applied for an hour to complete dissolution. Upon complete dissolution, the pH was adjusted to 7.4 with 5M NaOH solution before use (Table 3.13).

Table 3.13: Basal solution, pH 7.4

Composition	Amount per 100mL
BSA *	4.5g
Transport medium	100mL
Sodium hydroxide solution, 5M	QS to pH 7.4

3.4.4.3 D/P Experiment

Caco-2 cultured in a six well plate for 17 to 21 days was used as source of intestinal monolayer for this study. The plate was removed from the incubator, and the growing media was removed from the wells. 2.6mL of the basal solution were added to the basolateral sides first, before adding 1.5mL of the transport medium to the apical side. The plates were incubated at 37˚C under 5% CO_2 for 20 minutes to acclimatise the cells with the transport media. After 20 minutes, the plates were removed from the incubator and the media was decanted from the transwells. The transwell inserts were attached in between the apical and basolateral chambers of the D/P system so as to mount the membrane vertically, as shown in Figure 3.18. 8mL FaSSIF solution on the apical side and 5.5mL of the basal solution on the basolateral side were added. The magnetic stir bars were placed in both sides (apical and basolateral) rotating at 200rpm (150-250, adjusted using a tachometer) using multi magnetic stirrers. Once the chambers preparation was completed, they were transferred into an incubator to maintain temperature of 37˚C**.** The TEER values were measured prior the beginning of the experiment, and whenever a sample was withdrawn (set point 2000Ω, mode R- measured values varied between 300 to 400Ω during experiments). The drug or the formulation were added into the apical side of the D/P system, and 200µL aliquots of samples were withdrawn from the apical and the basolateral sides to measure the amount dissolved and permeated with time at 5, 15, 30, 60, 90, and 120 minutes. The apical samples were filtered via 0.45µm syringe filters. After taking the last sample, the TEER was measured again to confirm the integrity of the monolayer during the experiment. The apical samples were diluted 10 times for HPLC and 100 times if analysed by LCMS with acetonitrile. Basolateral samples were diluted 7 times (for HPLC) by adding 1.2mL of ACN in the tubes to precipitate proteins. The basolateral samples were vortexed and centrifuged at 15,000 rpm at 20 ˚C for 10 minutes to remove the precipitates.

3.4.4.4 Analytical Methods

- HPLC methods for furosemide and dipyridamole

Furosemide analysis was carried out using a HPLC system (LC-10A Shimadzu Co., Kyoto, Japan) with variable wavelength ultraviolet detector (SPD-10A, Shimadzu Co., Kyoto, Japan) and fluorescence detector (RP-10A, Shimadzu Co., Kyoto, Japan). The column was C18 YMC J'sphere H-80 4.6 × 75, with the following composition of mobile phase: 50mM Phosphate buffer (pH 2.5): Acetonitrile (72:28% V/V). The excitation and emission were at 333 and 415nm for and the UV wavelength was 280nm. The flow rate was 1.0mL/min and column temperature of 40˚C. The injection volume was 20µL.

Dipyridamole analysis was carried out using C18 Column YMC J'sphere H-80 4.6 × 75, with mobile phase composition of 50mM Phosphate buffer (pH 2.5): Acetonitrile (72:28% V/V). The UV absorbance was at 270nm with flow rate: 1.0mL/min and column temperature of 40˚C. The injection volume was 20µL.

- LCMSMS analysis of dipyridamole

The amounts of dipyridamole in the solutions from the basolateral side were determined by an UPLC system (ACQUITY® UPLC, Waters, Milford, MA) equipped with a tandem mass spectrometer (ACQUITY® TQD, Waters, Milford, MA). The reversed-phase Waters ACQUITY® UPLC BEH C18 analytical column of 50mm length × 2.1mm I.D. and 1.7µm particle size (Waters, MA) was used with a mobile phase consisting of 0.1% (V/V) formic acid in water (solvent A) and acetonitrile containing 0.1% (V/V) formic acid (solvent B) with a gradient time period. The initial mobile phase was 98% solvent A and 2% solvent B pumped at a flow rate of 0.3mL/min. Between 0 and 1.0 min, the percentage of solvent B was increased linearly to 95%, where it was held for 1.0 min. Between 2.01 and 2.5 min, the percentage of solvent B was decreased linearly to 2%. This condition was maintained until 3 min, at which time the next sample was injected into the UPLC system. All samples were injected as 5 µL into the UPLC system. Protonated precursor and production ions (*m/z*) for detection were 505.328 and 429.328, respectively.

3.4.4.5 Statistical and Data Analysis

Calculation of f_a based on permeation results were calculated as followed (Kataoka *et al*., 2012)

$$fa(\%) = \frac{\text{Absmax} * \text{PA}^{\gamma}}{\text{PA}_{50}{}^{\gamma} + \text{PA}^{\gamma}}$$

Equation 3.7: f_a estimations from the D/P system

Where Abs_{max} is the maximum absorption (defined as 100%), PA is the *in vitro* permeated amount in the D/P system (% of dose/2 h), PA_{50} is the permeated amount, which corresponds to 50% of the absorption *in vivo*, and γ is a Hill's coefficient. PA_{50} and γ were obtained by fitting the permeated amount (PA) of drugs in the D/P system and their oral absorption in human (on system validation).

The dissolution and permeation data were analysed by one way ANOVA repeated measurements followed by a tukey post- hoc analysis using Univariate General Linear Model tool in PASW statistics 22 (SPSS Inc., Illinois, USA).

3.4.5 Results & Discussion

The dissolution and permeation of dipyridamole were tested under different condition of sodium taroucholate/lecithin and pH (Figure 3.20 and Figure 3.21). As can be seen, the dissolution of dipyridamole in *m*FaSSIF in the apical side under different bile salts concentration gave a higher extent of release under 6mM concentration of sodium taurocholate ($p<0.05$), followed by 3mM and 1mM. As for the different pH conditions, at pH 7.4 the dissolution extent was the highest, followed by pH 6 and 6.5, although they were not significantly different from each other ($p>0.05$). The dissolution results obtained in this study confirmed that bile salt concentration has a more significant influence on the dissolution of dipyridamole, as the percentage of drug release after 2 hours was higher at pH 6.5 with 6mM taurocholate concentration than at pH 6 and 3mM taurocholate concentration (12 and 5% respectively). The differences in the extent dissolved under different conditions reported previously (Figure 3.8) in *m*Hanks were also more significant compared to the differences in the extent and dissolution rate found herein (60% release at 2 hours compared to only 12% release). This might be explained by the different composition of the media. As discussed, the *m*Hanks buffer resembles more closely the intestinal fluids in terms of ion composition and buffer capacity, and therefore the differences might be attributed to these factors. However, considering the significant effect of bile salt on the dissolution of dipyridamole and the basic nature of the drug, the differences in the bile salt composition of the two buffers might be better explanation for these differences in the extent of release. The bile salts used in *m*Hanks buffer are comprised of two bile salts, whereas in this study only the effect of taurocholate with lecithin was investigated. Interestingly, the permeation profile of dipyridamole under different bile salt concentrations did not differ ($p>0.5$), although increasing bile salt concentration increased the percentage of the drug dissolved on the apical side. However, a significant decrease was observed in the permeation amount of dipyridamole under pH 6. It seems that although bile salt concentration had a significant effect on the extent of dipyridamole dissolution, the pH was a more significant factor influencing permeation of the drug through the monolayer. On predicting the fraction absorbed in human, f_a was estimated to be around 30% with variation of 23% under all conditions (Figure 3.22)

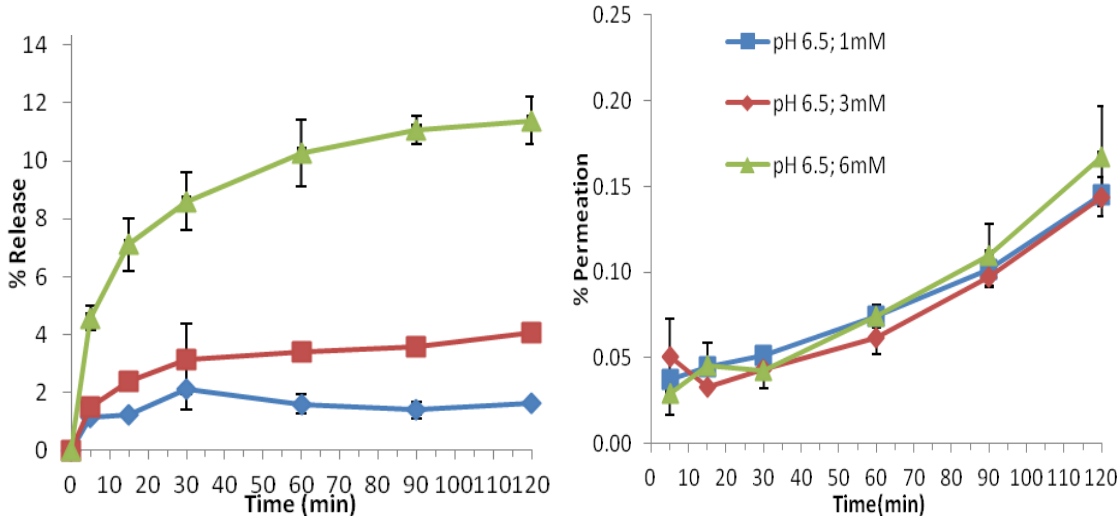


Figure 3.20: Dipyridamole dissolution and permeation under different bile salts concentration in the D/P system

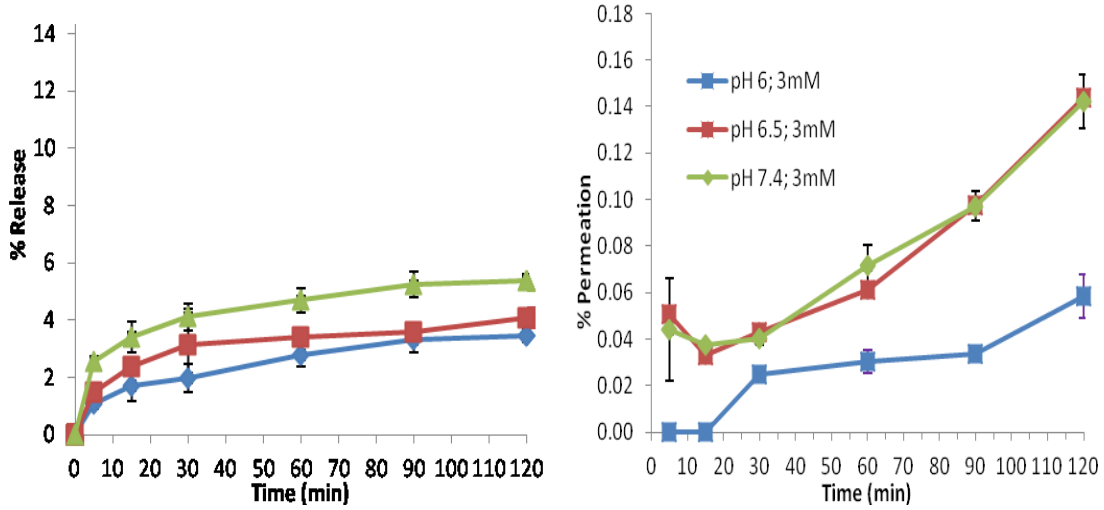


Figure 3.21: Dipyridamole dissolution and permeation under different pH conditions in the D/P system

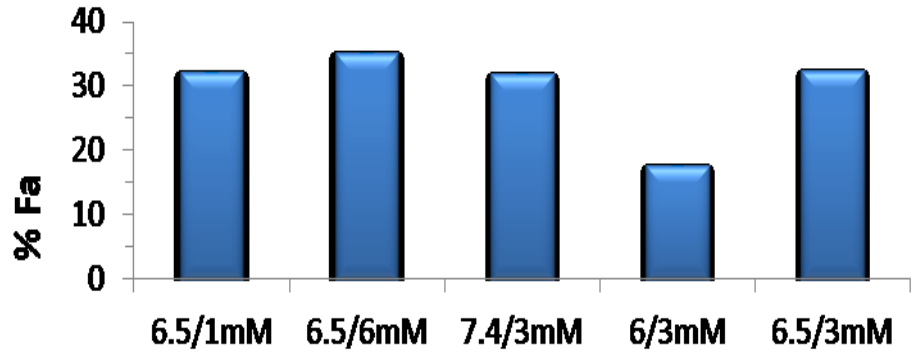


Figure 3.22: Predicted fraction absorbed based on D/P system.

Furosemide dissolution under different pH condition produced a general trend, with a higher extent of release at pH 7.4 and lower at pH 5.5. Significant differences were observed in the case of furosemide dissolution at pH 5.5 as compared to the other conditions ($p<0.05$). Similarly, at concentrations of 1mM taurocholate, the lowest extent of release was obtained. However, the extent released at taurocholate concentrations of 3 and 6mM did not differ significantly from one other similar permeation profiles which were obtained for furosemide under all conditions, with no significant differences (Figure 3.23 and Figure 3.24).

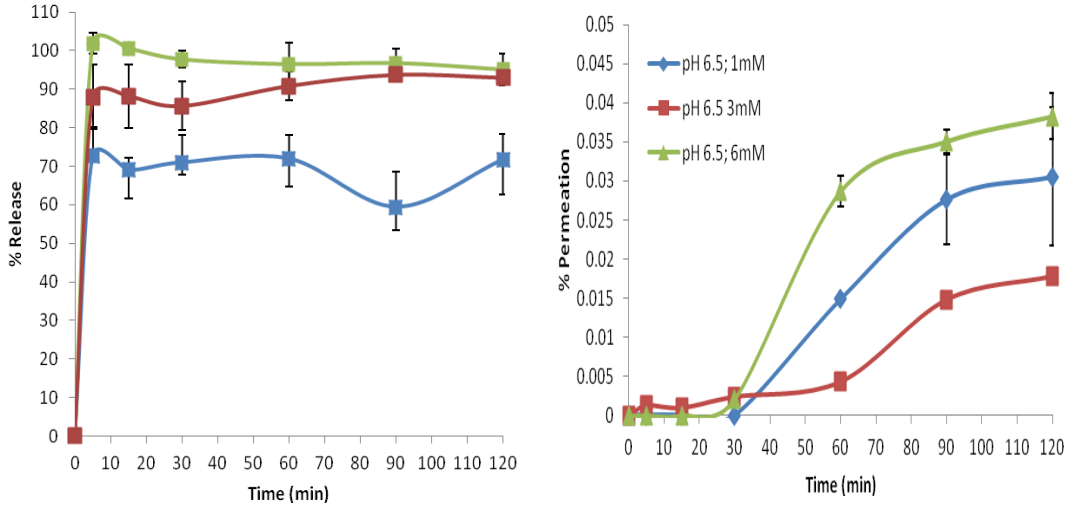


Figure 3.23: Furosemide dissolution and permeation under different bile salts concentration in the D/P system

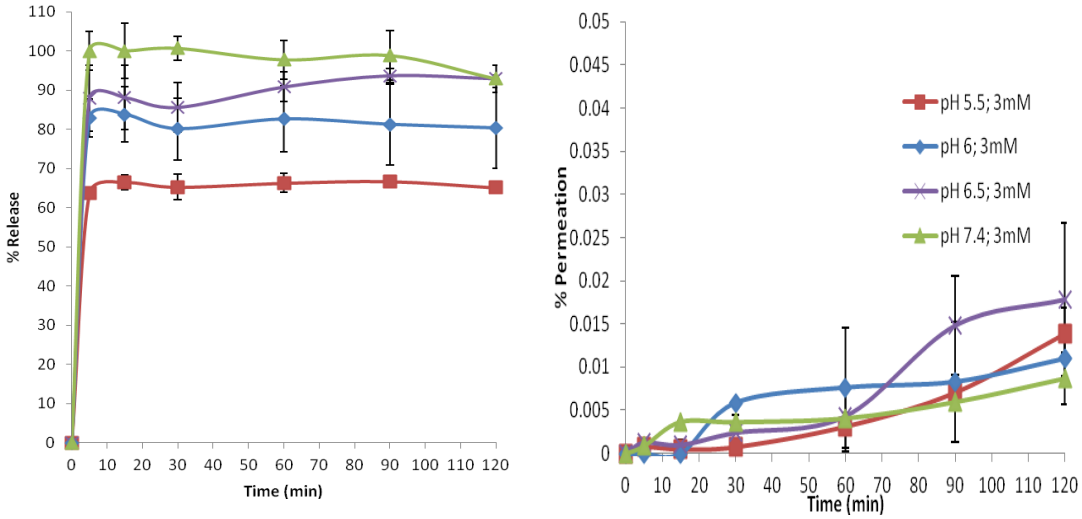


Figure 3.24: Furosemide dissolution and permeation under different pH conditions in the D/P system

Kataoka (2003) reported previously that addition of taurocholate facilitates both the dissolution and permeation of griseofluvin, although taurocholate was seen to be less effective on permeation than dissolution. With 6mM taurocholate, dipyridamole dissolved rapidly, and the dissolved amount reached a plateau level at around 10% of the dose. It can be suggested that micelle formation facilitates increased dipyridamole solubility, though the dissolved drug was not available to permeate through the monolayer since it was incorporated in the micelles. The pH effect was more significant for the permeation of dipyridamole. A compound diffuses across the lipid bilayer portion mainly as an uncharged and largely desolvated species, depending on its molecular size and affinity to the centre of the lipid bilayer. As mentioned, dipyridamole pka is 6.4, and so at that pH it is expected that around half of the applied amount will be present in the ionized form and the other half unionized; it is the unionized form which is available to be absorbed. With decreasing pH, the ionized form of the weak base will increase (therefore increased solubility/dissolution on the apical side). However, it is not available to permeate through the membrane, as only neutral species can penetrate the membrane in passive diffusion. In a different experiment, Kataoka *et al*. (2006) tested the *in vitro* permeation of propranolol, danazol, and albendazole with $FaSSIF_{mod}$ and $FeSSIF_{mod}$ as the apical media in the D/P system. The FaSSIF and the FeSSIF pH was adjusted to pH 6.5 and 5, respectively, based on the updated version published by Jantraid *et al*. (2008). It was found that although the dissolution of albendazole was much faster in $FeSSIF_{mod}$, the permeated amount only slightly increased by $FeSSIF_{mod}$. In addition, the permeated amount of propranolol was dramatically decreased when $FeSSIF_{mod}$ was applied. It was proposed that the lower pH of $FeSSIF_{mod}$ (pH 5.0) might facilitate the ionization of the basic drugs (propranolol and albendazole), and decreased its permeability through the Caco-2 monolayer.

As reported, the *in vivo* regional pH of the gastrointestinal fluid varies significantly. Moreover, it was suggested that an acidified (pH=5.3) microenvironment existed in the mucosal surface of enterocytes (Hogben *et al.*, 1959). Those results were further confirmed by direct microelectrode pH measurements in inverted intestinal segment *in vitro* experiments (Lucas *et al.*, 1975). Additional studies suggested the surface of cells was metabolically enriched with protons, predominantly by Na+/H+ antiporter (McEwan *et al*., 1988). All of these conditions might therefore affect the ionization of weak bases and acids, and need to be considered in order to better understand mechanisms of drug absorption. As described, most of the dissolution tests actually measure the drug concentration in the vessel; However, based

on the D/P system results presented herein, it is clear that the free drug available to be absorbed needs to be considered in order to predict absorption kinetics, and not only for predicting the luminal concentration in GI fluids.

As in the previous dissolution studies, highly variable dissolution ranging from 80 to 100% of furosemide release was attained under different conditions. This again supports the need for re-evaluating the classification of furosemide as BCS IV, considering its high dissolution in the given clinical dose. A possible explanation for the lack of correlation from the dissolution to the permeation profiles in the case of furosemide could be related to the accuracy and sensitivity of Caco-2 monolayers to detect small changes in the drug permeation. Considering the facts that the differences in the extent of release of furosemide in the apical side were not found to be significant, and furosemide is a very poorly permeable drug, the ability of the Caco-2 monolayer to detect these differences in the dissolution might be limited. The low sensitivity of the monolayer might be highly relevant to poorly permeable drugs including furosemide, which gave only a 0.01-0.02% permeated amount. Kataoka *et al*. (2012), for instance, tested different formulations of danazol (BCS II) and pranlukast (BCS IV) in the D/P system. For danazole (highly permeable drug), the differences that were obtained in the dissolution were well-reflected in the permeation profiles. However, for pranlukast (low permeable drug), although differences in the dissolution of the formulations were significant, the extent of drug permeation at 2 hours did not differ significantly for the different formulations, and ranged from 0.006 to 0.032 (%of dose/2h). Moreover, as discussed previously, the Caco-2 monolayer is derived from colon carcinoma cells, and usually characterized by tight junctions. Consequently, for poorly permeable drugs such as furosemide, it might be that other cell lines like MDCK will reflect better the changes in permeation and the prediction of *in vivo* absorption. It was also suggested that furosemide is a P-gp substrate and this effect in its permeation through the Caco-2 monolayer might be more significant than its effects on bile salt and pH. It will be interesting to test this hypothesis by adding P-gp inhibitor to the system.

3.4.6 Summary

The D/P system was utilised in this investigation to investigate the effect of bile salt concentrations and pH on the dissolution/permeation of dipyridamole and furosemide. The results presented herein emphasise the fact that investigation of both dissolution and permeability processes simultaneously is highly valuable as dipyridamole dissolution was significantly affected by the bile salt concentration, while permeability was significantly affected by the pH and the ionized and unionised form of the drug. For furosemide, poorly permeable drug, the adequacy of Caco-2 cell as measure of permeability was questioned to predict possible effects of different conditions.

3.5 Chapter Conclusions

As discussed, the complexity of the GI tract is difficult to mimic *in vitro*. In this chapter to simplify our understanding as for the possible factors affecting drug absorption, solubility, dissolution and permeability were investigated stepwise *in vitro*. In the first section, it was found that the saturated solubility of dipyridamole is pH and bile salt dependent. These results were confirmed by the dissolution test in the second section, where it was shown that bile salts at this concentration range increased the extent of dipyridamole release. The saturated solubility is an important factor in determining dissolution rate and extent. In this investigation, it is clear that bile salts have the greater effect on the saturated solubility, and hence the dissolution rate and extent of release. However, when both dissolution and permeability were tested simultaneously, it was found that although bile salt has a great impact on the solubility/dissolution of dipyridamole, pH plays an important factor in the permeation of dipyridamole. The results presented herein, in addition to the solubility data, can explain to some extent the variability between individuals in the absorption of dipyridamole. In addition, the results emphasis the fact that absorption is a continuous process and dissolution data alone might not always reflect the *in vivo* situation.

With regards to furosemide, BCS IV drug, solubility experiments showed that pH, buffer capacity and to a lesser extent bile salt affect its saturated solubility. Surprisingly, almost complete drug release was observed under all simulated conditions in this clinical dose. Similarly, the permeation of furosemide did not differ under different conditions. This might be attributed to the Caco-2 cell monolayer sensitivity to detect these changes.

Chapter 4 - Development of Three Formulations to Increase Drug Absorption and "*In Vitro*" "*In Vivo*" Evaluation

4.1 Chapter Overview

In the previous chapter, the effect of bile salt and pH in the GI tract was investigated in two model drugs with regards to solubility, dissolution and permeability. Once identifying the mechanism that might cause variation among individuals, it is desirable to find a solution to increase absorption and minimise these effects. Different formulation approaches are available to increase solubility and dissolution of drugs. In this chapter, it was our interest to compare different formulation approaches in increasing absorption and minimising the bile salts and pH effect in the GI tract. Moreover, usually formulation design for poorly soluble drugs is done based on the formulator experience and does not always take into consideration the API properties, the excipients fit and the preparation process. Therefore, comparison of different common formulation approaches is of great interest to minimise efforts and cost in designing the right formulation. Three formulation approaches were utilised to investigate how increasing solubility will increase absorption. The solid dispersion approach was chosen to represent the administration of a drug in its amorphous form, Self-Micro Emulsifying Drug Delivery System (SMEDDS) formulation represented the solubilisation of lipophilic compounds in oils and eventually nano-particle formulation was investigated to understand how reducing particles size affects solubility and hence absorption. Formulation evaluations were carried out by different techniques available in the lab. In addition, *in vitro* performance of the different furosemide and dipyridamole formulations were investigated using simple dissolution tests in *m*Hanks buffer, modified FaSSGF and in the D/P system. The formulations performance under different pH and bile salt concentration were also investigated. As described, to further validate the *in vitro* system for evaluation of formulation performance, *in vivo* trials need to be carried out. Here, the rat model was chosen as the most available and easy to execute for initial evaluation of formulation *in vivo*.

4.2 Development of Different Formulations and Evaluating their Performance *In Vitro*

4.2.1 Introduction

Recently, it was recognized that a significant percentage of the molecular entities undergoing evaluation as part of industrial drug development pipelines are poorly soluble drugs (Dahan *et al*., 2009; Ku and Dulin, 2012). Many attempts have been made in order to improve oral absorption and decrease absorption variability of these compounds by improving drug solubility and developing new dosage forms that enhance these characteristics. It was reported that for the same drug in different dosage forms, differences in the oral absorption can be expected to range widely (Block *et al*., 1981; Chiou and Riegelman, 1970; Levy *et al*., 1961; Weis *et al*., 1994). Those factors which can affect formulation performance and hence absorption typically include formulation excipients, disintegration characteristics, and the type of the dosage form (solution, dispersion, emulsion, gel, tablet, capsule and etc...). Many approaches to increase the solubility of crystalline drugs have been developed by the pharmaceutical industry and the scientific community (Figure 4.1): Arguably, the easiest approach currently utilised is the salt formation for weak bases or acids. However, this formulation has limitations, and does not always produce reliable release profiles - as such, other solutions need to be considered (Elder *et al*., 2013; Serajuddin, 2007). Some of these alternative approaches notably include cyclodextrins, solid dispersions, self-emulsifying drug delivery systems, solid nano-particle, liposomes, micelles, soft gelatine capsules, co-crystals and pH micro environmental modifiers. Of these, the most investigated methods in the scientific literature and the most commonly used in industry are solid dispersions in their amorphous forms, self-emulsifying drug delivery systems, and nano-particle delivery systems (Kawabata *et al.*, 2011).

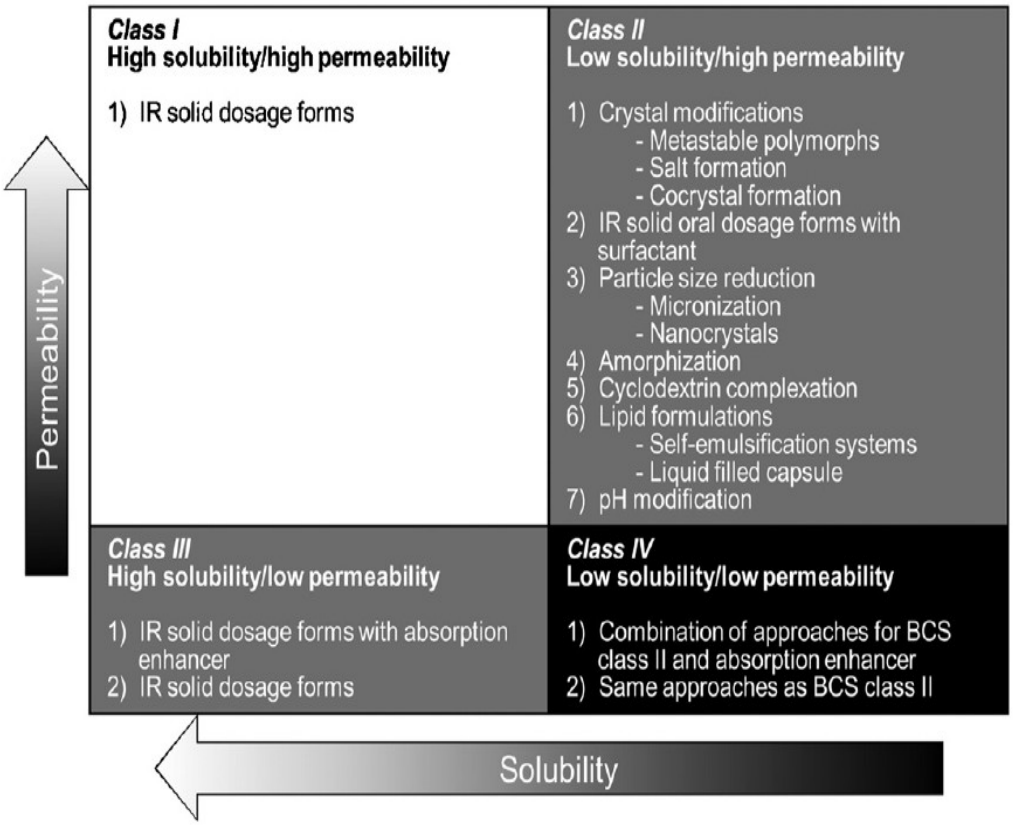


Figure 4.1: Formulation approaches considering the drug properties based on the BCS adapted from (Kawabata *et al*., 2011)

4.2.1.1 Solid Dispersion

An amorphous solid dispersion consists of an amorphous active API stabilized by a polymer, with the amorphous form of the drug providing increased apparent solubility (Leuner and Dressman, 2000). The advantage of solid dispersion in increasing drug solubility is related to the enthalpic energy. Lipinski *et al*. (2012) showed that the solubility (S) of a given solid solute is determined by the crystal packing energy (accounting for the energy necessary to disrupt the crystal pattern and remove isolated molecules), cavitation energy (accounting for the energy required to shift water in order to create cavity into which the solute molecule can penetrate) and salvation energy (accounting for the release of energy as favourable interactions are formed between the solvent and solute) (Equation 4.1).

$$S = f(\,Crystal\,packing\,energy + Cavitation\,energy + Solvation\,energy)$$

Equation 4.1: The factors affecting solubility adapted from Lipinski *et al*. (2012)

The crystal packing energy is the major driving force behind solubility: By formulating an amorphous form, this energy is reduced by disrupting the drug crystal pattern in the delivery form. This can be achieved by adding solubility-enhancing polymeric carriers to the drug. However, the decrease in this energy results in an unstable amorphous form comparatively to the crystalline form. Thus, when the amorphous form is placed in a media, its potential energy is released and the solid dispersion turns into its supersaturated solution state. It is well known that supersaturated states are thermodynamically unstable, and the drug is much more likely to precipitate into its crystalline form under these circumstances. Many publications have attempted to characterise this phase and understand how different excipients and polymers can prolong this super-saturation in order to increase the window for drug absorption (Brouwers *et al*., 2009; Higashino *et al*., 2014; Janssens and Van den Mooter, 2009; Lindfors *et al*., 2008; Sarode *et al*., 2013). Moreover, re-crystallization is a major concern for the appropriate storage of the solid drug, and can limit its shelf life. Not only can moisture promote drug crystallization, but the use of hygroscopic polymers may additionally result in phase separation or crystalline growth. Re-crystallization prior to administration will consequently decrease drug solubility, and no effect on absorption will be observed. Some of the other challenges in the development of solid dispersion include optimization of the preparation method and its formulation into a dosage form, reproducing its physicochemical properties, and scaling up manufacturing processes (Serajuddin, 1999) .

In order to avoid this, many efforts have been made to further optimize the formulation process through selection of an appropriate polymer, optimising drug load, and packaging selection, in addition to optimizing the manufacturing process (Brough and Williams III, 2013; Laitinen *et al*., 2013).

Amorphous dispersions can be prepared either by evaporating a solvent from a drug and polymer solution, or by melting/fusing methods in which the drug and the carrier solution is heated above their melting or glass transition temperature and then cooled gradually to keep the drug in its amorphous form. Both processes encompass challenges, and the end product might differ significantly in its stability or in the amorphous form (Agrawal *et al*., 2013). In solvent evaporating methods, the required temperature is usually much lower than in the melting methods. However, selection of the appropriate solvent is not always straightforward, and is typically highly dependent on the polymer and drug liphophilic/hydrophilic properties. Moreover, a second stage of drying any residual solvent needs to be carried out in order to avoid organic toxicity issues or formulation instability (Brough and Williams III, 2013).

Two common manufacturing processes of solid dispersions in the solvent method are spray drying or freeze drying. In spray drying, the feed solution (drug and polymer) is atomized into hot gas that causes the solvent to evaporate, resulting in spherical particles containing an amorphous drug. Process parameters to consider during a spray drying process include inlet temperature, drying gas properties (humidity, flow rate), feed rate, compressed air flow rate for a bi-fluid nozzle, pressure for a pressure nozzle, and disk/wheel speed for a rotary atomizer. On the other hand, formulation parameters to consider are the feed composition (API, carrier, solvent), solid content in the feed, solvent type, viscosity and surface tension of the drying solution (Paudel *et al*., 2013). Freeze drying is a process in which the feed solution is frozen and the solvent evaporates under vacuum (Betageri and Makarla, 1995). One common fusion method gaining increasing interest recently is that of hot melt extrusion. In this process, the drug and carrier are pumped through a heated barrel by one or more screws under pressure followed by discharging the extrudate through a dye (Crowley *et al.*, 2007; Lang *et al*., 2014). No solvents are involved in this process, and so all challenges generated from the use of solvent are not relevant here. Another fusion method used in industry is spray congealing where molten compositions are atomized into particles and then cooled to solid form (Fini *et al*., 2002).

Physicochemical characterization of solid dispersions such as the physical states of drugs, the drug–carrier interaction and the physical and chemical stability of drugs should be assessed in order to evaluate its pharmaceutical applicability and physicochemical stability. The crystalline state of drugs is commonly characterized by the following techniques: thermo-analytical techniques such as Differential Scanning Calorimetry (DSC), powder X-ray diffraction (PXRD) and Confocal Raman Spectroscopy. Moreover, it is possible to obtain a qualitative image about the crystalline state by microscopy techniques such as optical microscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM). Fourier Transformed Infrared spectroscopy (FTIR), solid state nuclear magnetic resonance, and Thermal Gravimetry Analysis (TGA) are used to investigate the chemical stability and molecular interaction of the drug and carrier.

Dissolution tests in bio-relevant media can also provide an insight into the solubility/dissolution enhancement mechanism of solid dispersion; however it is highly desirable to investigate the formulation performance *in vivo*. Newman *et al*. (2012) reviewed 40 research papers reporting active pharmaceutical ingredient (API) dissolution and bioavailability from various solid dispersion formulations. Generally, it was concluded that most amorphous dispersions produced improvements in bioavailability (~82% of the cases), with 8% of the amorphous dispersions exhibiting lower bioavailability than the reference material and 10% of the amorphous dispersions demonstrated similar bioavailabilities as the reference material.

4.2.1.2 Self-Emulsifying Drug Delivery System

Lipid based drug delivery systems include formulations such as oil solutions, emulsions, micellar systems and self (micro) emulsifying drug delivery systems (SMEDDS). Lipid-based drug formulations are presented to the GI in the solubilised formulation; as a consequence, no solubilisation from the solid state is required. Under these conditions, the solute-solvent interactions are reduced, resulting in enhanced solubility. It was suggested by Porter *et al*.(2007) that there are three possible primary mechanisms by which self-emulsifying drug delivery systems affect drug absorption. These include the alteration of the composition and character of the intestinal milieu (increase in bile secretion and easier partition of the drug into the mixed micelles that are believed to facilitate drug absorption), the recruitment of intestinal lymphatic drug transport, and the interaction with enterocyte-

based transport processes (increased intestinal permeability). The absorption mechanism was further developed to incorporate an explanation on the supersaturated state that lipid based formulation may promote (Williams *et al.*, 2013). It is important to note that the unstirred water layer forms a major diffusional barrier for lipids and lipophilic molecules, as their solubility in aqueous media is extremely low and needs to be considered when understanding the absorption mechanism of lipid-based formulations.

Recently, SMEDDS especially have attracted an increasing interest in the pharmaceutical community. Self-emulsification formulations are defined as "isotropic mixtures of oil, surfactant, co-solvent, and solubilised drug. Upon mild agitation followed by dilution in aqueous media, such as GI fluids, these systems can form fine oil−in−water (o/w) emulsions or micro-emulsions (SMEDDS)" (Neslihan Gursoy and Benita, 2004). The droplet size of SEDDS ranges between 100 and 300nm, whereas SMEDDS form transparent micro-emulsions with a droplet size of less than 100nm. Another form of SEDDS is the self-emulsifying nano-emulsion drug delivery system (SNEDDS), which does not differ in emulsion size from SMEDDS, but are also non-equilibrium systems and kinetically stable compared to SMEDDS, which are themselves thermodynamically stable; thus, by definition, will be in equilibrium in the solution. Moreover, in the case of SNEDDS, the droplet size is independent of dilution (Anton and Vandamme, 2011). This implication is not significant, however, given that the long-term stability of these formulations in the GI is not important.

The development of lipid base formulations is not simple, and investigation of the physical chemistry, thermodynamics and gastrointestinal digestion needs to be carried out. Usually, this includes a two-step process: first, a mixture of lipids, surfactants and co-solvents containing the drug in solution are chosen by assessing the drug solubility in these excipients. Then, the mixture is selected which forms an emulsion by addition of water or buffer and with the desired appearance and characteristics upon gentle agitation and with no precipitation of drug. Highly lipophilic drugs are generally the most suitable to formulate as SMEDDS thanks to their high solubility in lipids; however, non- lipophilic hydrophobic drugs can also be incorporated in lipid- based formulations with the addition of surfactant or co-solvents (Müllertz *et al*., 2010). Some of the factors to consider in the selection of excipients include regulatory issues (i.e. toxicity), solvent capacity, miscibility, morphology at room temperature, self-dispersibility and a role in promoting self-dispersion of the formulation, digestibility and fate of the digested products, and finally the chemical stability

and capsule computability (Pouton and Porter, 2008). Many scientists also utilise ternary or pseudo-ternary phase diagrams in the development of SMEDDS, which enables optimisation of the concentration ranges of different excipients, along with assessing the mixture, self-emulsification ability and drug loading.

As described in the second step of development, the ability of the oil mixture to form a microemulsion is assessed usually in water or simulated gastric/intestinal fluids. The water absorption and emulsification process during addition of an aqueous phase to the oil mixture can be characterised by viscosity and conductivity. Viscosity measurements help to determine the transition between mesomorphic structures, whereas conductivity measurements are able to determine the point of aqueous phase addition where the system changes from having a continuous oil phase to a continuous water phase (Kumar and Mittal, 1999). The rate of self-emulsification can be determined by a visual observation, or by monitoring the change of turbidity of a dispersion using a UV spectrophotometer after adding the oil mixture to aqueous media. Simple dissolution tests are conducted to assess the rate of dispersion and possible precipitation with dilution. However, it is important to note that the "usual" dissolution test cannot accurately predict the amount of available drug to be absorbed, given that some of the drug is incorporated into micelles. Dialysis bags can be utilised to this extent to determine the fraction dissolved and available for absorption. Particle size distribution in the formed nano- or microemulsion is measured by dynamic light scattering techniques (Müllertz *et al*., 2010). In addition to the above formulation assessments, it is important to understand the fate of the oil droplets and their digestion pathway in the gastrointestinal tract. It is possible that once the excipients in the SMEDDS are digested, the drug will precipitate and hence a decrease in solubility will be observed *in vivo*. This can be evaluated using *in vitro* lipolysis models (pH-stat model) (Kaukonen *et al*., 2004; Zangenberg *et al*., 2001). Some studies have shown that some surfactants are subject to digestion in the gastrointestinal tract, leading to the drug precipitation and a decrease in solubility (Dahan and Hoffman, 2008; Larsen *et al*., 2008; Sek *et al*., 2006). The above mentioned *in vitro* lipolysis setup is fairly complicated, suffering from low throughput and more importantly only partly mimics the gastrointestinal tract conditions. Kilic and Dressman (2013) have developed a simple method to mimic the lipolysis process by using their FaSSIF/FeSSIF recipes. Using a modified FaSSIF-V2, the same rank order in performance of four danazol formulations as previously observed in a pH-stat model was observed, and these results also reflected the *in-*

vivo study results in dogs. Using this method, it was also possible to identify whether precipitation of the drug is promoted by dilution or by lipolysis, with or without pancreatin.

Neoral®, a cyclosporin SNEDDS formulation, showed an increase in C_{max} and AUC compared with Sandimmune®, a coarse SMEDDS formulation, in human. This increase was attributed to the decrease in the droplet size (Mueller *et al*., 1994). Despite SEDDS formulations showing an increase in bioavailability in most of the clinical trials that were conducted (Fatouros *et al*., 2007), there are few approved SEDDS products currently on the market. Indeed, until 2010, only 9 SEDDS formulation were approved for use by patients (Kawabata *et al*., 2011). This might be related to the fact that there is still a gap in understanding of its absorption mechanism and an inability to accurately predict the fate of the formulation *in vivo* during early stages of development. Moreover, drug solubility in the oils/surfactants/cosolvents needs to be relatively high, given that the drug should be dissolved in a limited amount of oil. High chemical stability of the dissolved drug in oil phase would also be required for the lipid formulations. In addition, there is a lack of data on toxicity of some of the newly-developed excipients used. Therefore, more *in vivo* studies are required to investigate their effect on the gut membrane.

4.2.1.3 Nano-Particles

Nano-suspensions are defined as those which feature pure drug particles with a mean particle size of less than 1000 nanometers (nm), and which are suspended in an aqueous medium. Those particles that can exist in partially or fully crystalline states, are referred to as drug nano-crystals (Moschwitzer and Muller, 2007). The mechanism by which nano-particles improve solubility/dissolution is simply by reducing the particle size to a nano-size, hence increasing the surface area available for drug dissolution. Moreover, it has been reported that decreasing particle size will also reduce the thickness of the diffusion layer and thus eventually results in an increased dissolution rate: This is well reflected in Nernst-Brunner equation (Galli, 2006; Möschwitzer *et al*., 2011). In addition to the dissolution rate enhancement described above, an increase in the saturation solubility of the nano-sized API by reducing the particle size to less than 1µm as described by Ostwald–Freundlich's equation is also expected, as saturated solubility is affected by particle radius (Kesisoglou *et al.*, 2007; Müller and Peters, 1998). Similar to solid dispersions, nano-particles are less

thermodynamically stable then micro-particles, mainly due to change in Gibbs free energy and an increase in the surface energy. Therefore, nano-particles will tend to agglomerate in order to reduce their total energy (Van Eerdenbrugh *et al*., 2008). This phenomenon can be reduced by adding a stabilizer. A prerequisite for a good stabilizer is that it will increase the wetting properties of the hydrophobic surface, in addition to functioning as a barrier to agglomeration, likely achieved by electrostatic and steric forces (Van Eerdenbrugh *et al*., 2008).

Top-down and bottom-up technologies are the two primary technical approaches to drug nano-crystal production, and a combination of the two approaches may be applicable in some cases. The top-down methods are essentially high energy processes in which drug particles are broken down to nano size. Pearl milling (wet/dry), high pressure homogenization including piston gap homogenizer, and jet stream homogenizer are commonly used methods to decrease drug particle size (Möschwitzer, 2013). Though no harsh solvents are used in these techniques, some of the limitations of the top-down process include long process times for reducing particle sizes below 100nm, using a minimum amount of drug (which might not be available in early development stages), solid state changes, chemical degradation due to high heat during the milling, residual metal content production (zirconium, yttrium), and a usually low yield (Verma *et al*., 2009). Bottom-up approaches by contrast utilise the precipitation process from supersaturated solutions to grow crystals up to nano size. Sinha *et al*. (2013) reviewed the common techniques that are used, and classified them into four categories as follows: Precipitation by liquid solvent–anti-solvent addition, precipitation in the presence of supercritical fluid, precipitation by removal of solvent, and precipitation in the presence of high energy processes. The advantages of the bottom-up approaches include these being low energy processes, require simple instruments, are less expensive, and can be operated at a low temperature, making them particularly suitable for thermo-labile drugs. Most importantly, it is the ability to obtain smaller drug nano-crystals with a narrow particle size distribution that makes these technologies particularly useful. However, some of the issues related to the bottom-up methods include polymorphism, due to less time available for orderly molecular organisation. It is often very difficult to control the particle growth using this technique, and the formulator has to be careful in choosing the solvent, the antisolvent, the stabilizer (s) and the process parameters in order to obtain stable nano-suspensions with the desired particle size profile. Moreover, bottom-up approaches include dissolving the drug completely in an organic solvent, and the additional stage of removing organic residuals is

required. This may lead to further precipitation of the dissolved solute, which might be uncontrolled and could result in an increased mean particle size with wider particle size distribution (Sinha *et al*., 2013).

As for other formulations, it is essential to characterise nano formulations in terms of size and size distribution, particle charge, crystalline status, solubility/dissolution in bio-relevant media and stability (Müller *et al*., 2001). Moreover, it is highly desirable to investigate the effectiveness of these formulations *in vivo* on increasing oral absorption. Kawabata *et al*. (2011) reported that there are five nano-crystal oral formulations using NanoCrystal® (Elan Drug Technologies) and IDD-P® (SkyePharma) technologies which are available on the market and have proven efficacy. Moreover, numerous studies demonstrating the enhanced oral bioavailability of pharmaceuticals and neutraceuticals by nanocrystal technologies were published, and 1.7–60-fold and 2–30-fold enhancement in C_{max} and AUC respectively were found as compared to the crystalline formulations with micrometre particle size (Fakes *et al*., 2009; Hanafy *et al*., 2007; Hecq *et al.*, 2006; Jia *et al*., 2002; Jinno *et al*., 2006; Kondo *et al*., 1993; Liversidge and Cundy, 1995; Wu *et al*., 2004; Xia *et al.*, 2010).

Each of the approaches to improve the aqueous solubility of poorly water-soluble drugs, encompasses challenges for drug development and manufacturing. In general, salt formation, micronization, and pH modification in dosage forms are categorized into conventional technologies and usually are the first line to improve formulation performance. Other technologies, such as nano-crystal formation, amorphization, and SEMDDS, can be identified as non-conventional technologies. For formulation scientists, it is not always clear which method is preferable, since direct comparison between the methods are not available. Usually, the most commom method is utilised first, but this does not always indicate that this is the most useful method for increasing solubility, increasing absorption and the formulation stability. As such, this was addressed in the present study. The three formulation approches described herein were compared in terms of development and *in vitro* performance in order to predict which approach will best increase the dissolution/solubility and reduce inter-subject varibility caused by bile salts and pH.

4.2.2 Objectives

- To utilize three common approaches for increasing drug solubility to prepare the following formulations: solid dispersion, SMEDDS and nano-suspension for dipyridamole (BCS II) and furosemide (BCS IV).
- To evaluate formulation performance *in vitro* and assess the advantages and disadvantages in developing and preparing each formulation approach.
- To investigate formulation dissolution (dipyridamole and furosemide in *m*Hanks buffer and furosemide in FaSSGF) under different bile salts and pH conditions in order to understand which approach will increase the percentage of drug release and reduce the effects of pH and bile salts.

4.2.3 Materials

Furosemide, dipyridamole, polyvinylpyrrolidone K 30, polyvinylalchol (PVA) and mannitol were purchased from Sigma- Aldrich. Labrafac lipophile WL1349 (batch No. 135990) was kindly supplied as a gift from Gattfosse SAS. Kolliphor HS15 (batch No. 29749816KO) was kindly supplied as a gift from BASF. Povacoat Type F was supplied as a gift from Daido, NSK. Acetonitrile, ethanol and isopropyl alcohol and all solvents were HPLC grade and purchased from Fisher.

4.2.4 Methods

4.2.4.1 Formulation Development

A. Solid Dispersion

Furosemide and dipyridamole solid dispersions were prepared using a solvent method by spray-drying. PVP-K30 was chosen as the model polymer based on previous experience in developing furosemide and dipyridamole solid dispersion in various methods. Furosemide and dipyridamole were dissolved separately in 500mL ethanol, and PVP-K30 was added to prepare a feed solution for the spray-drying process. The solutions were mixed until clear solutions were obtained, and then subsequently spray-dried using a Niro SD Micro spray dryer (GEA Pharma systems Inc., Switzerland) with an inlet temperature of 40°C, outlet temperature of 40°C, and a feed rate of 18% (out of a maximum 5L/h). With all formulations, the following parameters remained constant: air flow rate 600 L/h, atomizer flow rate: 2.5 kg/h, chamber flow rate: 25/2.5 kg/h, and nozzle pressure: 1.5 bar. All spray dried material was kept in an oven for overnight drying at 25 ◦C. The dried solid dispersions were stored in a sealed vial.

B. Self-Micro Emulsifying Drug Delivery System (SMEDDS)

Dipyridamole SMEDDS formulations were adopted from a publication by Guo *et al*. (2011). The original published formulation was prepared as described in the publication; however, the size measurements were not consistent with the published results, and so further modifications to the formulation were carried out. Based on the solubility test of dipyridamole in different oils and the emulsifying characteristics of the mixtures of various oils and surfactants published by Guo *et al.* (2011), the following ingredients were selected; Labrafac lipophile WL 1349 and Isopropyl alcohol as surfactant and co-surfactant respectively. Solutol (kolliphor) HS 15 was selected as the oil phase. Different ratios of ((surfactant: co-surfactant): oil) were tested, as described in Table 4.1. In addition, different drug loads were tested in the following ratios 1:10, 1:25 and 1:50 (w/w). The oil, surfactant and co-surfactant at finite proportions were mixed in screw-capped glass vials. Thereafter, dipyridamole was added into the mixture and the formulation was left to mix overnight. After the drug was dissolved completely by vortex and mixing, a clear and transparent solution was obtained.

Table 4.1: Composition of tested SMEDDS formulation

Formulation	**1**	**2**	**3**	**4**	**5**	**6**
S_{mix} (surfactant:cosurfactant)	3:1			1:1		
Oil	9:1	4:1	2:1	9:1	4:1	2:1

The furosemide SMEDDS formulation was initially adopted from Zvonar *et al*. (2010). Similar to dipyridamole experiments, the size results were not consistent with the published results, and a higher micelle size was attained. Further experiments were carried out to optimise this formulation. S_{mix} of labarsol and plurol oleique in the ratio of 4:1 was kept constant. Based on solubility tests of furosemide in different oils, the following oils were tested in combination of S_{mix}: soybean oil (3:1), castor oil, tocopherol acetate, oleic acid and iso propyl alcohol. In addition, the formulations developed for dipyridamole were tested for furosemide. Furosemide final formulation was carried out in the same manner as described for dipyridamole.

C. Nano-Particles

Nano formulations were developed in Setsunan University, Osaka, Japan, with the kind guidance of Mr. Kayo Yuminoki. A rotation/revolution mixer (Nano Pulverizer NP-100, Thinky Co., Ltd., Tokyo, Japan) was used to pulverize the compounds.

I. Preparation of Solutions

For the furosemide suspension, 10g of povacoat was added to 100mL of DI water, and after 1 hour of mixing, 10g of mannitol was then added to the solution. For the dipyridamole suspension, 5g of PVA was added to 100mL DI water, and again after 1 hour of mixing, 10g of mannitol was then added to the solution. Povacoat and PVA were used to stabilise the nanomilled API, and mannitol was used a stabiliser in the freeze drying process.

II. Nano Suspension Preparation

100mg furosemide was added to 2.5g zirconium beads (0.1mm diameter) in a zirconium container. 0.5mL of 10% povacoat and mannitol solution was added and milled at 2000 rpm for 2 min at -20 ˚C (milling speed, orbital to axial ratio, 1:1). 4.5mL of the same solution was then added and mixed at 500 rpm for an additional 1 min.

200mg dipyridamol was added to 2.5g zirconium beads (0.1mm diameter) in a zirconium container. 1mL of 5% PVA and D-mannitol was then added and milled at 2000 rpm for 5 min at -20˚C (milling speed, orbital to axial ratio, 1:1). An additional 19mL of 5% PVA and D mannitol solution was then added and mixed at 500 rpm for 1 min.

III. Freeze Drying

Prior to freeze drying, the suspensions were sonicated for 10 minutes. The suspensions were then frozen at -80 ˚C for 30 minutes in an acetone dry ice bath, and thereafter freeze dried at a pressure of 10 μm Hg (under vacuum) at 25 ˚C for 48 hours.

4.2.4.2 Formulation Evaluation

I. Differential Scanning Calorimetry

A DSC 7 Differential Scanning Calorimeter (PerkinElmer Instruments, Beaconsfield, UK) calibrated with indium was used to assess the presence of crystalline drug in the solid dispersions and nano-particles. Formulation powder (3–5mg) was accurately weighed and placed in a non-hermetic aluminium pan. Furosemide samples were scanned from 25 to 300°C at a rate of 10°C/min or 100°C/min in 2 cycles. Dipyridamole samples were scanned first from 40 to 120°C at a rate of 10°C/min and a second cycle from 40 to 300°C at the same rate. Pyris Thermal Analysis Software was used to record and analyse the data.

II. X-Ray Powder Diffraction

X-ray diffraction patterns were obtained for the samples using an X-ray diffractometer Rigaku MiniFLEX 600 (Rigaku, Kent, UK) to perform qualitative and quantitative analysis of polycrystalline materials. The data sets obtained were processed and scaled using PDXL (full-function powder diffraction analysis software suite- minflex guidance). The data were scanned at a step size of 0.2 theta from 2 to 40 degrees at a speed of 5 deg/min.

III. Size Analysis

The median volume diameter of each formulation suspended in water or in simulated intestinal fluids was measured in triplicate using laser light scattering using a Malvern Mastersizer with a 45mm lens (Malvern Instruments Ltd, Malvern, UK). The particle size is reported D(v,0.9), D(v,0.5) and D(v,0.1) where the particle diameters are at the 90th, 50th and 10th percentile, respectively, of the microsphere size distribution curve. Particle size analysis of each formulation was carried out in triplicate.

A Zetasizer (Malvern Nano ZS, Malvern Instruments Ltd, Worcestershire, UK) was used to analyse the mean particle size of the nano-carriers and self-emulsifying drug delivery systems using dynamic light scattering (DLS).

IV. Transmission electron microscope (TEM) & Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to analyze the morphology of the API, solid dispersion and the physical mixtures of the solid dispersion formulations. Samples were coated with gold using a K550 sputter coater (Emitech, Ashford, Kent, UK) and observed using a Philips/FEI XL 30 SEM (Phillip, Cambridge UK) at 10 kV.

Nano suspension and self-emulsifying drug delivery systems morphology was analysed using a FEI CM 120 Bio Twin transmission electron microscope (TEM, Philips Electron Optics BV, Netherlands). Approximately 1 drop of the preparation was placed on a copper grid with a nitrocellulose covering and negatively stained with 1% uranyl acetate.

V. Dissolution Tests

Dissolution tests under different conditions were carried out as specified in the previous section. All formulations were tested in *m*Hanks buffer under different conditions of bile salts and pH (in the range of 1-6mM and 6.4-7.4 respectively) as described in chapter 3. In the case of nano-particles, samples were taken manually at 5, 15, 30, 60, 90 and 120 min, filtered via 0.45µM PTFE syringe filters and immediately centrifuged at 25˚C 13,000 RPM for 15 min. Samples were then diluted 10 times with mobile phase and were analysed by HPLC.

In addition, further dissolution tests were carried out to evaluate the precipitation effect from the stomach to the duodenum. An unpublished recipe from the laboratory of Prof. Yamashita (Setsunan University, Osaka) was adopted. The study was performed in pH 1.6 fasted state simulated gastric fluids (FaSSGF), followed by in-situ transfer to a pH 6.5 fasted state simulated intestinal fluids (FaSSIF). The study was also performed using small volumes, with proportional clinical doses of the drug. The drug dose was calculated as follows (
Equation **4.2**):

Clinical concentration= Clinical dose \ Gastric volume

Equation 4.2: Calculated dose tested in the dissolution test

Where: gastric volume is a sum of gastric residual volume (30mL) and volume administered with drug (250mL)

Preparation of FaSSGF and pre-FaSSIF is given in Table 4.2 and Table 4.3, respectively. For FaSSGF preparation, sodium chloride was added and dissolved in DI water, followed by addition of SIF powder (mixture of 0.75mM sodium taurocholate and 3mM lecithin). The volume with DI water was made up to 100mL, and pH was accurately adjusted to 1.6 using 5M HCl. For pre-FaSSIF preparation, KCl and KH_2PO_4 were completely dissolved in DI water, followed by the addition of SIF powder and NaOH solution. The solution was mixed and sonicated until clear solution was obtained. MES was then added and dissolved, and the volume made up to 50mL with DI water. The final pH of this media was between 9 and 9.5, which on addition to FaSSGF gives a final pH of 6.5. This was prepared *in-situ,* when 5mL of pre-FaSSIF was added to 10mL of FaSSGF using a syringe pump at a rate of 0.5mL/min over 10 minutes.

Table 4.2: Composition of FaSSGF

Composition	**Amount per 100 mL**
Sodium chloride	200
SIF powder	5.8µg
DI water	QS to 100 mL
5 M HCl	QS to pH 1.6

Table 4.3: Composition of Pre-FaSSIF

Composition	**Amount per 50 mL**
Potasium chloride	1.155 g
KH_2PO_4	0.585 g
SIF powder	0.327 g
5 M NaOH	1.025 mL
MES *	0.15975 g
DI water	QS to 50 mL

* (n-morpholino) ethane sulfonic acid (Nacalai Tesque, 216-23)

Experimental Procedure

An experimental dose was added to 10mL FaSSGF in a glass vial, placed on a magnetic stirrer and mixed continuously at 200 RPM. The temperature was maintained at 37 ˚C by placing the stirrer in an incubator. After 1 minute the first sample was withdrawn and the syringe pump was started immediately to transfer 5mL of pre-FaSSIF at 0.5mL per minute. Aliquots of 100µL were withdrawn at 1, 3, 5, 7, 9 and 11 minutes. Sampling was continuous for 4 hours, and the samples were subsequently filtered via 0.45µm 4mm PTFE syringe filters (Millex LH, SLLHH04NL, Millipore) and further diluted by use of an ACN: water mixture (1:1). The samples were analysed using HPLC by methods described in the previous section. Figure 4.2 shows a schematic of the experimental run and sampling frequency.

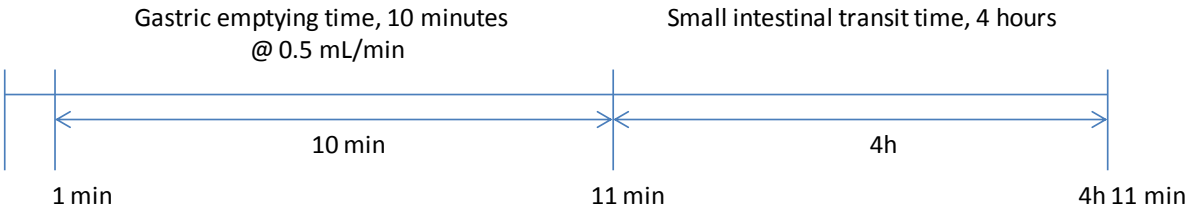


Figure 4.2: A schematic of the experimental run and sampling frequency of the dissolution test.

4.2.4.3 Statistical and Data Analysis

The dissolution and permeation data were analysed by one way ANOVA repeated measurements followed by a tukey post- hoc analysis using Univariate General Linear Model tool in PASW statistics 22 (SPSS Inc., Illinois, USA).

4.2.5 Results & Discussion

4.2.5.1 Solid Dispersion Preparation and Evaluation

Furosemide and dipyridamole were successfully prepared in their amorphous forms using the spray drying method. The preparation was relatively straightforward, involving a simple procedure of dissolving the drug in an organic solvent, adding the carrier and spray-drying it. The yield for dipyridamole and furosemide solid dispersions was 52% and 33% respectively. This relatively low yield is related to the low batch size used (15-30g) as compared to the minimum solid material usually used for Niro SD micro (200g). Moreover, a high percentage of the powder was left on the walls of the spray cylinder, cyclone and tubing due to the static properties of the polymer, and only a limited amount of the powder reached the collecting container.

A reduction in the particle size was observed in the case of dipyridamole. Dipyridamole and furosemide solid dispersion particle sizes were measured at 0.88, 2.84 and 4.87µm and 0.74, 3.34 and 5.79µm at the 10, 50 and 90 percentile, respectively (compared to 5.15, 39.20 and 69.57µm and 1.09, 3.33 and 8.53µm for dipyridamole and furosemide API). The SEM micrographs of dipyridamole, furosemide, their solid dispersion formulations and physical mixture are presented in Figure 4.3 and Figure 4.4.

As can be seen, both drugs produced sharp, long particles; structures not produced in the solid dispersion formulations characterised by the presence of spherical particles. This may indicate a possible interaction between the polymer and the drugs in the solid-dispersion formulations.

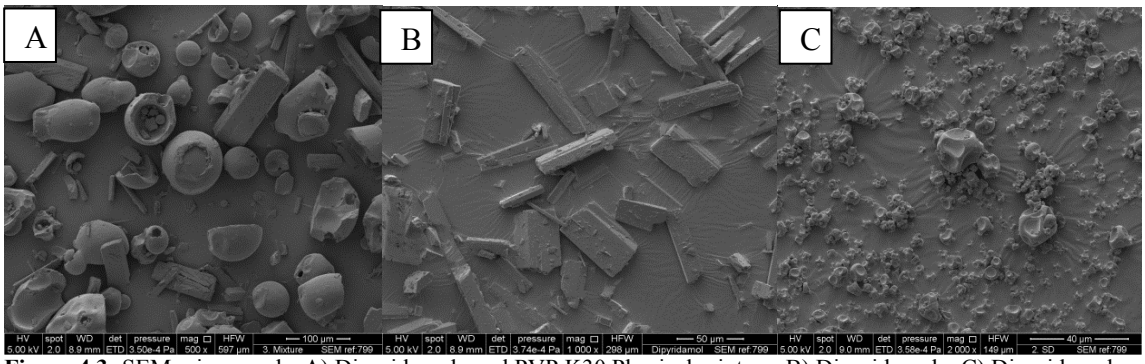


Figure 4.3: SEM micrographs A) Dipyridamole and PVP K30 Physical mixture, B) Dipyridamole, C) Dipyridamole solid dispersion.

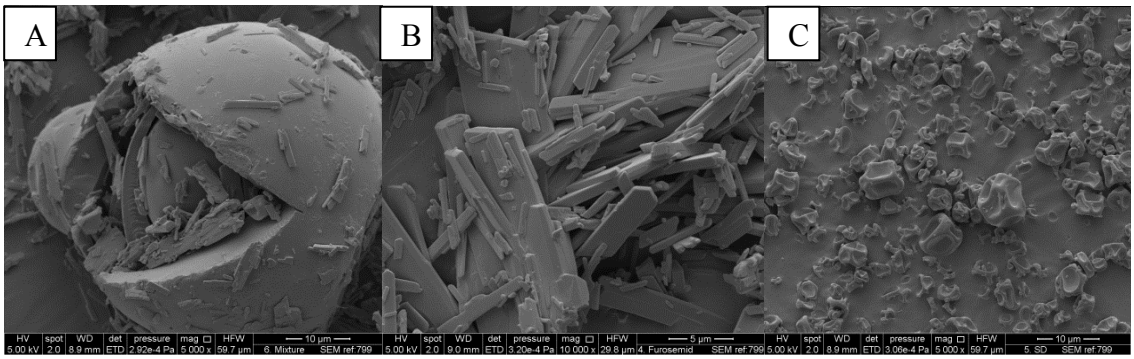


Figure 4.4: SEM micrographs A) Furosemide and PVP K30 Physical mixture, B) Furosemide, C) Furosemide solid dispersion.

Powder X-ray diffractograms of furosemide, dipyridamole, their physical mixture and solid dispersion formulations are shown in Figure 4.5 and Figure 4.6. The presence of numerous distinct peaks in the PXRD spectrum indicates that both furosemide and dipyridamole are presented in their crystalline form. The spectrum of PVP was characterized by the complete absence of a diffraction peak, otherwise characteristic of an amorphous compound. The diffraction patterns of solid dispersion formulations show a broad peak due to PVP present in the formulations, and an absence of major diffraction peaks corresponding to furosemide and dipyridamole, with most of the diffraction indicating the drugs were present as amorphous material inside the PVP matrix. In the case of the physical mixtures, in both diffractograms of furosemide and dipyridamole, the peaks indicated the detection of crystallinity, and the absence of the interaction of the drug with its carriers in the physical mixture.

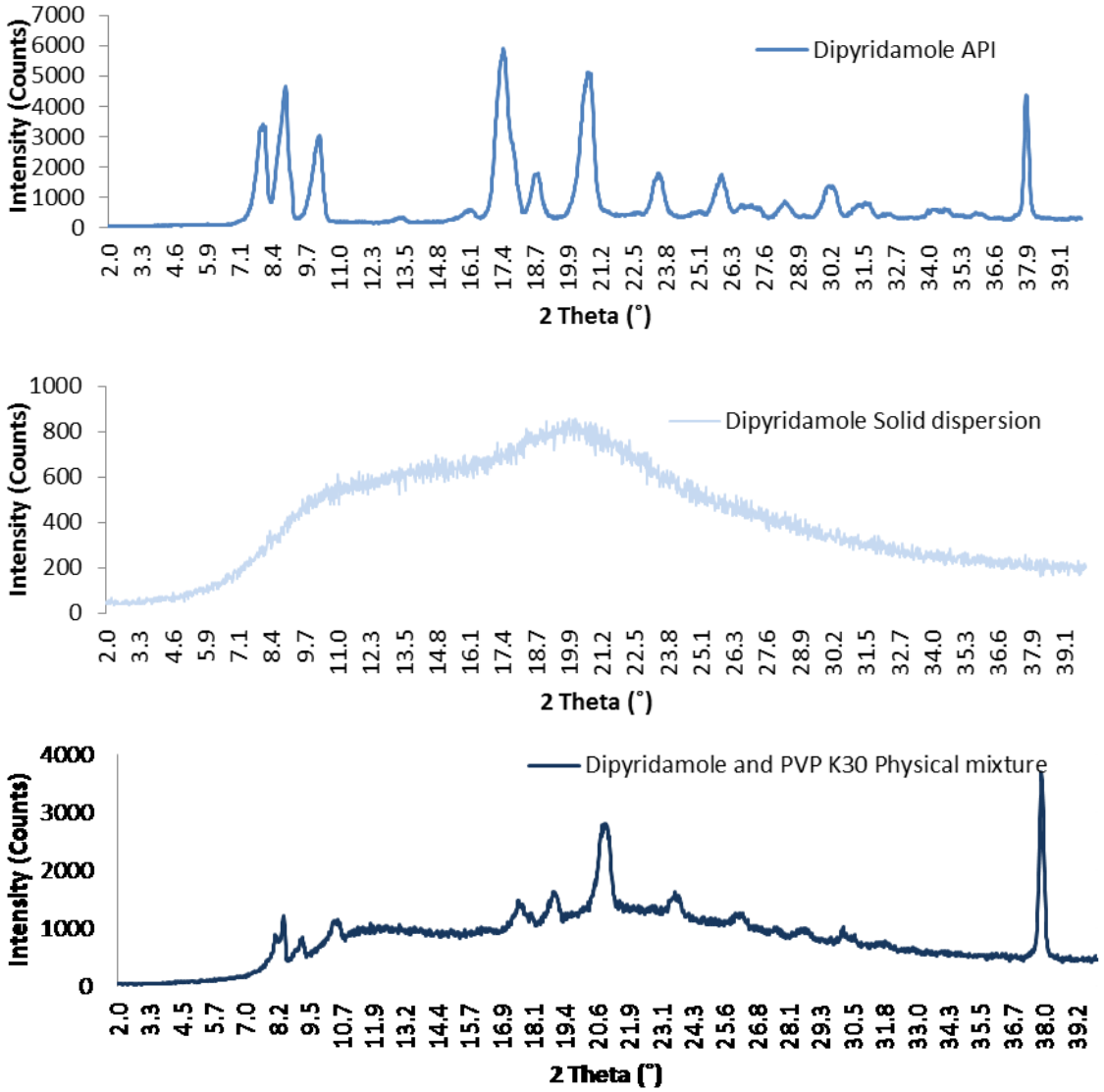


Figure 4.5: Powder X-ray diffractograms of A) Dipyridamole, B) Dipyridamole solid dispersion, C) Physical mixture of Dipyridamole and PVP-K30.

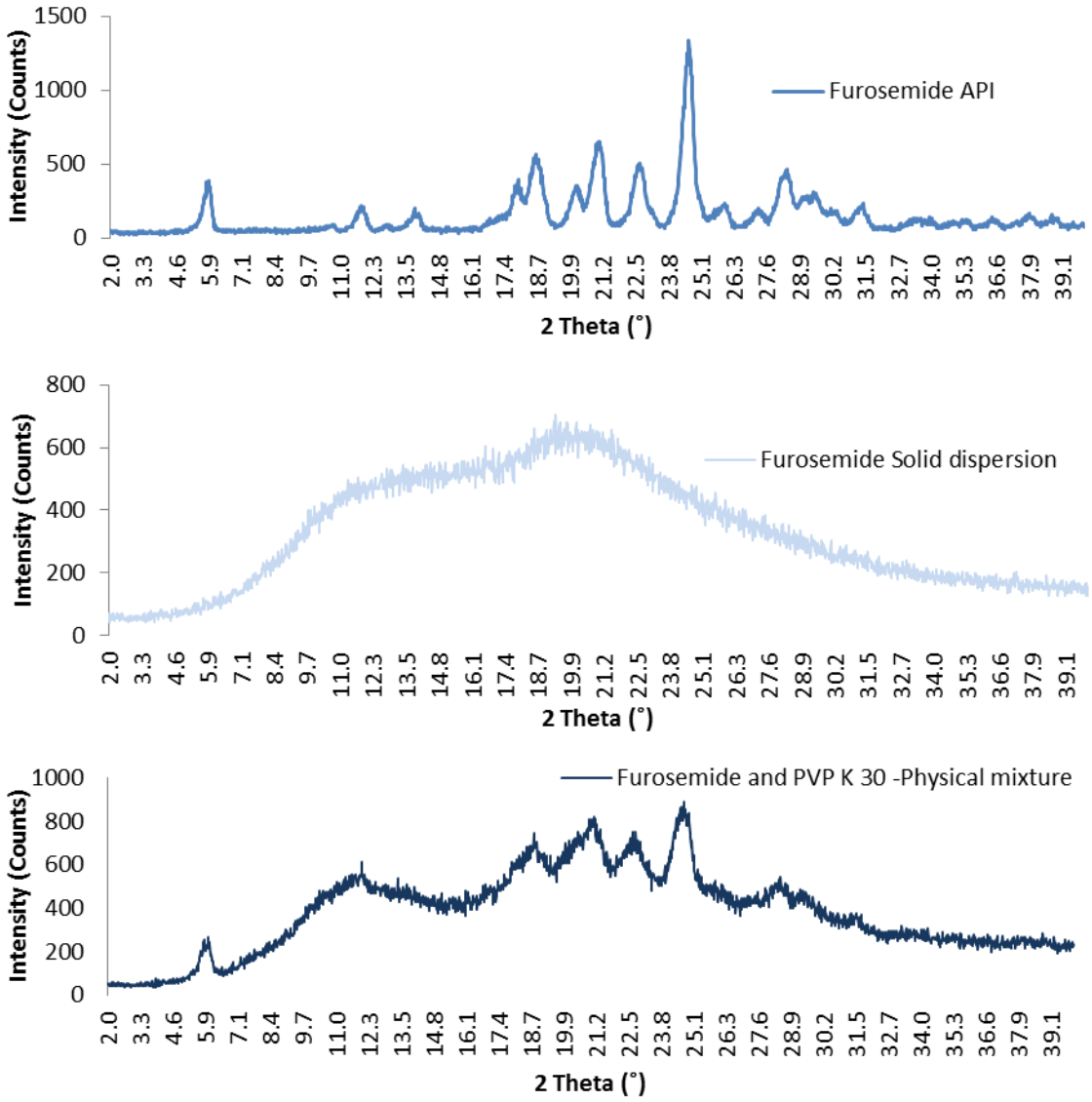


Figure 4.6: Powder X-ray diffractograms of A) Furosemide, B) Furosemide solid dispersion, C) Physical mixture of furosemide and PVP-K30.

The DSC thermographs for pure furosemide, dipyridamole, PVP-K30, their physical mixtures and solid dispersion formulations are shown in Figure 4.7 and Figure 4.8. Dipyridamole showed a sharp endothermic peak at 166˚C, corresponding to its melting point. The DSC scan of PVP showed a broad endotherm peak, ranging from 80 to 120°C due to the presence of residual moisture in PVP in the first cycle. The T_g at 146˚C could be observed when the sample was further heated. Furosemide showed a small melting peak at 220°C followed by an endothermic peak. Another exothermic peak was observed at 270°C. Similar DSC thermographs were reported by Patel *et al*. (Patel *et al.*, 2008). Samples of solid dispersion showed a complete absence of drug peak at the aforementioned melting points with T_g at 90°C. This complete absence of peaks indicates that the drugs are amorphous, or are in a solid solution inside the PVP matrix. This further type of interaction confirmed the results which were observed in the PXRD studies.

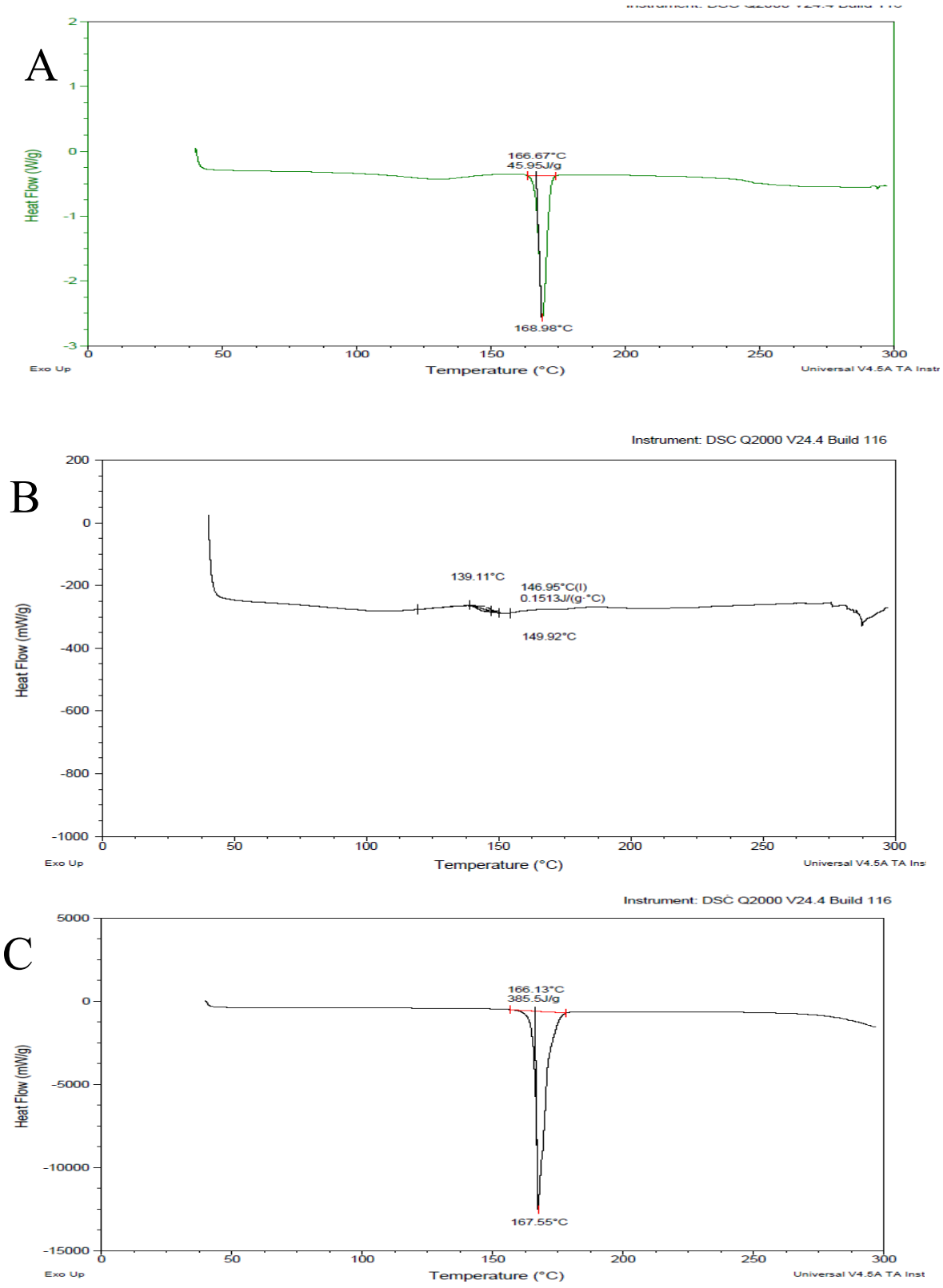


Figure 4.7: DSC thermographs for A) Dipyridamole, B) Dipyridamole solid dispersion, C) Physical mixtures of dipyridamole and PVP-K30

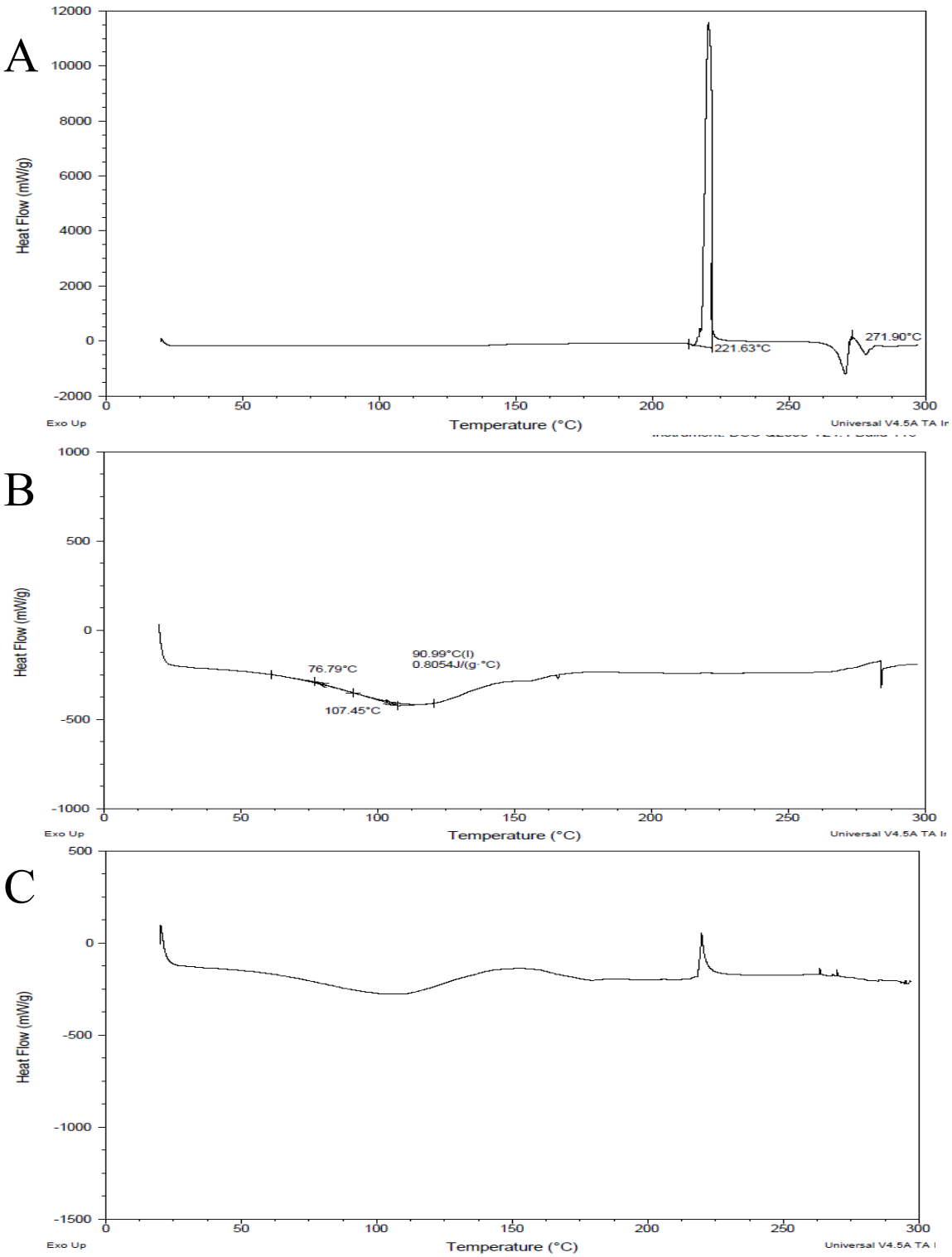


Figure 4.8: DSC thermographs for A) Furosemide, B) Furosemide solid dispersion, C) Physical mixtures of furosemide and PVP-K30

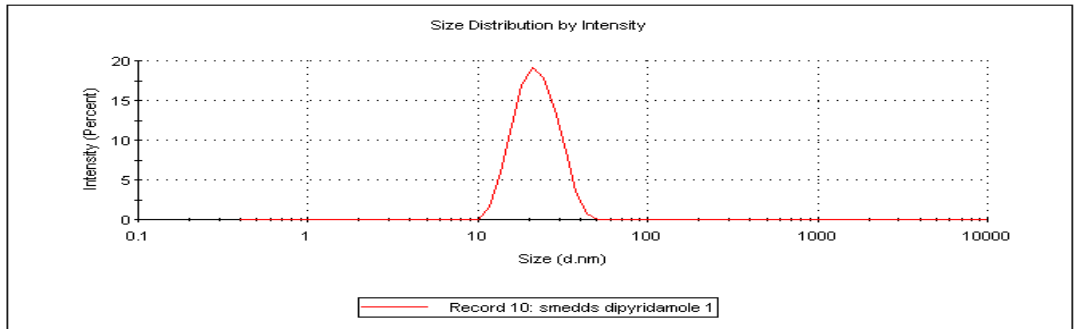
4.2.5.2 Self- Micro Emulsifying Drug Delivery System Preparation and Evaluation

The process of developing SMEDDS formulations for both drugs was more complicated. The attempt to adapt published SMEDDS formulations was not successful, and was not consistent with the published results. Guo *et al*. (2011) developed SMEDDS formulation for dipyridamole with the following composition: oleic acid, labrfac, kolliphor and iso propyl alcohol at the ratio of 18, 12, 42 and 28% w/w respectively. It was reported that the average droplet size was 89nm, and the size of all droplets was below 100nm. However, when the formulation was repeated in our lab, the average droplet size was 320nm and polydispersed. Thus, further modifications were carried out. The oleic acid and the labrfac represented the oil phase in the origin formulation. It was reported that the purpose of adding oleic acid to the original formulation was to increase dipyridamole solubility in the oil phase. However, the addition of the oleic acid also increased the droplet size, and decreased the emulsifying capability. For this investigation needs, it was decided to omit the oleic acid from the formulation and decrease the drug load (compromising dipyridamole solubility in the oil formulation).

The effects of changing labrfac and kolliphor ratios in the formulations on droplet size and emulsifying capabilities were investigated. The droplet size of the dipyridamole micro-emulsion decreased with a reduction in oil content (labrfac) in SMEDDS. When S_{mix}: oil ratio was 2:1 and 4:1, a bigger particle was formed in comparison with ratio 9:1 of S_{mix}: oil (formulations 2, 3, 5 and 6- Table 4.1). Moreover, the emulsifying capability increased at the ratio of 1:9 of S_{mix}: oil, and both formulations 1 and 4 were dispersed within seconds under gentle conditions of stirring to produce clear solutions. The average droplet size of formulations 1 and 4 was around 20 to 25nm, and the size of all droplets was below 100nm. The droplet size did not differ when the formulation was dispersed in water, SGF or SIF (Figure 4.9). As seen in Figure 4.10, the SMEDDS formulation containing dipyridamole, following self-emulsification observed under TEM, was spherical in shape and uniform in size. The following drug loads were tested as ratios of 1:10, 1:25 and 1:50. It was found that the drug load did not affect the droplet size (around 20nm) nor the emulsifying capability. As such, larger ratios of 9:1 (S_{mix}: oil) and 3:1 (surfactant: co-surfactant) were used for further studies.

A

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 21.71	Peak 1:	22.66	100.0	6.424
PdI: 0.167	Peak 2:	0.000	0.0	0.000
Intercept: 0.943	Peak 3:	0.000	0.0	0.000
Result quality : Good				



B

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 24.00	Peak 1:	23.29	93.4	7.386
PdI: 0.271	Peak 2:	352.6	4.2	96.20
Intercept: 0.955	Peak 3:	5188	2.4	481.8
Result quality : Good				

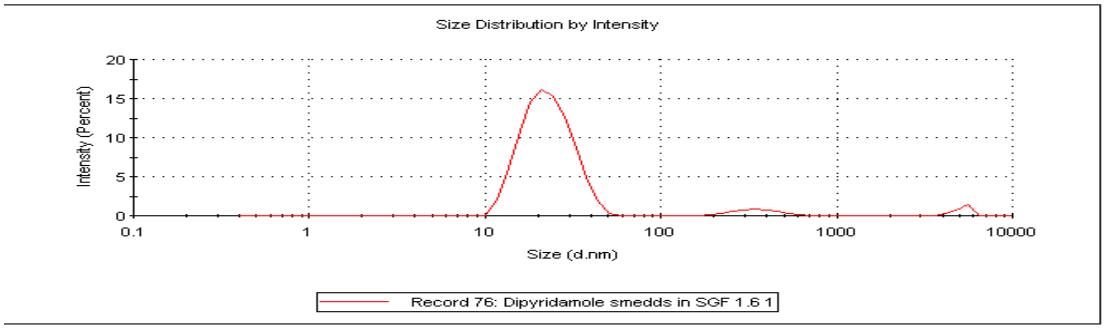


Figure 4.9: Micelles size measurements for dipyridamole SMEDDS after self-emulsifying A) In water and B) In SGF

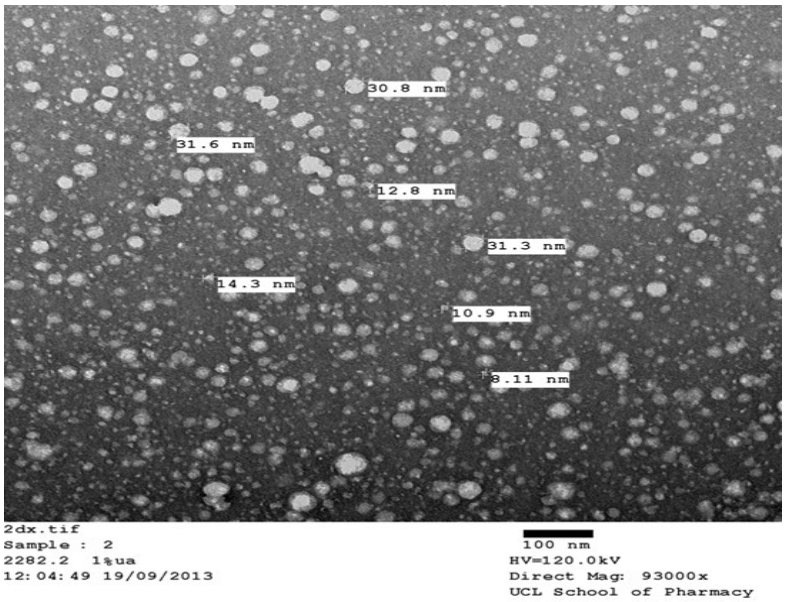


Figure 4.10: Dipyridamole SMEDDS formulation, following self-emulsification observed under TEM.

Zvonar *et al*. (2010) developed SMEDDS formulation for furosemide. The formulation included the following ingredients: labrasol, plurol oleique and mygliol 812 as the surfactant, cosurfactant and the oil phase respectively. As with previous attempts to replicate dipyridamole SMEDDS formulations from published literature, preparing furosemide SMEDDS formulation with this composition proved to be unsuccessful, with a droplet size of more than 4μm attained with polydispersed micro-emulsion. To improve upon this furosemide SMEDDS formulation, a S_{mix} of labarsol and plurol oleique (as described by Zovenar *et al*. (2010) in the ratio of 4:1 was tested. The following oil phases were tested: soybean oil, castor oil, tocopherol acetate, oleic acid and IPA. Soybean oil and oleic acid as the oil phase did not yield one oil phase solution. With tocopherol acetate and castor oil, precipitation of the drug was observed when water was added, and the emulsions were not unambiguous. With IPA, a clear micro-emulsion was obtained; however, on measuring the droplet size, values around 100nm were attained with polydispersed micro-emulsions. Based on the success of developing a dipyridamole formulation, the composition for dipyridamole SMEDDS formulation was investigated for furosemide. This evaluation produced a droplet size similar to that of the dipyridamole formulation at around 20-30nm, and a monodispersed microemulsion was attained. The droplet size also did not alter when the formulation was dispersed in water, SGF or SIF (Figure 4.11). As seen in Figure 4.12, the micro-emulsion of the SMEDDS formulation containing furosemide, following self-emulsification observed under TEM, was spherical in shape and uniform in size.

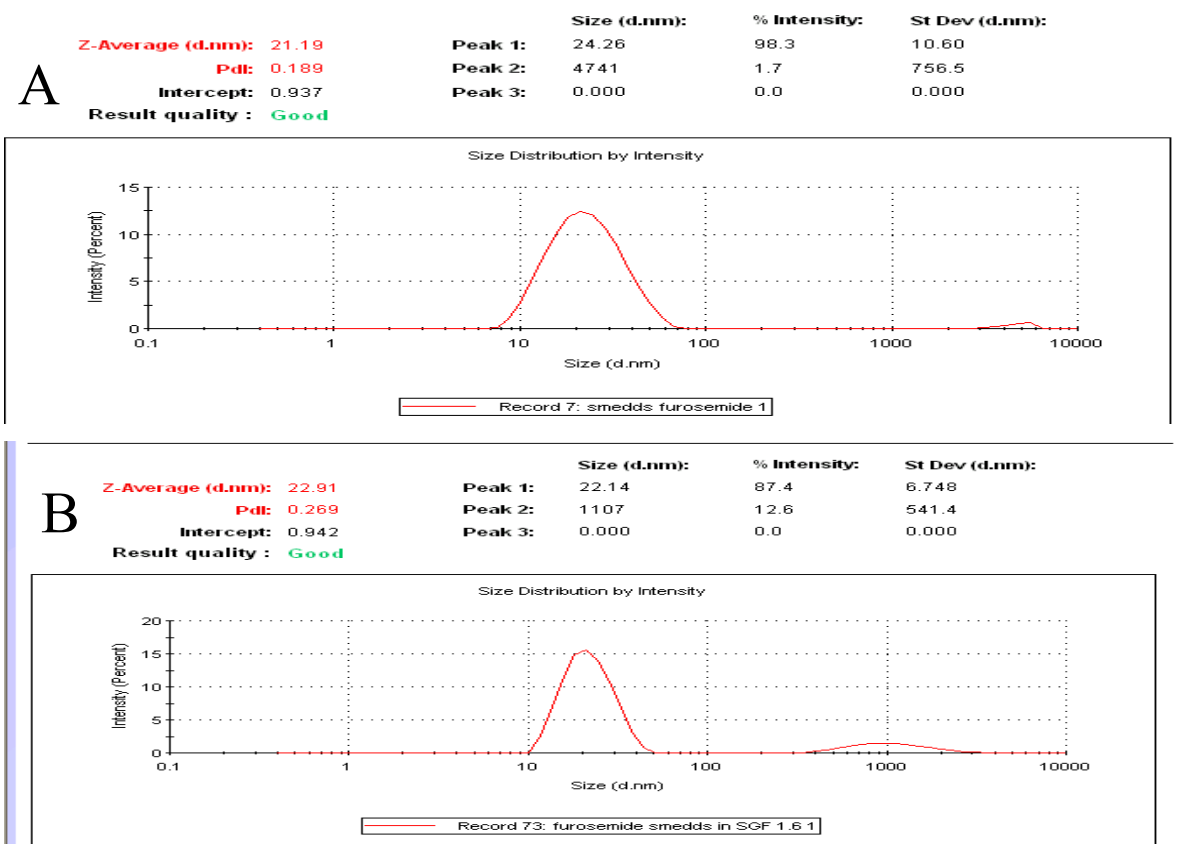


Figure 4.11: Micelles size measurements for dipyridamole SMEDDS after self-emulsifying A) In water and B) In SGF

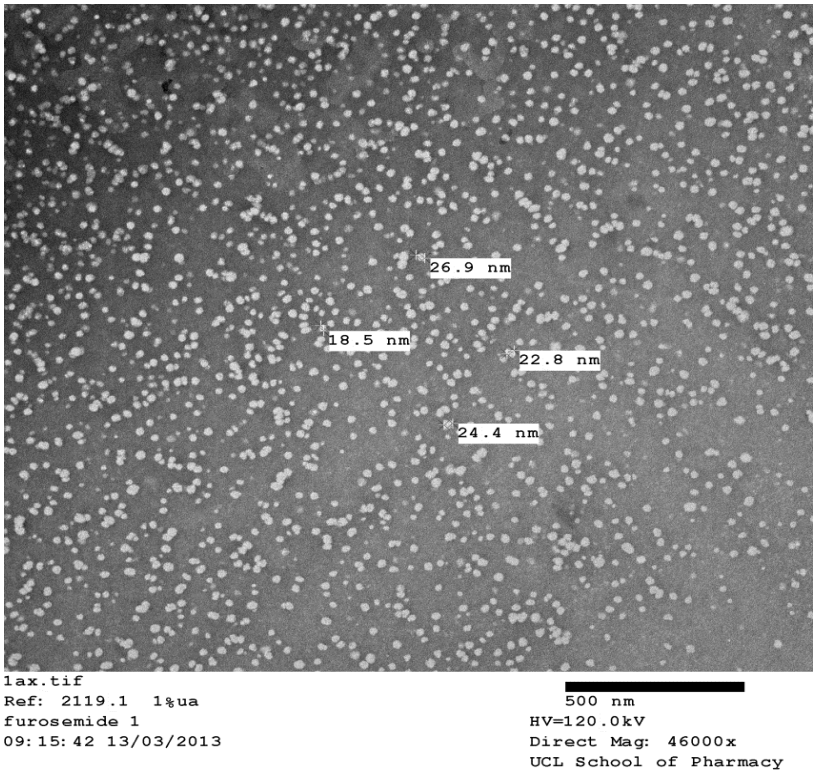


Figure 4.12: Furosemide SMEDDS formulation, following self-emulsification observed under TEM.

4.2.5.3 Nano -Particle Preparation and Evaluation

The development of furosemide and dipyridamole nano suspensions and the choice of excipients was based on the kind guidance of Mr. Kayo Yuminoki at Setsunan University labs (Takatsuka *et al.*, 2009). Particle reduction to nano size was successful for both furosemide and dipyridamole by this approach. For furosemide and dipyridamole suspended in water, particle size was measured as 0.0573 6.95 and 34.4µm and 11.7, 36.1 and 116µm at 10, 50 and 90 percentiles, respectively. Nano particle sizes for furosemide and dipyridamole were measured as 0.071, 0.152 and 0.485µm and 0.068, 0.139 and 0.289µm at 10, 50 and 90 percentiles respectively. As freeze drying processes might otherwise promote crystal growth, the particle size was also measured after freeze drying. The powder was then re-suspended in water, and it was found that the particle size measurements were not affected by the freeze drying process. In addition, particle size was measured after 6 days of storage as suspension, and no aggregation was observed either for furosemide or dipyridamole suspensions (Table 4.4). Size measurement was also carried out 6 months after storage of the freeze dried powder. Re-suspension the freeze dried powder after storage of 6 months in water gave particle size measurements of 0.0238, 0.0881 and 0.341µm and 0.0248, 0.0908 and 0.315µm for dipyridamole and furosemide at 10, 50 and 90 percentile accordingly, indicating the particle size was stable after six months of storage.

Table 4.4: Particle size measurements for nano suspension formulations

	Particle size (nm)		
	10%	50%	90%
Dipyridamole nano-suspension	68	139	298
Dipyridamole nano-suspension after 6 storage days	89	216	622
Freeze dried nano-particles dipyridamole	76	174	450
Furosemide nano-suspension	70	149	494
Furosemide nano-suspension (after 6 storage days)	70	146	356
Freeze dried nano-particles furosemide	73	163	639

The freeze-dried powders after resuspension of dipyridamole and furosemide were analysed by TEM (Figure 4.13). For dipyridamole, the same range of particle size was observed under the microscope; in addition, some of the particles (bigger size) retained their elongated and sharpened shape, while others (smaller size) were identified as spherical but well-defined. Furosemide sample was more uniform in size and shape. Most of the particles were identified as being spherically-shaped.

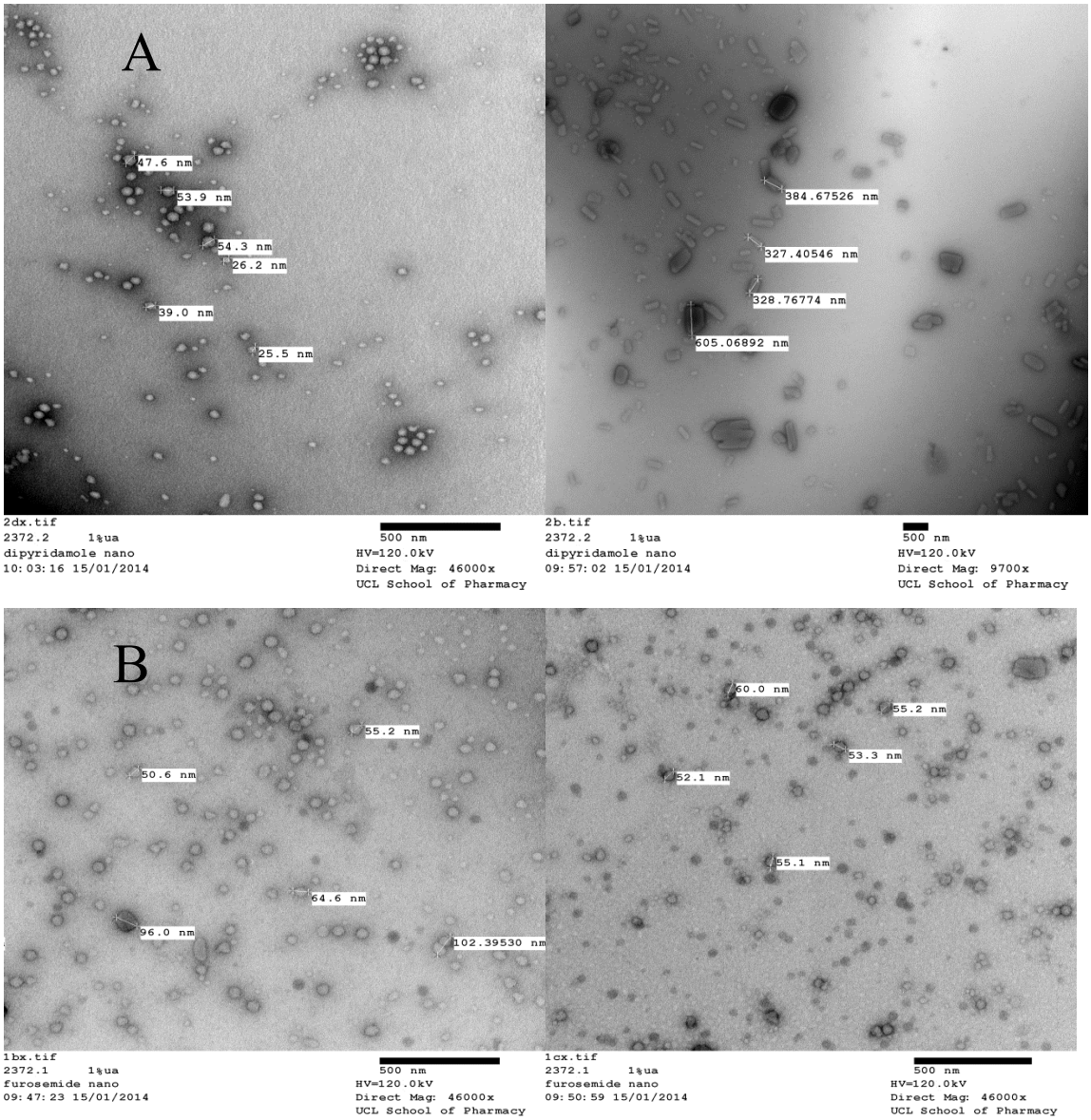
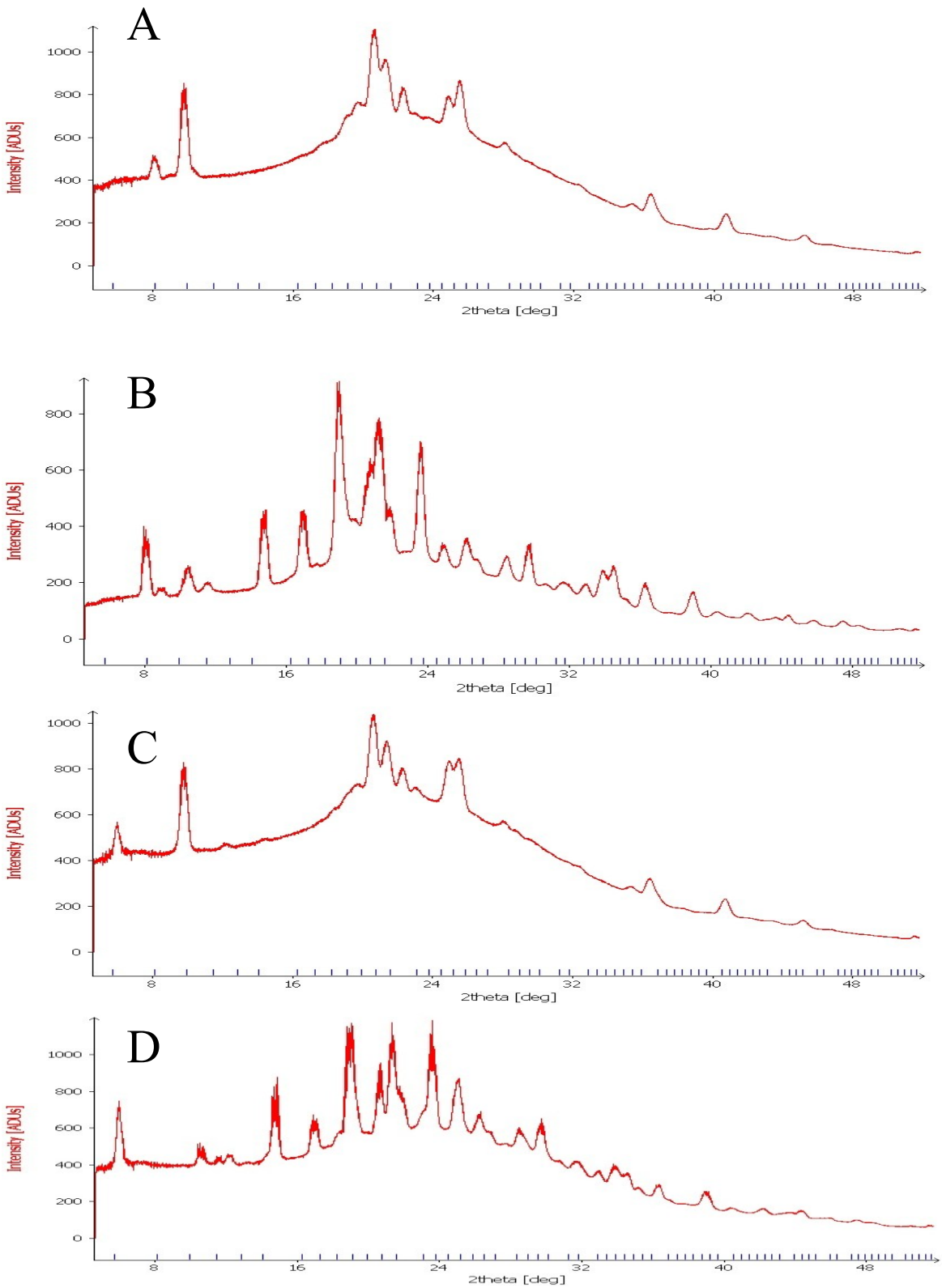


Figure 4.13: A) Dipyridamole nano suspension and B) Furosemide nano suspension under TEM.

PXRD was performed to investigate the crystalline form of furosemide and dipyridamole nano particles. Figure 4.14 shows the X-ray powder diffraction of the formulation excipients, their physical mixture, and the freeze-dried samples of the pulverized dipyridamole and furosemide. It can be seen that PVA and povacoat generate broad peaks which are reflected in the freeze dried samples. However, other peaks can also be seen in the spectrum of dipyridamole and furosemide in the freeze dried samples, and can be found in the API spectrum alone. This may indicate that some of the drug in the nano- particle formulation is found in its crystalline form.


A
B
C
D
Intensity [ADUs]
2theta [deg]
0
200
400
600
800
1000
8
16
24
32
40
48

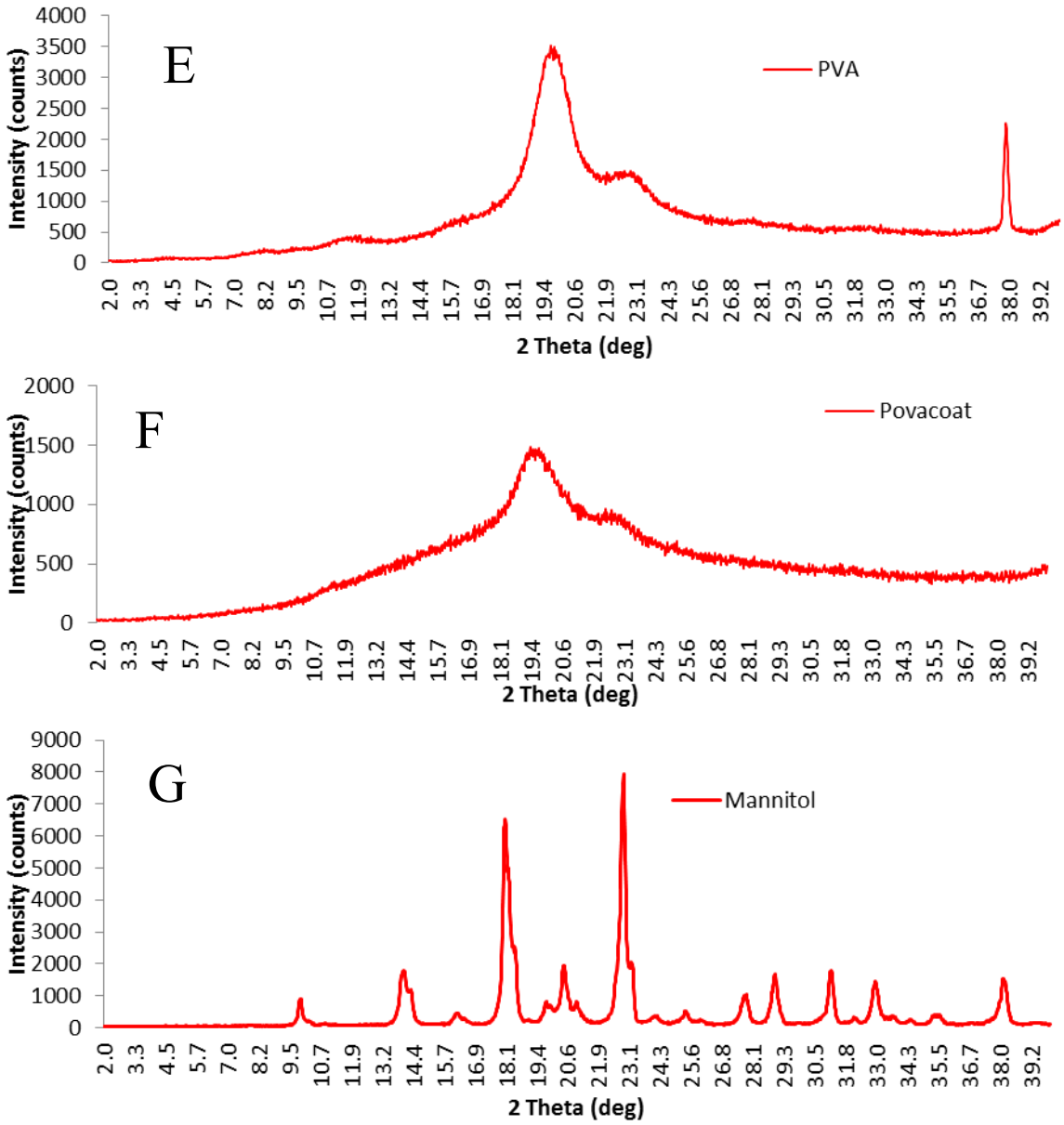
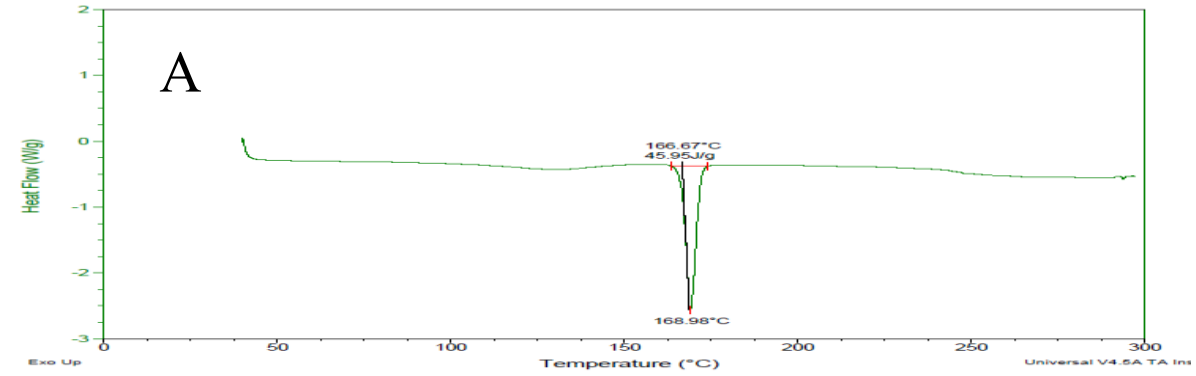
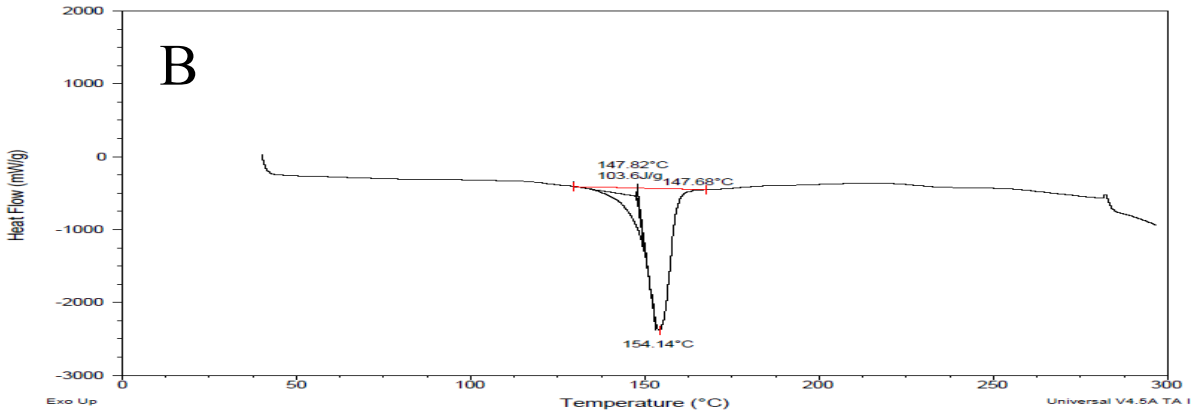
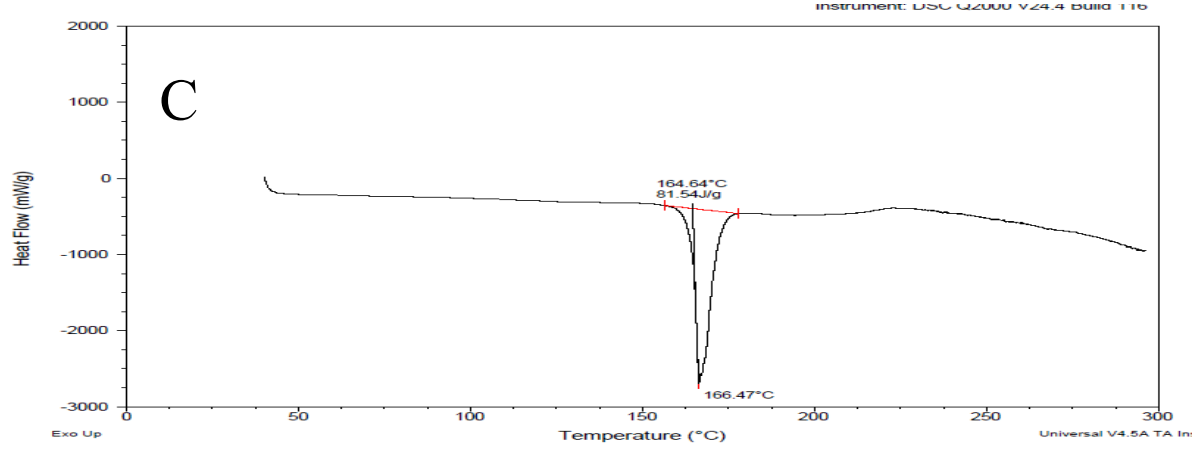


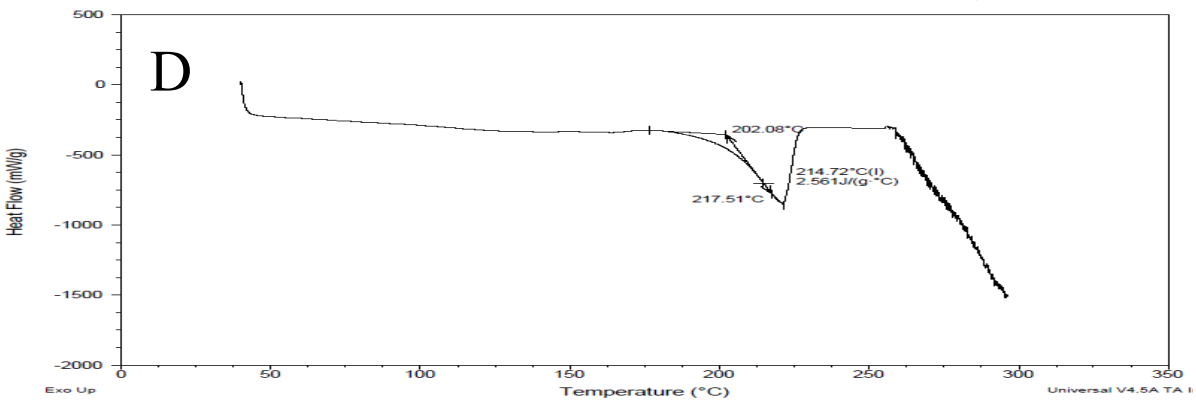
Figure 4.14: Powder X-ray diffractograms of A) Dipyridamole nano particles B) Physical mixture of dipyridamole, mannitol and PVA, C) Furosemide nano particles D) Physical mixture of furosemide, mannitol and povacoat, E) PVA, F) Povacoat, G) Mannitol

The freeze-dried sample of the pulverized dipyridamole and dipyridamole were analysed in DSC. DSC thermographs of the formulations excipients, dipyridamole, furosemide, their physical mixtures and freeze dried formulations are presented in Figure 4.15 and Figure 4.16. For dipyridamole, it can be seen that the melting point for the freeze dried sample decreased from 166˚C to 154˚C. An acceptable explanation is that, less energy is required to melt the particles due to the reduction in the particle size and a higher surface area. Another possible explanation is that it might be that the inclusion of PVA and mannitol affected the melting point of the composition, and the melting point decreased due to a decrease in the composition eutectic temperature. A similar effect was observed for freeze dried phenytoin nano particles with methyl cellulose (Takatsuka *et al*., 2009), whereby the exothermal peak was not found. The physical mixture also showed a similar melting point to dipyridamole alone. The decrease in melting point was not observed in the physical mixture, probably due to the fact that the physical mixture was only ground together and not freeze dried. Therefore, the interaction between PVA, mannitol and dipyridamole was not observed in the physical mixture thermograph. As for furosemide, the pattern of melting point at 220˚C followed by a big endothermic peak (recrystallisation) and a second peak at 270˚C of melting point followed by a second re-crystallisation may indicate on solid solid transition. This pattern disappeared for the nano-particles formulation. Similar to dipyridamole nano-particles, a melting point at 153˚C was observed. Considering the DSC thermographs similarity between furosemide and dipyridamole nano-particles formulations, it might be that only mannitol melting point can be observed in the DSC. The physical mixture gave an exothermic peak at 167˚C similar to the peak observed for mannitol, which might further emphasise the fact that the interaction in the case of the nano-formulation might affect the sensitivity of the DSC to observed further phase transitions for nano-formulations. To further understand this, the heating rate was increased to 100°C/min. It can be seen that for furosemide, the pattern of solid solid transition was kept the same (big endothermic peak followed by exothermic peak at 236°C, and second transition at 277°C). Similar pattern was observed for the physical mixture and the nano-formulation (Figure 4.17). This may indicate that part of furosemide exits in its crystalline form in the nano-formulation.


A
166.67°C
45.95J/g
168.98°C
Heat Flow (W/g)
Temperature (°C)
Exo Up


B
147.82°C
103.6J/g
147.68°C
154.14°C
Heat Flow (mW/g)
Temperature (°C)
Exo Up


C
164.64°C
81.54J/g
166.47°C
Heat Flow (mW/g)
Temperature (°C)
Exo Up


D
202.08°C
214.72°C(I)
2.561J/(g·°C)
217.51°C
Heat Flow (mW/g)
Temperature (°C)
Exo Up

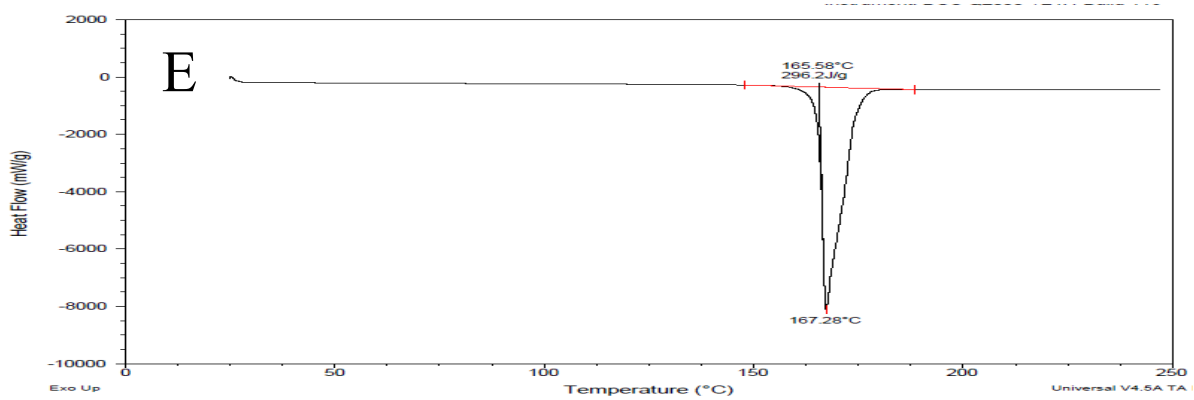
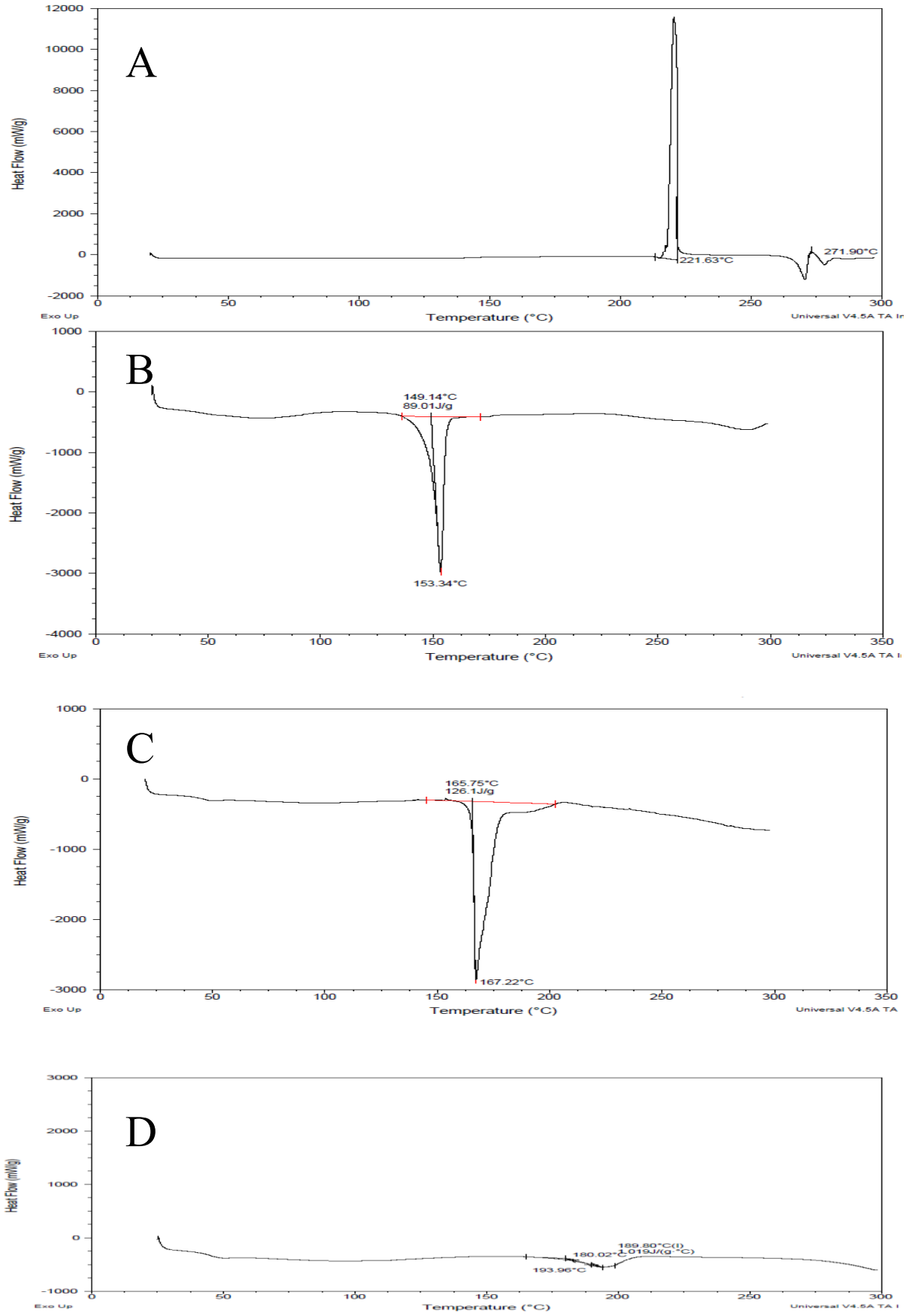


Figure 4.15: DSC thermographs for A) Dipyridamole, B) Dipyridamole nano particles, C) Physical mixtures of dipyridamole, PVA and mannitol, D) PVA E) Mannitol


A
Heat Flow (mW/g)
Temperature (°C)
221.63°C
271.90°C
Exo Up
B
149.14°C
89.01J/g
153.34°C
C
165.75°C
126.1J/g
167.22°C
D
189.80°C(I)
193.96°C

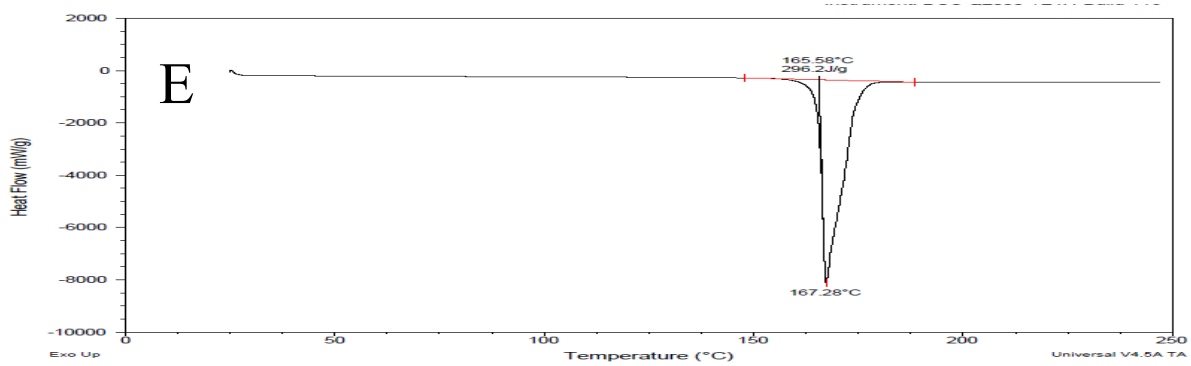


Figure 4.16: DSC thermographs for A) Furosemide, B) Furosemide nano particles, C) Physical mixtures of furosemide, povacoat and mannitol D) Povacoat, E) Mannitol at 10°C/min heating rate

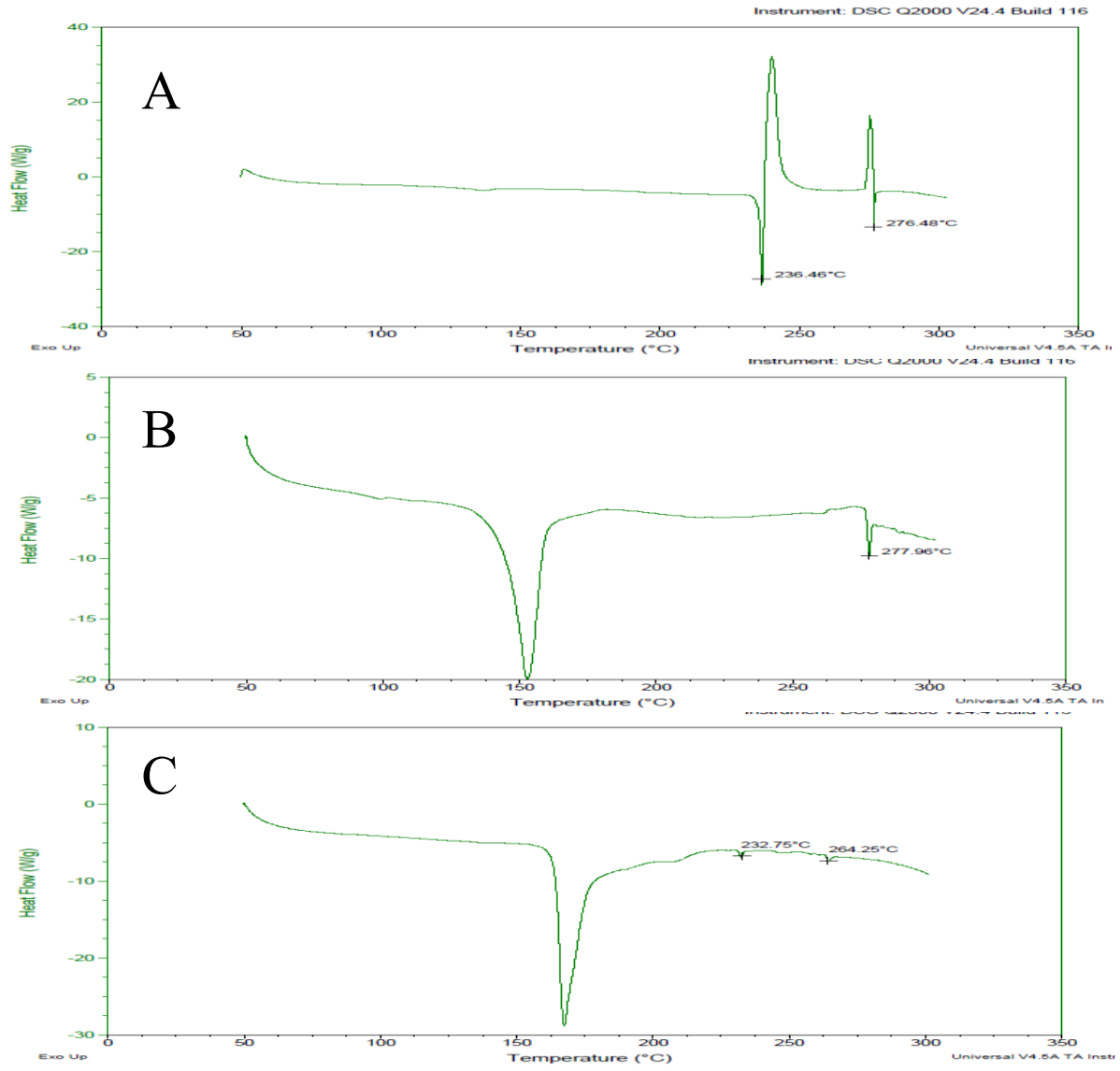


Figure 4.17: DSC thermographs for A) Furosemide, B) Furosemide nano particles, C) Physical mixtures of furosemide at 100°C/min heating rate

4.2.5.4 Dissolution Tests

The extent of dipyridamole release from the solid dispersion under different conditions of pH and bile salts concentration increased significantly, and was around 80-95%. Moreover, no effect of bile salts or pH was observed (Figure 4.18), as was observed in the case of the API alone or the marketed tablet in the previous section (Figure 3.8) Similar results were obtained for dipyridamole as SMEDDS (Figure 4.19). No drug precipitation was observed during 2 hours of experiments, and the percentage of drug release at 2 hours was around 80-90% under all conditions. For the dipyridamole nano-formulation, no increase in drug dissolution was observed. Moreover, the bile salt concentration affected the extent of drug release, with higher dissolution observed at 6mM, followed by 3 and 1mM. Similarly to the API, it seems that pH had a slight effect on the dissolution (Figure 4.20).

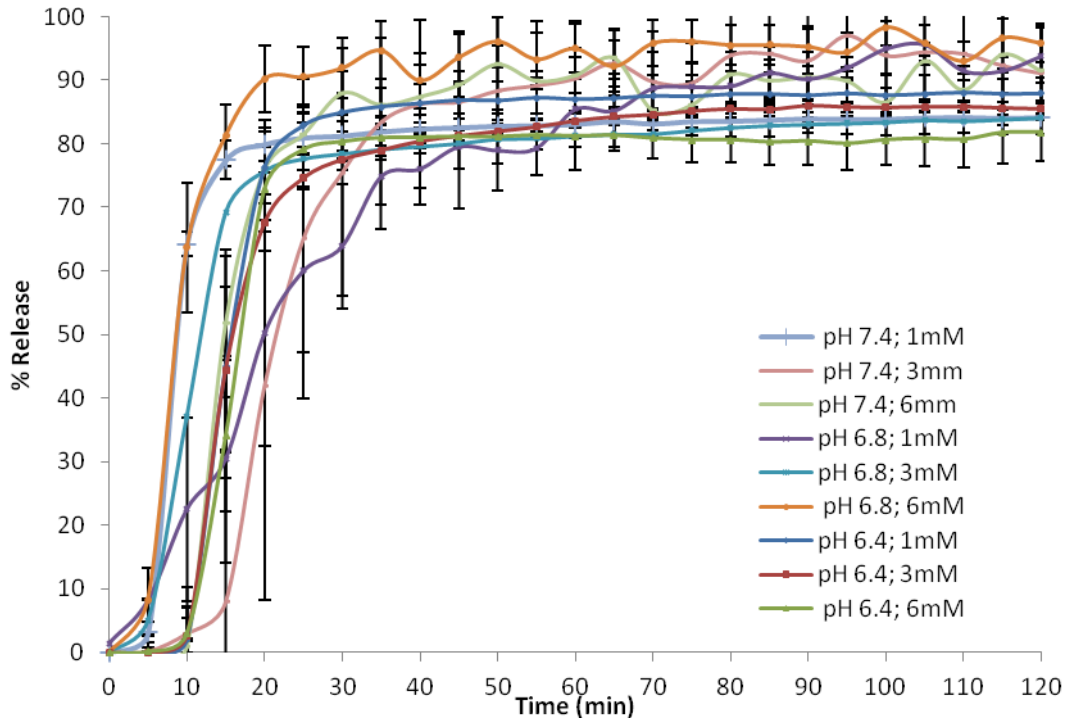


Figure 4.18: Dissolution of dipyridamole solid dispersion under different conditions of bile salt and pH in *m*Hanks buffer

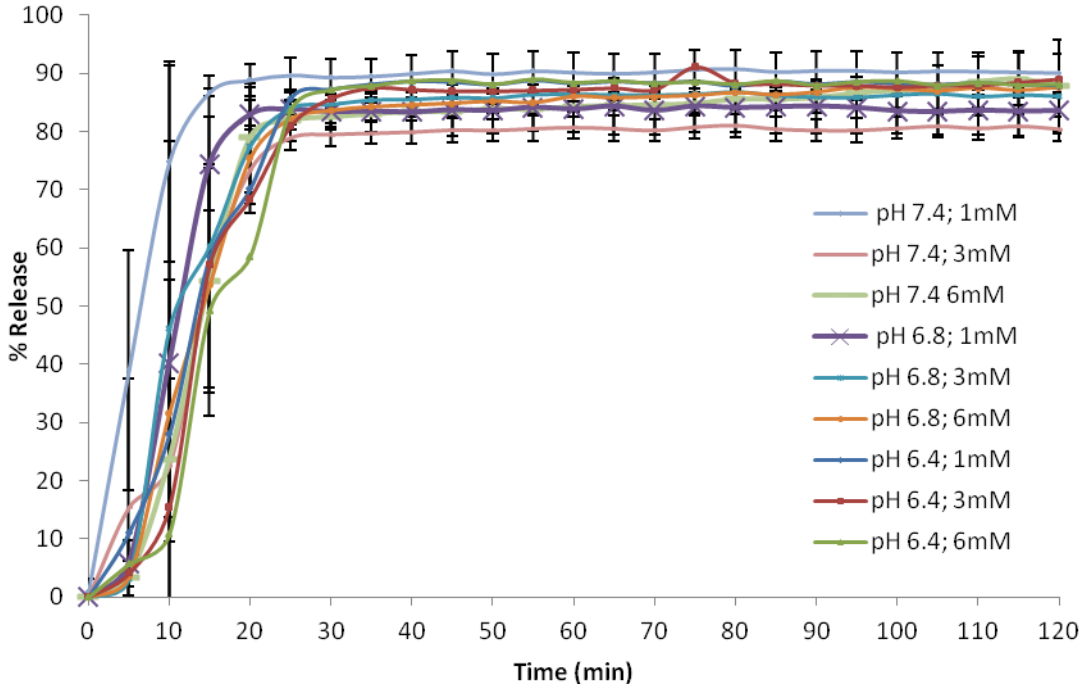


Figure 4.19: Dissolution of dipyridamole SMEDDS under different conditions of bile salt and pH in *m*Hanks buffer

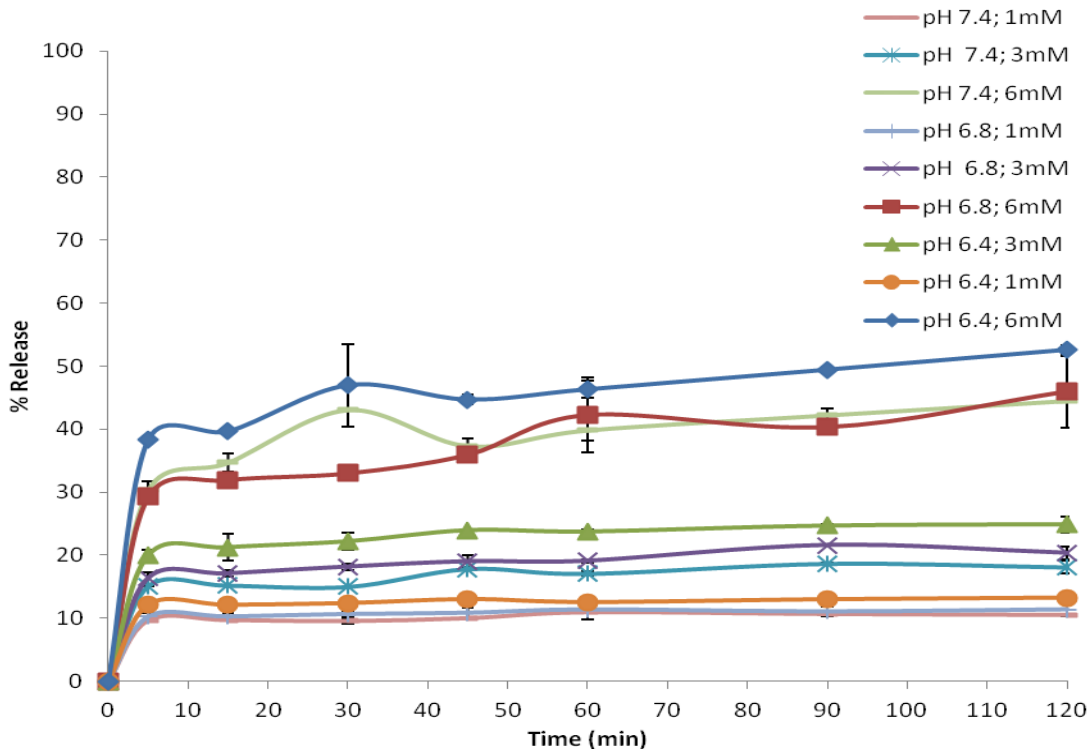


Figure 4.20: Dissolution of dipyridamole nano particles under different conditions of bile salt and pH in *m*Hanks buffer

Furosemide solid dispersion gave similar values of percentage of drug release to the API alone. The variability in dissolution rate and extent of release was not reduced in the case of the solid dispersion formulation (Figure 4.21). For SMEDDS, it can be seen that the range of the extent released was narrow relatively to the other formulations; however, the dissolution rate still appeared to be variable (Figure 4.22). The furosemide nano-formulation, gave similar results to that of the SMEEDS formulation (Figure 4.23).

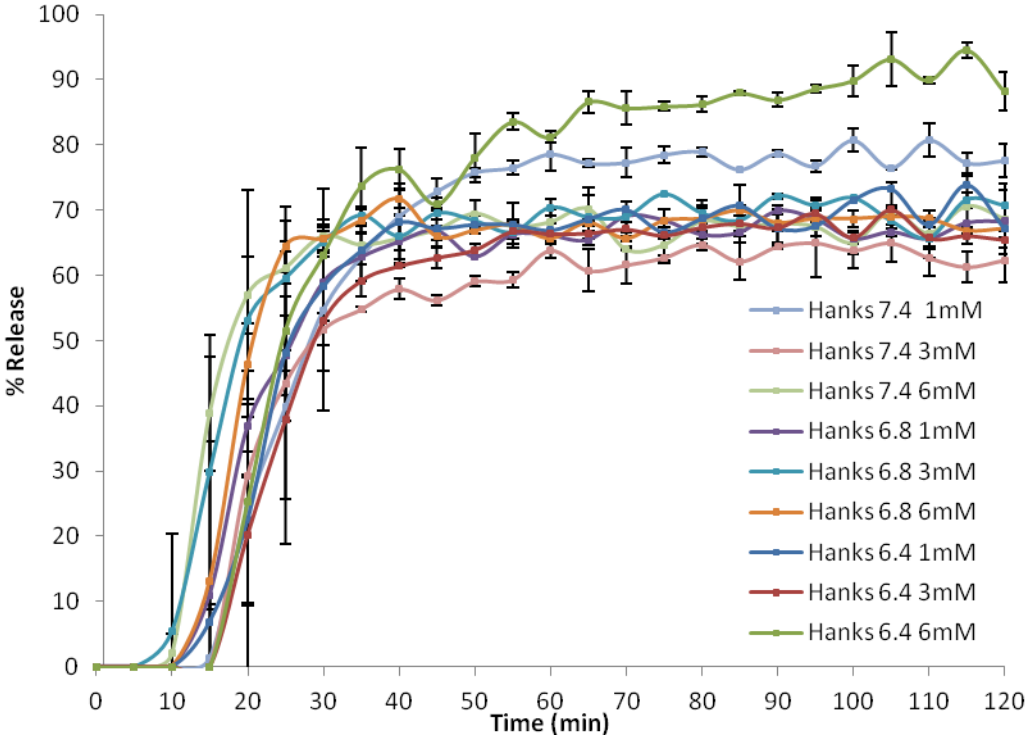


Figure 4.21: Dissolution of furosemide solid dispersion under different conditions of bile salt and pH in *m*Hanks buffer

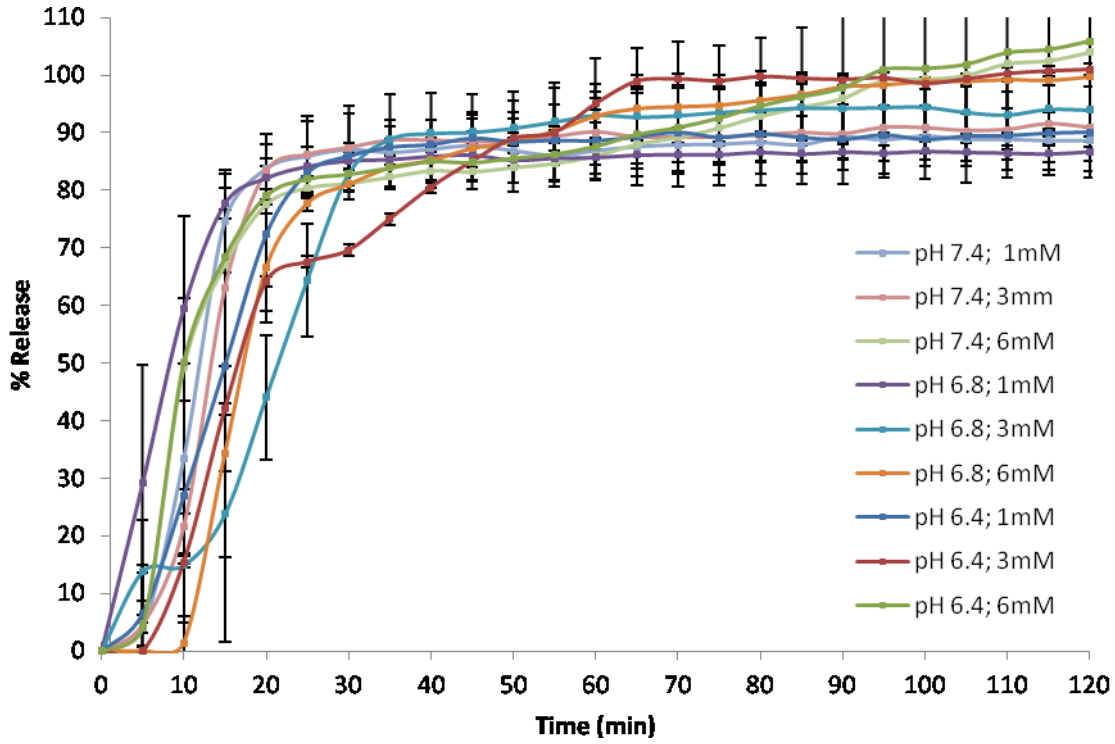


Figure 4.22: Dissolution of furosemide SMEDDS under different conditions of bile salt and pH in *m*Hanks buffer

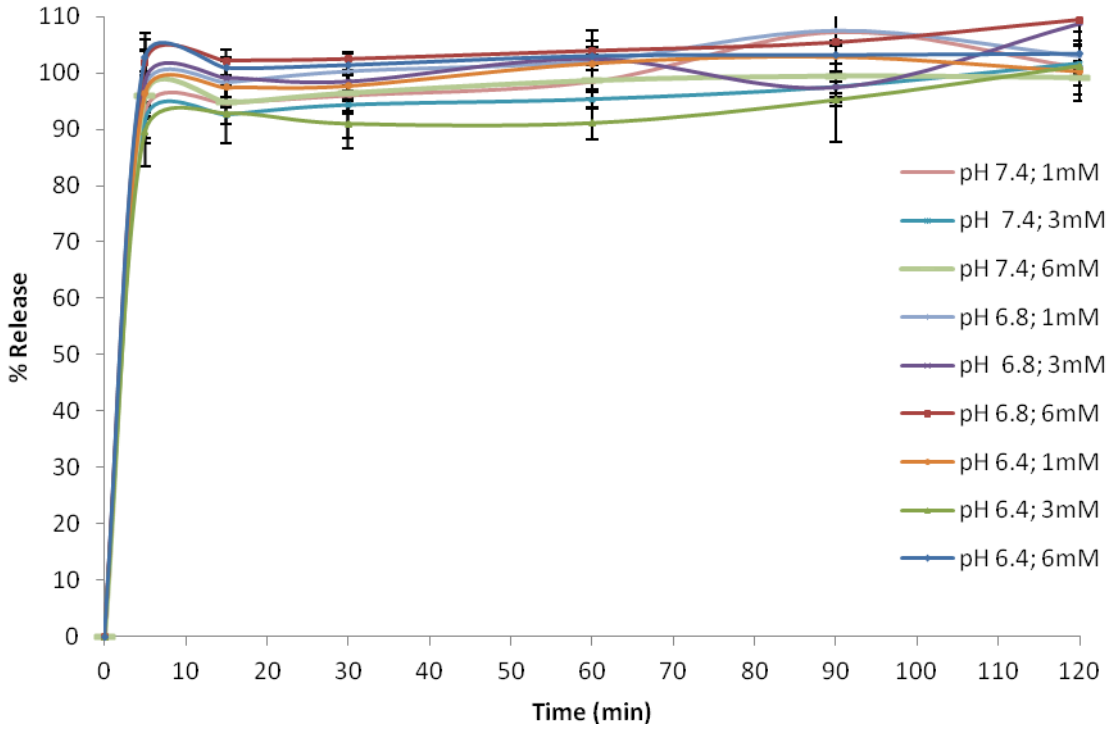


Figure 4.23: Dissolution of furosemide nano-particles under different conditions of bile salt and pH in *m*Hanks buffer

As described previously, the percentage of furosemide release from the API alone in FaSSGF under different conditions ranged from 8 to 16%. For the furosemide SMEDDS formulation in acidic conditions, no drug precipitation was observed, and the percentage release was very high (90%) at 2 hours (Figure 4.24). The nano particle formulation gave similar results to that of the API and the marketed tablet, for which a low percentage of release was observed (Figure 4.25). Interestingly, the solid dispersion increased the percentage of the drug release (80-100% under all conditions) until one hour, and precipitation of approximately 20% was observed at 2 hours (Figure 4.26). In terms of variability in the extent of release and dissolution rate, it seems that only SMEDDS reduced the variability under different conditions.

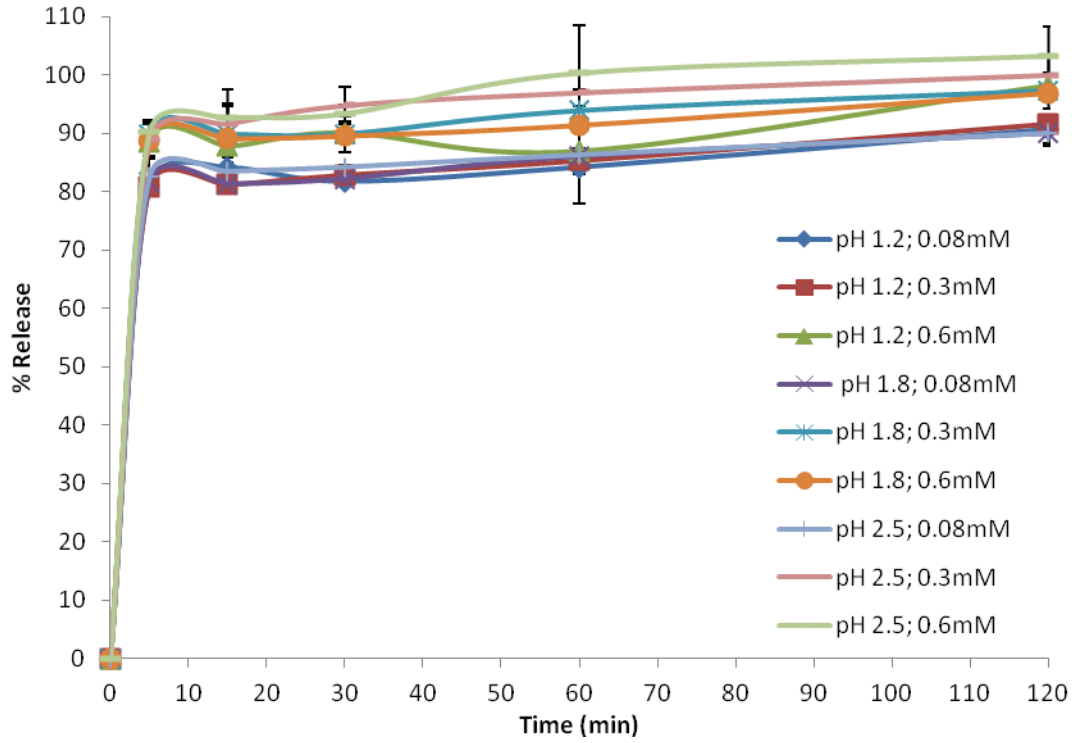


Figure 4.24: Dissolution of furosemide SMEDDS under different conditions of bile salt and pH in FaSSGF.

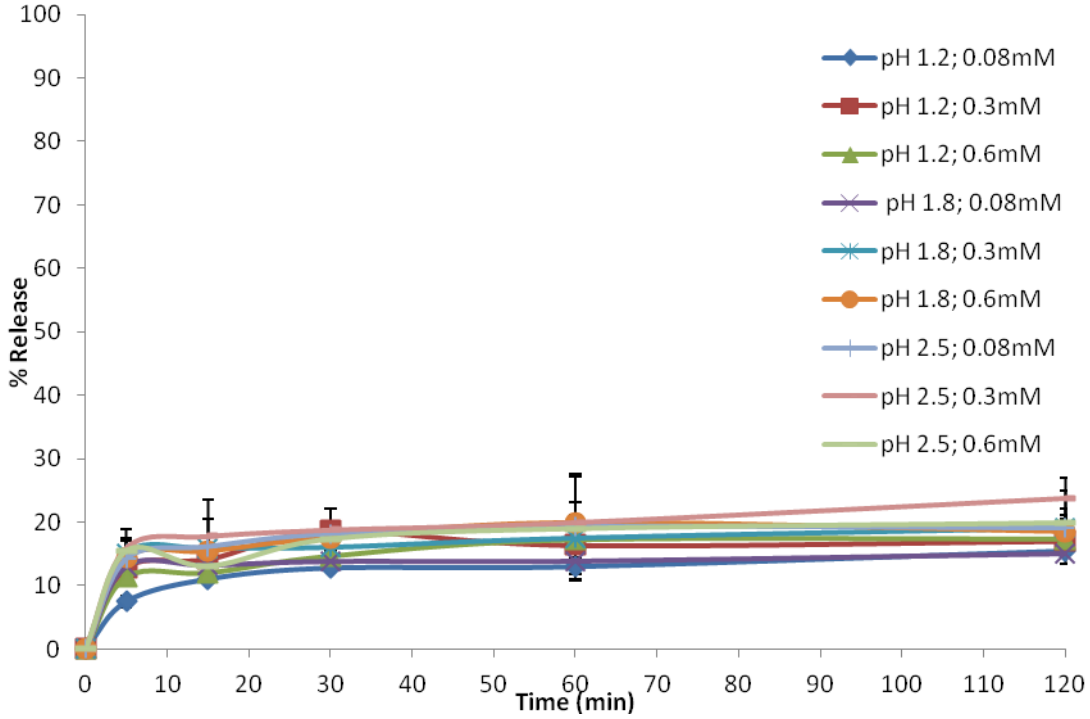


Figure 4.25: Dissolution of furosemide nano particles under different conditions of bile salt and pH in FaSSGF

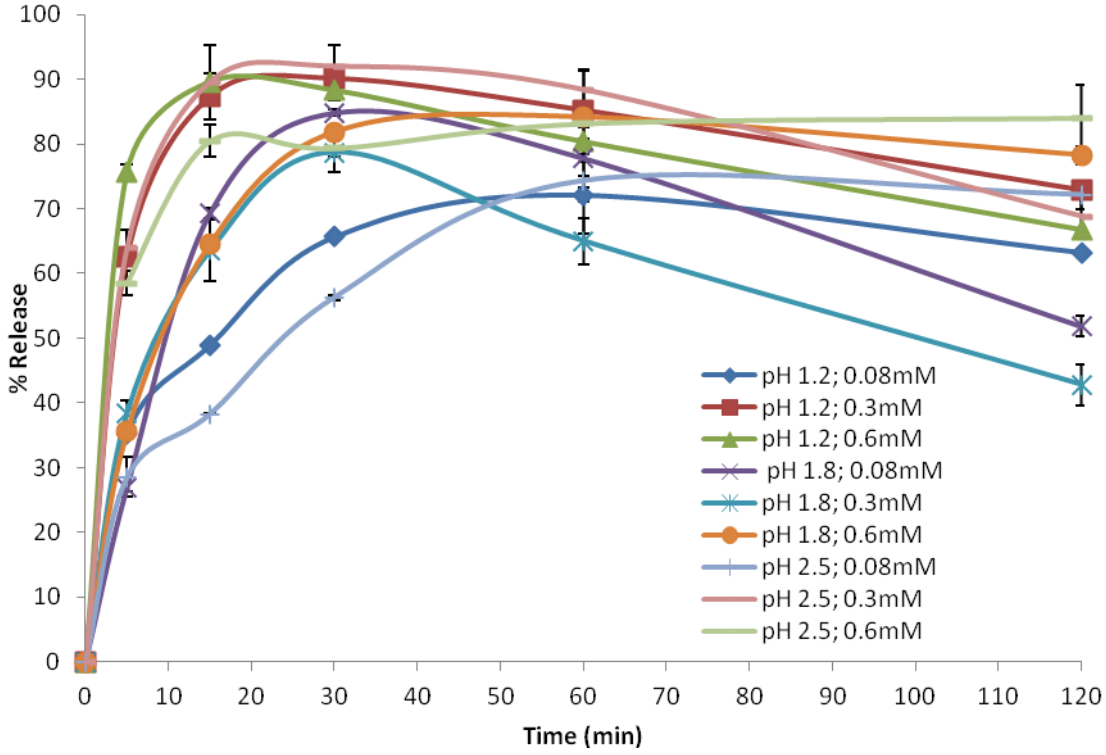


Figure 4.26: Dissolution of furosemide solid dispersion under different conditions of bile salt and pH in FaSSGF.

For weak acids and bases, it is important to investigate the change in drug release when the drug transfers from the acidic conditions of the stomach to the basic pH in the duodenum. Dissolution of all formulations was tested under changing pH conditions from 1.6 to 6.5. The dissolution profiles for dipyridamole and furosemide under these conditions is presented in and Figure 4.27 and Figure 4.28. It was found that precipitation of the dipyridamole-marketed tablet was fast, and the percentage of drug dissolved at 10 min after changing the pH to 6.5 was 10%. Precipitation was observed for all dipyridamole formulations. Solid dispersion and nano-suspension formulations precipitated at approximately the same rate, and after 20 min in the neutral phase, only 20% of the dose was dissolved. The precipitation rate decreased, and at the end of 4 hours, only 10% of the dose was dissolved. For SMEDDS, it can be seen that precipitation of 30% of the dose was observed with the addition of pre-FaSSIF; however, after 10 minutes, 50% of the dissolved drug was dissolved and no further precipitation was observed up to 4 hours.

For furosemide, the marketed tablet gave the lowest dissolution in the stomach conditions (less than 10%), followed by the nano-particle formulations (10%), solid dispersion (50%) and SMEDDS (90%). Upon increasing pH, the percentage of drug release for all formulations increased, and ranged between 70-80% without a significant difference. The marketed tablet dissolution rate was slower as compared to the other formulations, however, at around 5 min the same extent of release was observed.

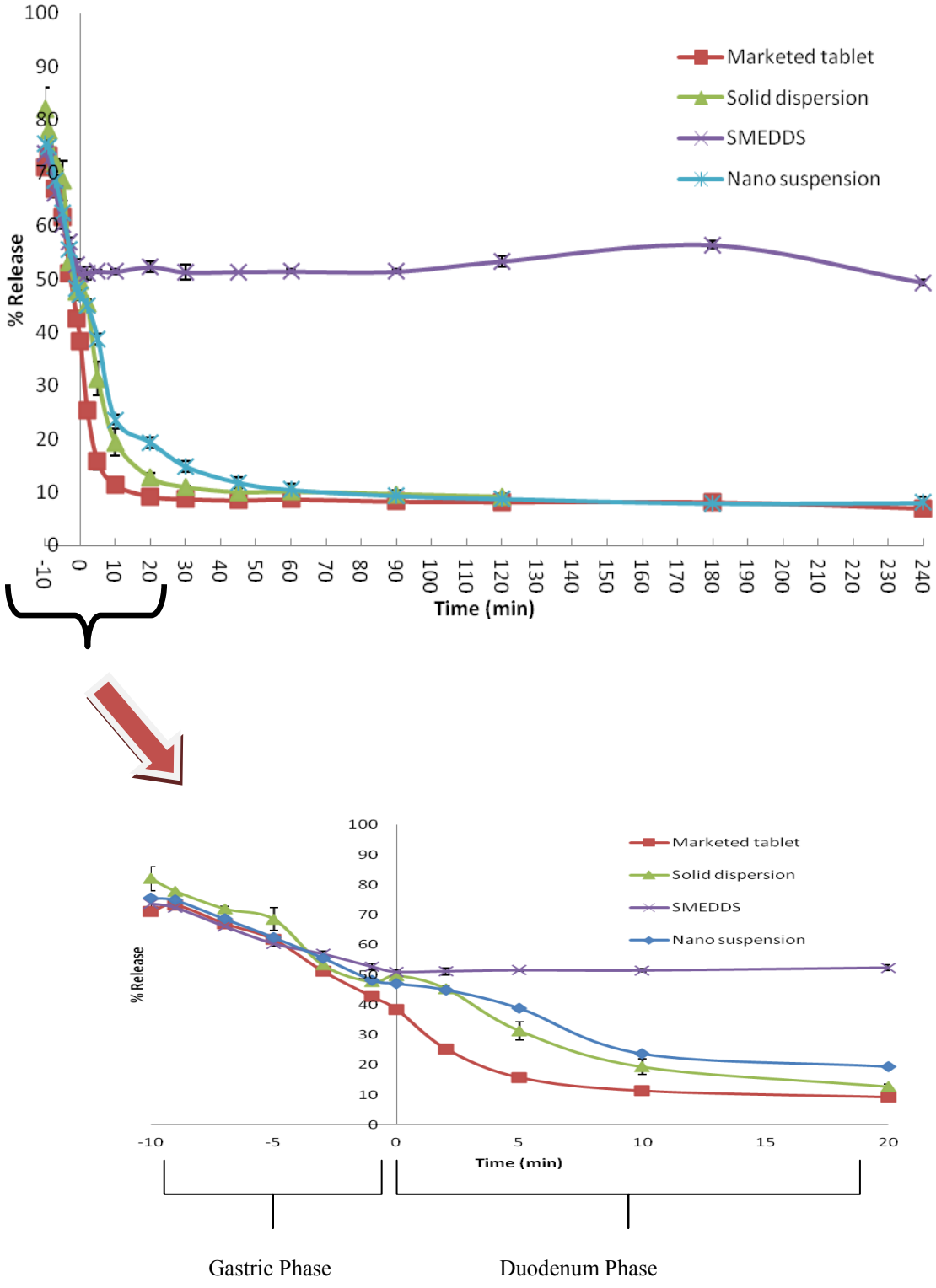


Figure 4.27 : Dissolution of dipyridamole formulations in modified FaSSGF (pH=1.6) followed by FaSSIF (pH=6.5)

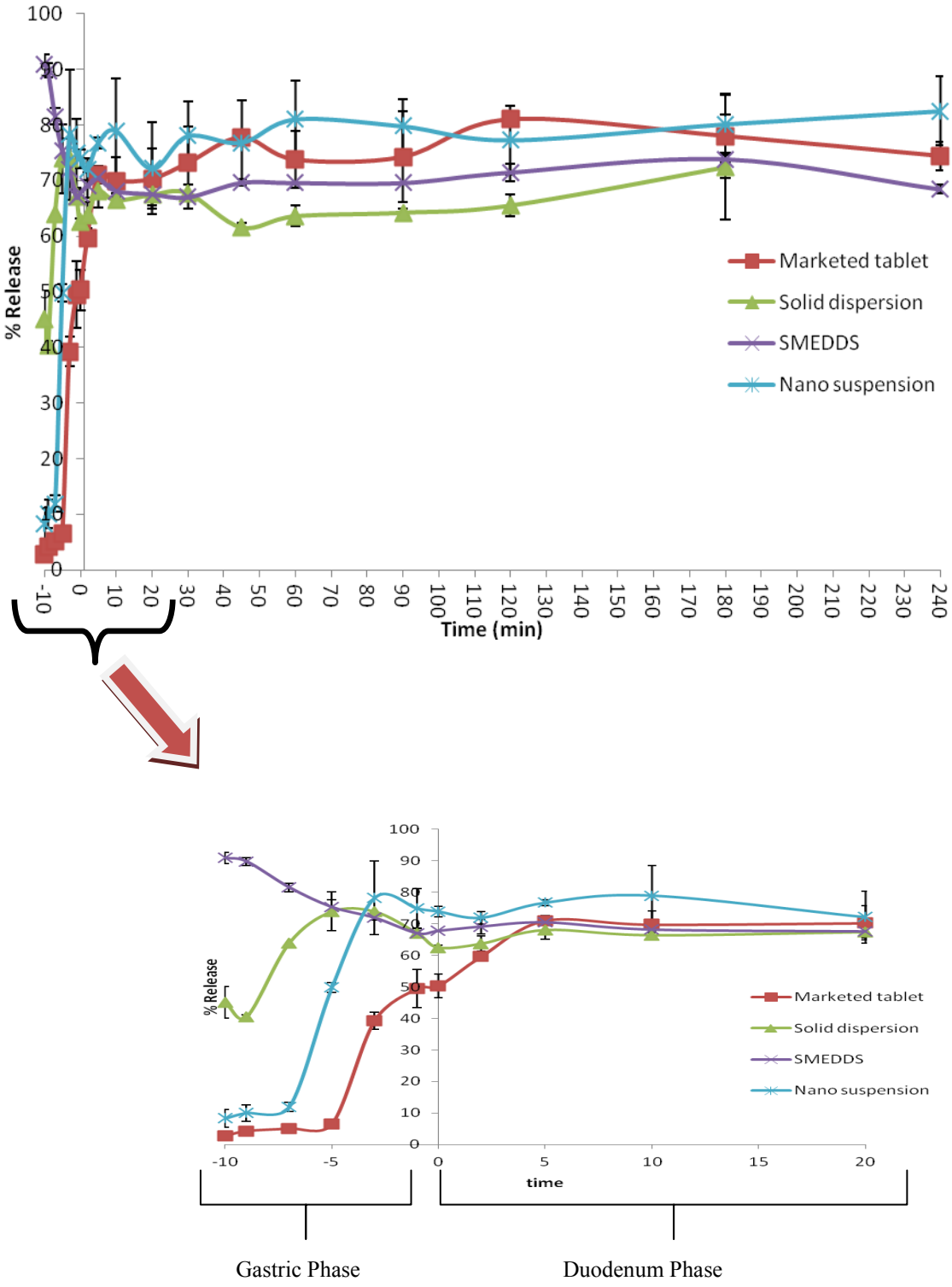


Figure 4.28: Dissolution of furosemide formulations in modified FaSSGF (pH=1.6) followed by FaSSIF (pH=6.5)

An attempt to interpret dissolution results in the context of the *in vivo* situation is not straight forward, and the interplay of many factors needs to be considered. Formulating dipyridamole as a solid dispersion in the amorphous form or as SMEDDS increases its solubility and dissolution, as can be seen from the dissolution tests. However, as dipyridamole is a weak base and precipitation on transferring from the stomach to the duodenum was observed, a formulation that is able to maintain dipyridamole in its supersaturated state for a longer period of time in the duodenum will eventually increase its chances for absorption. Based on the results presented herein, it appears that SMEDDS offers the possibility for longer absorption *in vivo*, as 50% of the drug was dissolved. However, one has to bear in mind that the 50% dissolved will also be readily available for absorption, as some of dipyridamole will be incorporated into oil micelles. Solid dispersion and nano-suspensions were precipitated at lower rates than the marketed tablet, which can offer a comparatively longer time for the dissolved drug to be absorbed.

For furosemide, dissolution of the clinical dose was high in the simulated intestinal fluids; the formulations did not increase the dissolved amount of drug in the duodenum. However, the extent of drug release from the solid dispersion and SMEDDS increased under the acidic conditions of the stomach. For drugs with a narrow absorption window, this is especially relevant and important. The shift from low pH to high pH therefore needs to be considered for these weak acid drugs. All formulations increased the dissolution rate of the drug as compared to the marketed tablet; a higher dissolution rate upon transfer of the formulation from the stomach to the duodenum will offer more of dissolved drug available to be absorbed earlier. Based on this study, SMEDDS will offer the highest amount of dissolved drug upon emptying from the stomach, followed by the solid dispersion and by nano particle formulation.

In terms of furosemide variability, in the dissolution test no effect of bile salt or different pH (either in the gastric or intestinal simulated fluids) was observed. It can be assumed that a different concentration of bile salts and different pH conditions will not affect the dissolution of furosemide *in vivo* in the upper GI at this clinical dose. However, the variable dissolution rate might have significant effects in the case of drugs with narrow absorption windows. The formulations tested did not show an improvement in the dissolution rate variation, which can be attributed to the wetting properties of the formulation powder. Moreover, in the previous chapter, it was reported that the excipients in the marketed tablet or compressing the powder

to tablet reduced the dissolution rate variation. It can be assumed that further development of the tested formulations by adding simple excipients or compressing the powder to a tablet form will give similar results.

4.2.5.5 Comparison of Formulation Development and Preparation

Each method presented herein might encompass challenges in development, and will not be suitable for some drugs depending on their physicochemical properties. For instance, preparing nano-crystal particles using the wet ball method will not be suitable for drugs with low melting points, given that during the milling process, the generation of friction heat results in amorphization, and lead to instability of the formulation. With regards to SMEDDS, administering a liquid formulation would not be an appropriate choice for those drugs with low solubility in lipid excipients, and with low stability in the liquid state. Due to inherent stability problems associated with the amorphous form, the solid dispersion approach might also be unsuitable for those drugs with low chemical and physical stability (Kawabata *et al.*, 2011). Therefore, it is clear that the physicochemical properties as well as the clinical dose could affect the strategic selection of a formulation to improve drug solubility and performance *in vivo*.

Based on the experience in this study, and considering the development stages of the different formulations, the solid dispersion preparation was fairly straightforward in terms of planning and choosing the excipients. There is a great deal of knowledge and published literature for planning, executing and assessing solid dispersion formulations. The SMEDDS development involves many stages. Moreover, the fact that the repetition of the published formulations was not successful in this study indicates that small changes in the preparation due to human practice and differences in excipient batches might affect the end product and its performance. Unlike the literature for solid dispersion, the published literature for SMEDDS is limited, although an increasing interest in the pharmaceutical community is becoming more apparent. Moreover, *IVIVCs* for SMEDDS are scarce, mainly due to a lack of understanding of the absorption mechanism. Common dissolution tests cannot predict the free fraction available to be absorbed, and as such, the use of dialysis bags is required during dissolution tests. However, the transfer of drugs from dialysis bag can take a long time, and is not always reflective of the *in vivo* dissolution process. The published setups which assess the *in vitro*

lypolysis are also fairly complicated and not available and further *IVIVC* validation for these methods is required. All of these factors consequently make the SMEDDS formulation a less attractive strategy. Nano-milling by contrast has gained a great deal of interest in recent years: Nano particle development also includes many stages, however, and excipients choice is not always simple. On the other hand, assessing the formulation performance can be done by the same tools as used for solid dosage forms, and interpretation is not complicated. Assuming that all three methods increase solubility to the same extent, a solid dispersion formulation will be the preferred strategy based on the method limitations described herein.

A comparison of all three approaches increasing dipyridamole solubility/dissolution rates *in vitro* indicates that the highest solubility for the longest time and after increasing pH was attained with SMEDDS, in addition to reduced variability due to bile salt and pH. However, as mentioned previously, it is difficult to extrapolate these results to the *in vivo* absorption results, given that the free fraction to be absorbed out of the 50% dissolved drug is not known. Similarly, solid dispersion appears to increase the solubility and decrease the effects of intra-luminal differences on solubility. When precipitation was assessed for the solid dispersion, it produced similar percentages of dissolved drug at the end of 4 hours to those of the nano-particle and the marketed tablet formulation. However, the precipitation rate was slower in the case of solid dispersion than the marketed tablet, which might give a longer window for the dissolved drug to be absorbed *in vivo*. A reduction in particle size did not yield an increase in the solubility of the drug.

In the case of furosemide, dissolution in the intestine is not, however, a limiting step for the tested clinical dose. Considering the limited dissolution in the stomach, SMEDDS was shown to increase the extent of drug release in the stomach, followed by the solid dispersion. In contrast, nano-particles did not yield an increase in drug release. Again, the implication of increasing drug release in the case of SMEDDS is not clear due to the limitation of the dissolution test for SMEDDS formulations. The high extent of release from the solid dispersion might offer a better chance for furosemide to be absorbed in the upper parts of the intestine. Similar effect but to a lesser extent might be observed in the case of the nano-particle formulation thanks to the higher dissolution rate of the nano-particles compared to the marketed tablet during the pH change. It is important to note that most of the efforts in improving drug physicochemical properties are focused on improving drug solubility and less focused on improving drug permeability (BCS III/IV). Some investigations have shown that

the addition of permeation enhancers such as fatty acid, bile salts, surfactants, and polysaccharides play a role in enhancing the permeability of drugs via the paracellular pathway; however, some of them are known to have membrane-damaging effects, and therefore the formulation approaches in the case of BCS III is limited to an immediate-release tablet (Kawabata *et al*., 2011).

4.2.6 Summary

Different formulation approaches to increase solubility and dissolution of poorly soluble drugs are available in the scientific community and pharmaceutical industry. Herein, three different formulation approaches were compared in increasing solubility/dissolution. Solid dispersion appeared as the most convenient method for preparation and increased the extent of release of dipyridamole and minimised the effect of bile salt and pH on the drug dissolution. SMEDDS formulation also increased the extent of release, however due to the limitation in the *in vitro* dissolution tests to estimate the percentage of drug available to be absorbed, it is difficult to draw a conclusive conclusion compared to the other formulations. Reduction in the drug particle size did not yield an increase in the extent of dissolution under different bile salt and pH conditions. Different *in vitro* tools can be utilised to investigate formulation performance *in vivo.* Due to ethical consideration, it is difficult to conduct *in vivo* studies in humans to assess the formulation absorption, therefore in the next section the formulation pharmacokinetics will be assessed in animal model.

4.3 *In Vivo* Evaluation of Formulations in a Rat Model

4.3.1 Introduction

4.3.1.1 *In Vivo* Studies to Evaluate Formulation Performance

There are several biopharmaceutical tools used in pharmaceutical development to characterise formulation performance, including *in vitro* tests, animal studies and clinical trials in healthy human subjects (and sometimes in patients). There is no doubt that the information gained from *in vivo* human clinical trials is much more valuable and reflects the real situation more closely. The key limitations of human studies are providing ethical justification for the trial in addition to limitations in throughput and cost. *In vitro* tests can circumvent these limitations but fail to adequately mirror the complexity of the gastrointestinal environment *in vivo*. Conditions such as volume and composition and static environment in the compendial dissolution tests do not take permeability, metabolism or the dynamic nature of the gut into consideration, and can contribute to lack of *IVIVC*. Moreover, to validate *in vitro* models, *in vivo* data in humans is also needed. Therefore, a proof-of-concept study in a suitable laboratory animal is important in order to gain more information of formulation performance in a living body before it is subject to any human trials.

The choice of the right animal model is not always straightforward. A recent review article summarizes the current knowledge on anatomy and physiology of the human gastrointestinal tract compared with that of common laboratory animals (dog, pig, rat and mouse) with emphasis on *in vivo* methods for the testing and prediction of oral dosage form performance. Needless to say, there is no definite conclusion as for the best model to predict human *in vivo* data, and each decision needs to be taken based on drug properties, the physiological differences between the animal model and human, and its possible effect on the formulation performance *in vivo* (Sjögren *et al*., 2014).

4.3.1.2 Rat GI Physiology Compared to Humans

The rat is the most investigated animal model to yield *IVIVC*. However, there are some remarkable differences between the GI physiology of the rat and humans, namely that rodents feature a well-defined caecum, whereas in humans the caecum is very small and is continuous with the colon. Moreover, gastric volume adjusted to body weight of rats is larger than in man, which might feature implications for drug solubility when dosing rats based on body weight (Davies and Morris, 1993).

In recent research by our group, the gastrointestinal environment in rats and how it affects drug solubility was investigated, knowing that differences in the GI milieu such as pH, buffer capacity, osmolality and surface tension may lead to differences in drug solubility. In rats, the highest buffer capacity was measured in gastric fluid, which decreased down the small intestine and increased again in the caecum and colon. The buffer capacity from human jejunal and ileal fluids (Fadda *et al*., 2010b) were appreciably lower than those in rats. The inter-species differences in buffer capacity are important aspects for consideration, especially for the administration of pH-responsive formulations and ionisable drugs.

The osmolality of the gastrointestinal contents of rats in turn was low in the stomach, increasing in the proximal small intestine and decreasing in the distal gut. This pattern of osmolality in general is in agreement with the physiology of the rat digestive system, whereby most of its food is digested and broken down into component molecules such as glucose, amino acids and fatty acids in the small intestine, hence producing a higher fluid osmolality. As nutrients are absorbed further down the gut, osmolality of the contents decrease.

Surface tension of rat gastrointestinal fluids was significantly lower than that of water throughout the gut. Surface tension in rats was found to be higher in the stomach and lower in the small intestine, increasing again in the distal gut. Surface tension of the human gastric aspirates was reported to be ~30mN/m in the fed state, which was relatively constant over a period of time and was lower than the surface tension of the rat (Kalantzi *et al., 2006)*. A similar observation was noted with human duodenal aspirates, where surface tension was lower (~28mN/m) than in rats. Surface tension of the supernatants from human ascending

colon fluids was also lower (39.2mN/m) (Diakidou *et al*., 2009) as compared to the surface tension of the proximal colon fluids from the rat.

McConnell *et al*. (2008b) reported that the gastric pH in rats was found to be higher than in humans, and especially in the fasted state (4 compared to 1.6). In addition, the intestinal pH of rats did not reach the pH values reported in man in the distal small intestine, caecum and colon. The low intestinal pH in rats has implications for the *in-vivo* testing of oral pharmaceuticals in rats. For example, drugs which require a basic pH to dissolve may precipitate at lower pH values seen in the rat. Moreover, lack of correlation and ability to extrapolate *in vivo* results from rats to humans may occur where pH-responsive drug carriers are being investigated.

Similarly to human, taurocholate is the major bile acid at a total concentration of 8–25mM in rats, and bile salt secretion is also induced endogenously by food intake. As for species differences, the total bile acid and phospholipid concentration in rat GI tracts were found to be much higher than those of humans. This might have significant implications for rat *in vivo* results for lipophilic drugs, where an improvement in oral absorption might not be observed due to the high concentration of bile salts (Tanaka *et al*., 2012).

In situ perfusion of intestinal segments of rats is frequently used to study the permeability and absorption kinetics of drugs. In situ study provides the advantage of isolating comparisons to the level of the intestine, focusing on the epithelial permeability in small and large intestines. P_{eff} from rat jejunal studies were found to correlate strongly with the corresponding human jejunal P_{eff} (Cao *et al*., 2006; Fagerholm *et al*., 1996). Moreover, good correlation was found between the expression levels of transporters and metabolic enzymes in rat and human jejunum. However, a moderate correlation for the transporter expression levels in duodenum and no correlation in metabolizing enzyme levels were found (Cao *et al*., 2006). This can explain the lack of correlation in plasma clearance of 54 extensively metabolized drugs between humans and rats (Chiou and Barve, 1998). Characterisation of the GI tract of rats is summarised in Table 4.5 (adapted from Sjögren *et al*. (2014)).

Table 4.5: Charctersation of the GI tract of rats adapted from (Sjögren *et al*., 2014)

Parameters	Location	Rat
pH fasted	Stomach	4–5 (glandular region); 7 (anterior region) (Davies and Morris, 1993; Kararli, 1995)
	SI	4.5–7.5 (Davis and Wilding, 2001; Lennernäs and Regårdh, 1993)
	LI	N\A
pH fed	Stomach	3.8–5.0 (Davies and Morris, 1993)
	SI	6.5–7.1 (Davies and Morris, 1993)
	LI	6.6–6.9 (Davies and Morris, 1993)
Transit time fasted	Stomach	15–30 min (T1/2) (Langguth *et al*. 1994) 5–65 min (t1/2) (Maerz *et al*. 1994)
	SI	3–4 h (Davis and Wilding, 2001; Lennernäs and Regårdh, 1993)
	LI	10–11 h based on a total GI transit time of 15 h (DeSesso and Jacobson, 2001)
Transit time fed	Stomach	N\A
	SI	N\A
	LI	N\A
Length SI		102–148 cm (Kararli, 1995)
Length LI		26–26 cm (Kararli, 1995)
Bile concentration	SI	33.5–61.3 mM (fasted) (Staggers *et al*. 1982) 17–18 mM (fasted) (Kararli, 1995) Compared to man higher BS/PL ratio but PL concentration similar to man
Metabolic activities		CYP related activities (Takemoto *et al*. 2003) In general not correlated to humans
Major drug transporters		Similar transporter expression patterns as in humans (Cao *et al*. 2006)
Permeability		Less than in humans, good correlation
Water volumes	Stomach	2.4 mL (Takashima *et al*. 2013)
	SI	3.0–4.6 mL (Takashima *et al*. 2013)
	LI	N\A

In this section, the *in vivo* and the *in vitro* performance of the different furosemide and dipyridamole formulations were investigated in the rat model and the D/P system. In addition, an attempt to assess the inter-subject variability in bioavailability was carried out for the different formulations in rat model.

4.3.2 Objectives

- To evaluate *in vitro* performance in terms of dissolution and permeability of three formulation approaches (solid dispersion, SMEDDS and nano-particles) using the D/P system.
- To compare three different formulation approaches in increasing bioavailability *in vivo* in the rat model.
- To estimate the effectiveness of the formulations in decreasing *in vivo* bioavailability variability in rats.
- To establish the *in vitro in vivo* correlation.

4.3.3 Materials

Dipyridamole (D9766) and furosemide (F4381) were obtained from Sigma Aldrich Chemicals (Poole, UK). Solvents used in HPLC and LCMS were: HPLC\LCMS water, acetonitrile and phosphoric acid. All were of HPLC grade or LCMS grade and purchased from Fisher Scientific (Loughborough, UK).

4.3.4 Methods

4.3.4.1 D/P System Set Up

A more detailed method is described in Chapter 3. Briefly, the drug or the formulations were added to the apical side of the D/P system, and 200µL aliquots of samples were withdrawn from the apical and the baso-lateral sides to measure the amount dissolved and permeated with time at 5, 15, 30, 60, 90, and 120 minutes. The dipyridamole dose was equivalent to 1mg, and furosemide absorption was tested at dosages of 0.4mg and 1.4mg.

4.3.4.2 Animal Experiments

All animal experiments were approved by the UCL School of Pharmacy Ethical Review Committee, and were conducted in accordance with Home office standards under the Animals (Scientific Procedures) Act, 1986. Male Wistar rats (average weight app. 200-250g) were supplied by Harlan (Oxfordshire, UK). Studies were performed in 6 rats for each formulation. The animals were restrained in the laboratory for one week before the commencement of any experiment to allow the animal to adjust to a new environment and to avoid any dramatic change in feeding behaviour. Twelve hours prior to dosing, the rats were fasted but were allowed free access to water.

Rats were given an oral gavage of the sole API and solid dispersion (The Size 9 Dosing Kit for rats - Dosing Syringe) in capsules with an additional 0.4mL of water immediately after capsule administration. SMEDDS and nano-suspension and the preparation of nano-formulation with un-milled API were administered as a 0.4mL suspension/solution using the oral gavage. For furosemide, drug suspensions and solutions were administered as 0.4mL. All administered doses were equivalent to 10mg/kg for both drugs. To calculate the bioavailability of these formulations, an IV bolus injection was administered to 6 rats to the

tail in a dose of 16mg/kg. Blood samples (200µL) were collected from the rat tail at time points of 20 ,40 ,60 ,90 ,120 ,150, 180, 240, 360 and 400 minutes, and transferred into EDTA BD microtainer capillary blood tubes (New Jersey,U.S.) before being immediately vortexed for 20 sec. Blood tubes were centrifuged for 10 min at 13,000 rpm using Centrifuge 5415D (Eppendorf AG, 22331 Hamburg, Germany). Plasma was placed into labelled Eppendrof tubes and stored at -20˚C for HPLC/LCMS assay.

4.3.4.3 Plasma Samples

Furosemide and dipyridamole extraction from rat plasma samples were carried out following a method developed based on previous work (Bauza *et al*., 1985; Qin *et al*., 2010). For both furosemide and dipyridamole samples, 300µL of acetonitrile were added to 100µL of plasma samples. The samples were vortexed for at least 1 minute, and centrifuged for an additional 10 min at 13,000 rpm at 4˚C. 300µL of the organic phase was taken and transferred into a 2mL Eppendorf tube. The tubes were then transferred to a vacuum centrifuge (Speed Vec) to evaporate the organic solvent for 300 min at room temperature, and reconstituted with 100 µl of the mobile phase in the case of furosemide, and 300µL mobile phase in the case of dipyridamole.

4.3.4.4 Furosemide HPLC analysis

The equipment consisted of an integrated HP 1200 Series HPLC system comprising an HP1200 autosampler, a HP 1200 pump and a HP 1200 multiple wavelength detector system, a UV Vis spectrophotometric detector and fluorescence detector (Agilent Technologies, West Lothian, Scotland). The detector was interfaced with a pv with PC/Chrom + software (H&A Scientific Inc, Greenville,NC, USA). Furosemide was assayed using a 150x4.6 mm particle size 3µm reserved- phase C18 column Hypersil Gold (Fisher scientific) at 40˚C. The mobile phase used for analysis consisted of acetonitrile (A) and 0.05M phosphate buffer adjusted pH to 2.5 (B). The following gradient was applied: 80:20 B:A (V/V%) gradually changed to 60:40 (B:A) for 15 minutes, followed by a change to initial conditions 80:20 (B:A) for 5 minutes, and thereafter conditioning the column to initial conditions for 4 minutes, constituting a total run time of 24 min. The flow rate was 1mL/min and the injection volume was 80µl. The detection wavelength for vis-UV was 238nm, and for fluorescence detection,

excitation and emission were set to 233 and 389nm, respectively. The drug retention time was 12.5 min.

The standard curves were linear in the range of 0.1 to 10µg/mL (R^2=0.998). The HPLC method was tested for accuracy and precision at low, medium and high concentrations. The extraction rate ranged between 80-105%, with a limit of detection as 0.05µg/mL and the limit of quantification being 0.1µg/mL. Chromatograms of blank plasma sample and furosemide-spiked plasma sample from a Wistar rat are shown in Figure 4.29.

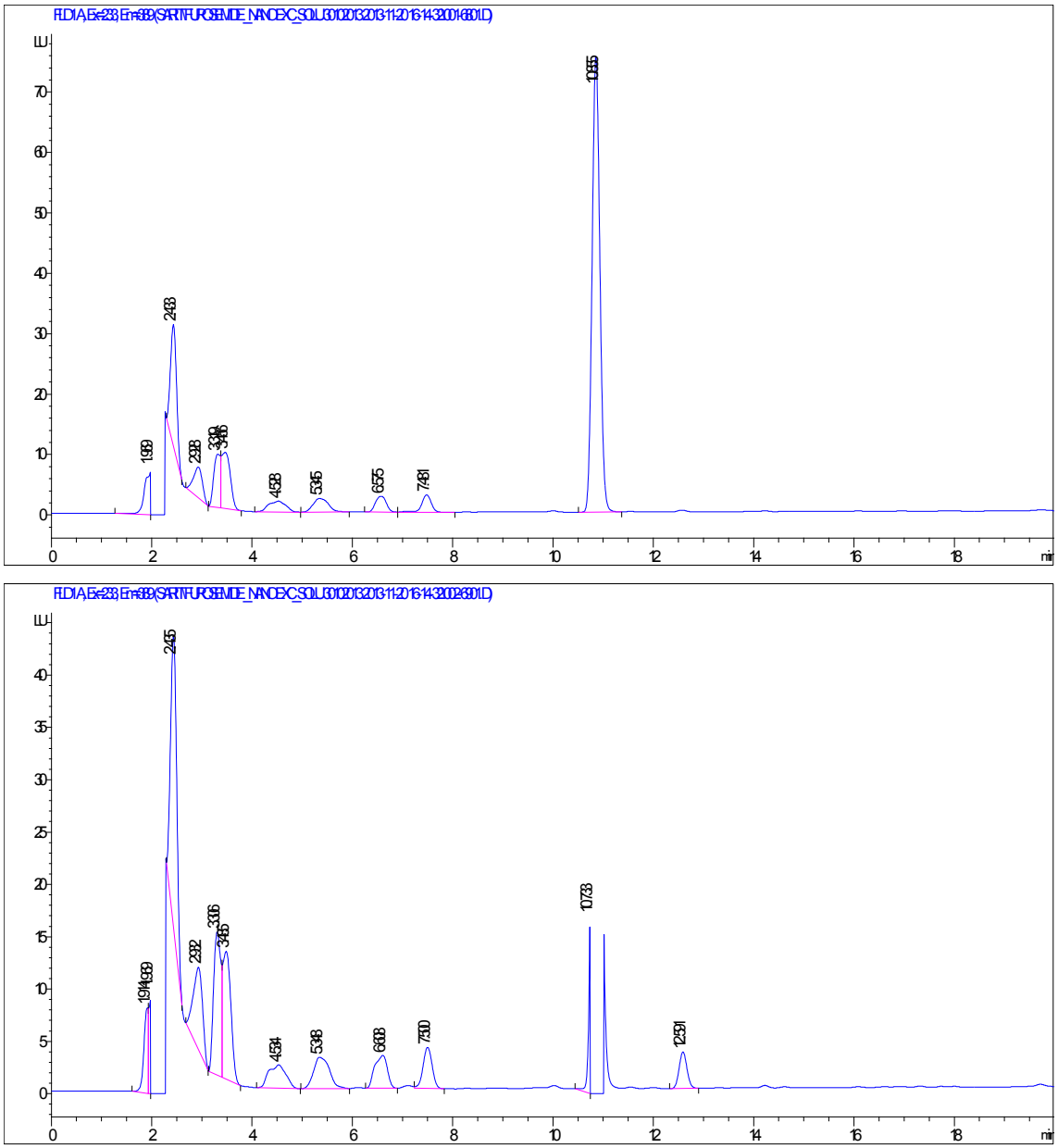


Figure 4.29: Chromatogarms of A) Blank Wistar rats and b) Furosemide spiked (0.1µg/ml) Wistar rat's plasma

4.3.4.5 Dipyridamole LCMS analysis

Dipyridamole LCMS was developed at King's College University, MS unit. The method was used in equipment consisting of a HPLC system comprising an HP1260 autosampler, a HP 1260 pump and 6400 Series Triple Quadrupole LC/MS (Agilent Technologies, West Lothian, Scotland). Separation of dipyridamole was achieved with a 150x4.6 mm particle size 3µm reserved- phase C18 column Hypersil Gold (Fisher scientific) at 40˚C. The mobile phase used for analysis consisted of 30:70% (V/V), acetonitrile 0.1 formic acid and 0.1% formic acid in water. The flow rate was 0.4 ml/min and the injection volume was 5µL. Drug retention time was 8 min. Protonated precursor and production ions (*m/z*) for detection were 505.328, 429.328 and 385.5, respectively. Ionization conditions for analysis of dipyridamole were as follows: electrospray ionization, positive mode; source temperature 250°C; cone voltage 4000V; and collision energy 52 and 36 for 429.328 and 385.5, respectively;

The standard curves were linear in the range of 10 to 1000ng/mL (R^2=0.99). The LCMS method was tested for accuracy and precision at low, medium and high concentrations. The extraction rate ranged between 90-120%. The limits of detection and quantification were 5ng/mL and 10ng/mL respectively. Chromatograms of blank plasma sample and dipyridamole-spiked plasma sample from a Wistar rat are shown in Figure 4.30.

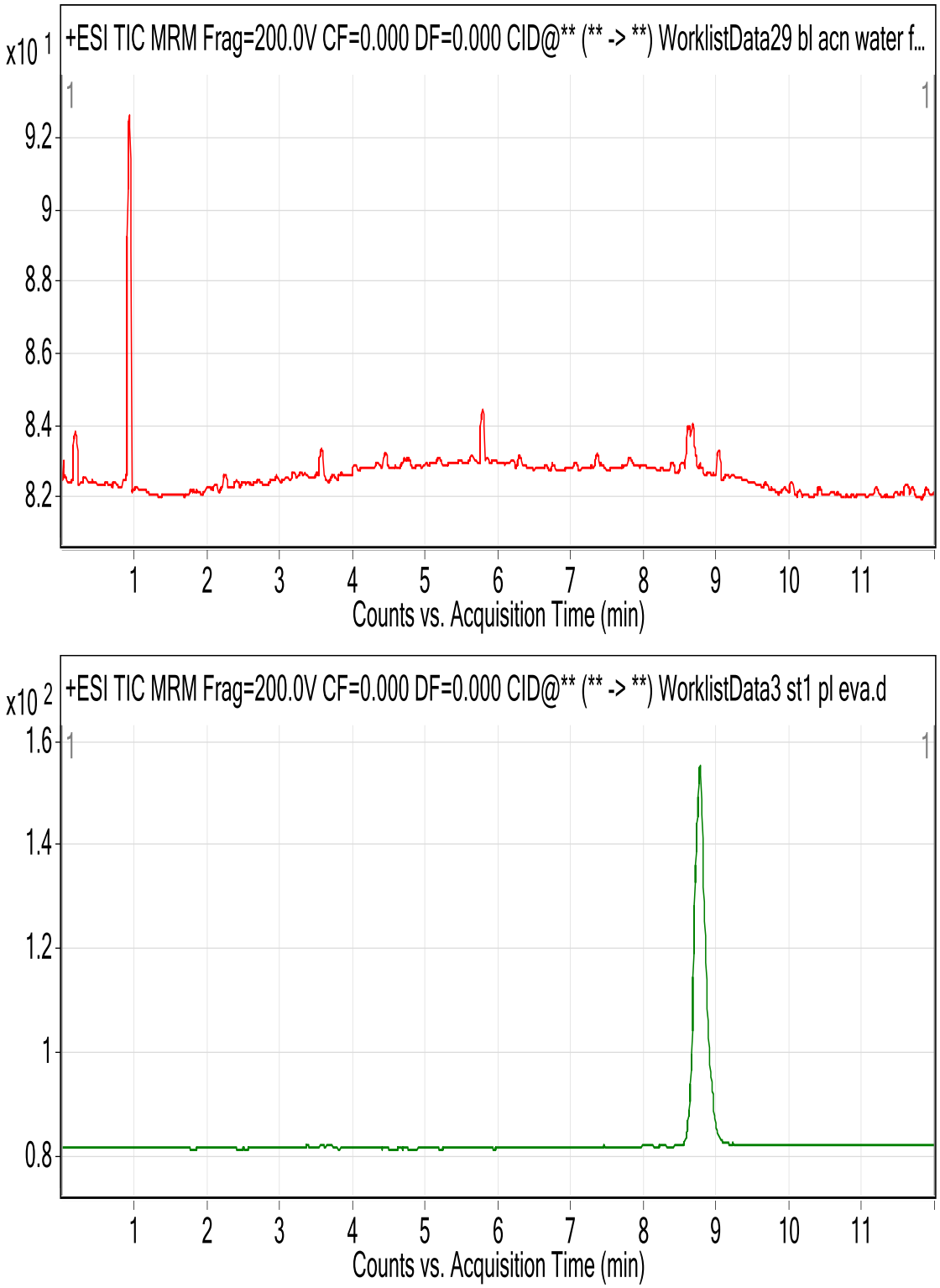


Figure 4.30: Chromatogarms of a) Blank Wistar rats and B) Dipyridamole spiked 10ng/mLWistar rat's plasma

4.3.4.6 Statistical Analysis

All results are presented as mean ± SEM. AUC_{0-8} calculations were done based on the Trapezoidal Rule. Bioavailability was calculated according to Equation 4.3. The variation coefficient (%CV) was calculated in the same way as indicated in Section 2.3.3.4

$$F = \frac{AUC\ oral}{AUC\ iv} * \frac{Dose\ iv}{Dose\ oral}$$

Equation 4.3: Bioavailability calculation

The plasma concentration vs. time data and the permeation and dissolution profiles of the different formulations were analysed by one way ANOVA repeated measurements, using a linear model followed by a tukey post- hoc analysis (SPSS Inc., Illinois, USA). AUC_{0-8} and bioavailability values were analysed by one way ANOVA with post hoc tests using PASW statistics 22 (SPSS Inc., Illinois, USA).

4.3.5 Results & Discussion

4.3.5.1 Furosemide Formulations Assessment *In Vitro* Using the D/P System

Evaluation of furosemide formulations *in vitro* using the D/P system revealed that at the apical side, all formulations gave a similar extent of drug release of around 80% after 2 hours. This is in agreement with the formulation dissolution results under different conditions using *m*Hanks buffer described previously herein. Although a high percentage of dissolved drug was attained on the apical side, a very low permeated amount was achieved at 2 hours for all formulations (less than 0.1%). All formulations showed similar permeation profiles, while furosemide SMEDDS resulted in higher permeation (Figure 4.31). This emphasises the fact that the rate-limiting step in furosemide absorption is permeation through the gut membrane, and that the classification of furosemide in this clinical dose (40mg) as BCS IV needs to be reconsidered. Calculations of the predicted f_a in humans are presented in
Figure **4.32**. The SMEDDS furosemide formulation showed an increase in the fraction absorbed relative to the other formulations ($p<0.5$). As discussed, these formulation approaches are intended to increase the solubility/dissolution of poorly soluble drugs and as such, no effect on the drug permeability is expected. In the case of furosemide, no effect on solubility/dissolution of the different formulations in this clinical dose was observed *in vitro*, as the extent of release was already high. The increase in the permeation and hence absorption of the drug in the oil formulation might be attributed to a direct partitioning of the drug from the micelle to the membrane. A number of absorption mechanisms were summarised by Yano *et al*. (2010); Firstly, that the absorption of micelle drugs involves collisional transfer to the glycocalyx of the gut enterocytes. Secondly, the micelles can assist in transport of solubilised (incorporated) solutes across the aqueous diffusion layer to the surface of the cell membrane, which reduces the effect of the unstirred water layer on absorption. In addition, it was suggested that some of the surfactants in SMEDDS formulation might have the ability to inhibit transporters like P-gp. It was reported that furosemide might be sensitive to efflux transport and therefore the SMEDDS formulation was successful in increasing the permeation by inhibiting P-gp transporters.

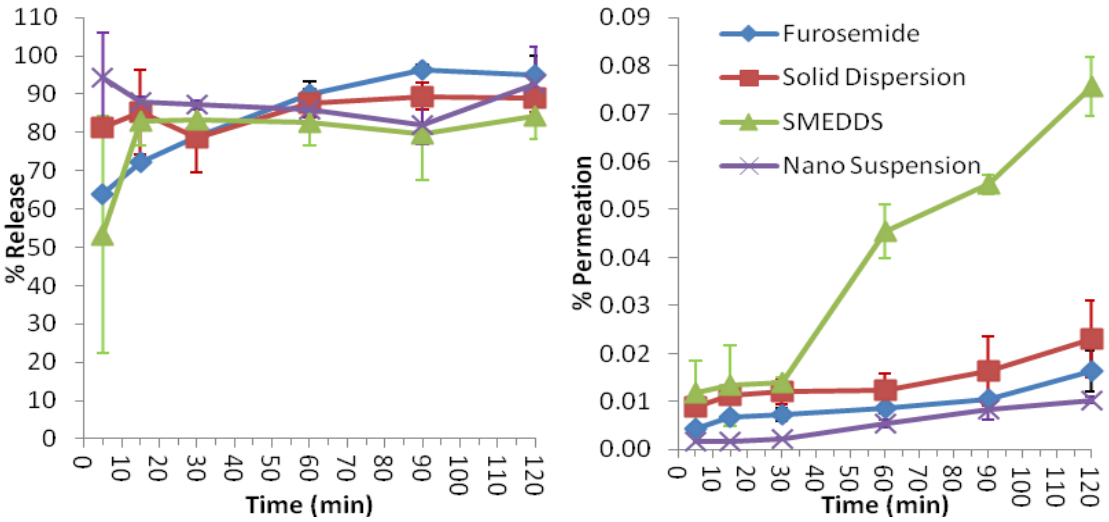


Figure 4.31: Dissolution and permeability of furosemide formulations-D/P system

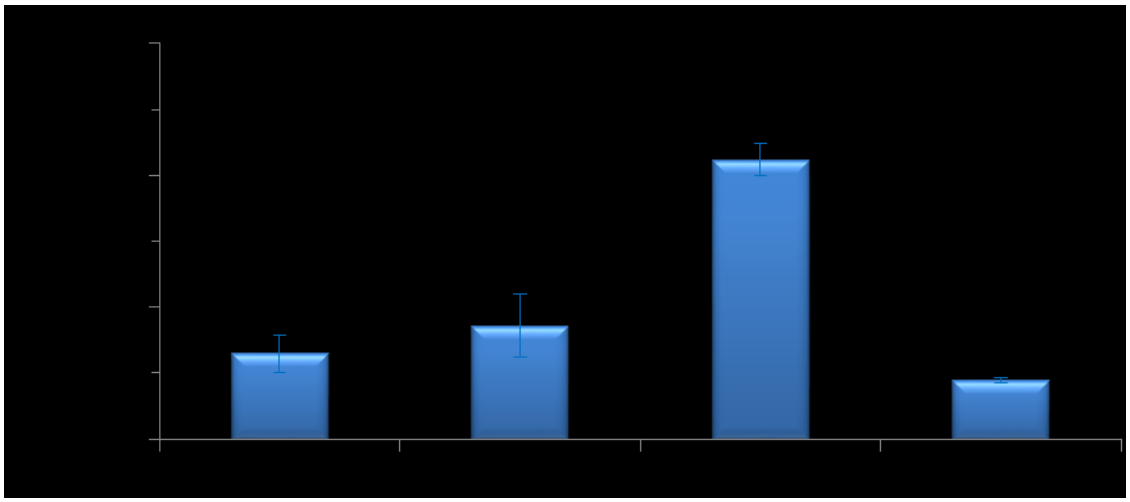


Figure 4.32: Furosemide fraction absorbed calculated based on the D/P system

4.3.5.2 Dipyridamole Formulations Assessment *In Vitro* Using the D/P System

The extent of drug release on the apical side was the highest for the dipyridamole SMEDDS formulation, followed by the solid dispersion. A reduction in the drug particle size did not yield any increase in dipyridamole release, and instead gave a similar dissolution to the API alone. On the baso-lateral side, it was observed that the highest permeated amount was for the solid dispersion formulation, followed by SMEDDS. No increase in the permeation was attained in the case of nano suspension (Figure 4.33). This is not surprising, however, considering the fact that the extent of drug release in the case of SMEDDS formulation does not reflect the free drug amount available for permeation through the membrane due to the micelle structure created by the oil-in-water phase. In terms of the f_a (Figure 4.34), it can be seen that solid dispersion increased absorption to 80%, followed by 70% to the SMEDDS formulation, with no increase in the case of the nano-suspension (30%).

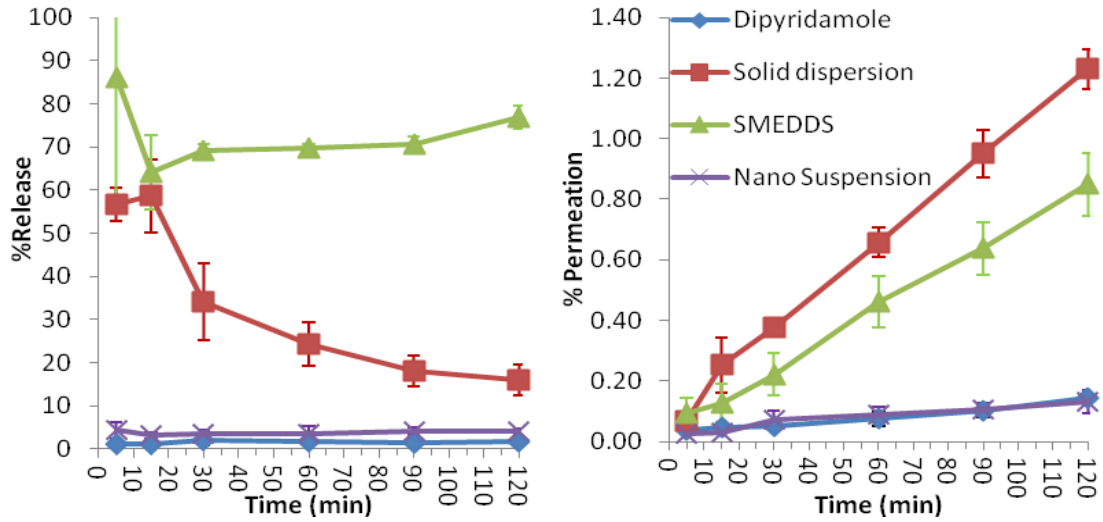


Figure 4.33: Dissolution and permeation of dipyridamole formulations- D/P system

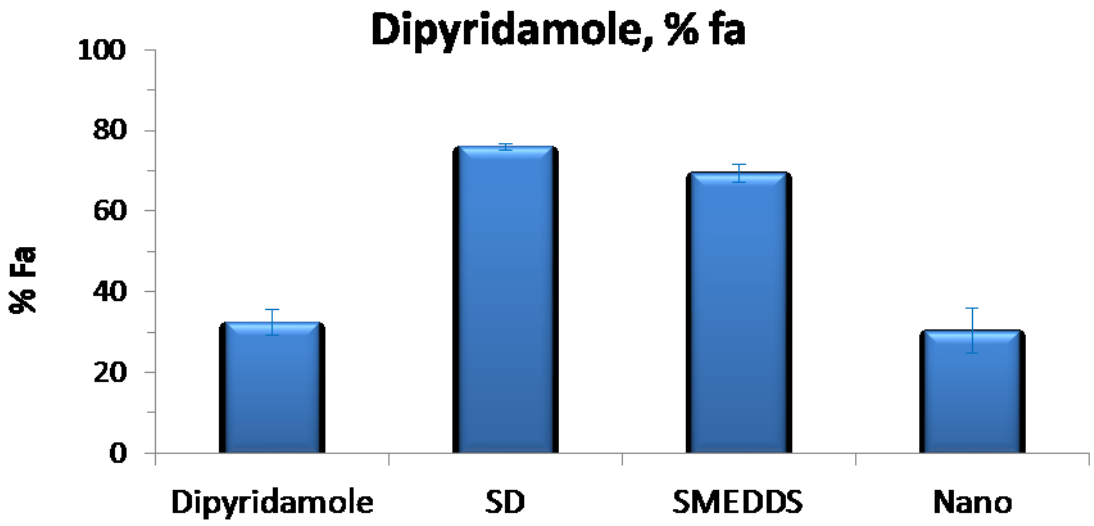


Figure 4.34: Dipyridamole fraction absorbed calculated based on the D/P system

4.3.5.3 Furosemide Formulations *In Vivo*

The pharmacokinetics of furosemide in rats was evaluated after oral administration of furosemide as a solution, oral suspension and the sole API in capsule (Figure 4.35). No significant differences were observed between all three dosage forms in AUC_{0-8} ($p>0.05$). T_{max} of furosemide caps was around 1 hour, while the T_{max} of the suspension and the oral solution, came earlier at around 20-30 minutes. The calculated bioavailability of furosemide in rats was very low at 5, 6.5 and 10% for the suspension, oral solution and API capsules, respectively.

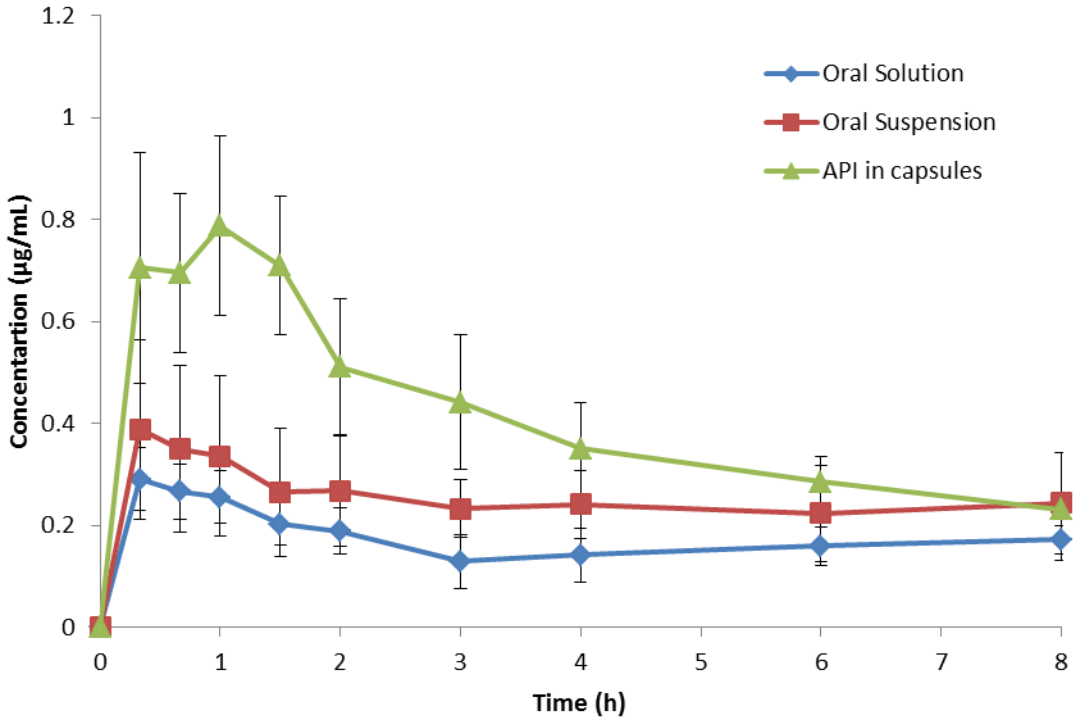


Figure 4.35: Plasma concentrations vs. time of oral solution, oral suspension and API in capsules of furosemide

To investigate the effect of different formulations on furosemide bioavailability, the following were administered to the rats: solid dispersion, SMEDDS and nano-suspension (Figure 4.36). AUC_{0-8} and bioavailability values are presented in Table 4.6. Solid dispersion plasma concentration vs. time did not differ significantly compared to the API capsules, and generated similar AUC_{0-8} values ($p>0.05$) around 3µg*h/mL. Thus, no increase in furosemide bioavailability was observed in the case of the solid dispersion (11%). The nano-suspension gave the highest AUC_{0-8} value, followed by SMEDDS, and for both formulations, drug exposure was significantly different from the API capsules ($p<0.5$). With respect to bioavailability, the nano-suspension formulation increased furosemide bioavailability 3-fold, calculated as 33%, whereas the SMEDDS formulation increased bioavailability only moderately to a value of 15%. High variability was observed for both formulations (30-40%). Variability in AUC_{0-8} was also slightly lower for the solid dispersion as compared to API capsule administration (24 vs. 40%). T_{max} values for SMEDDS and nano-suspension (administered as a solution) were similar at around 30 minutes. The furosemide solid dispersion T_{max} was recorded around 1.5-2 hours; slightly later from the T_{max} recorded for the furosemide API.

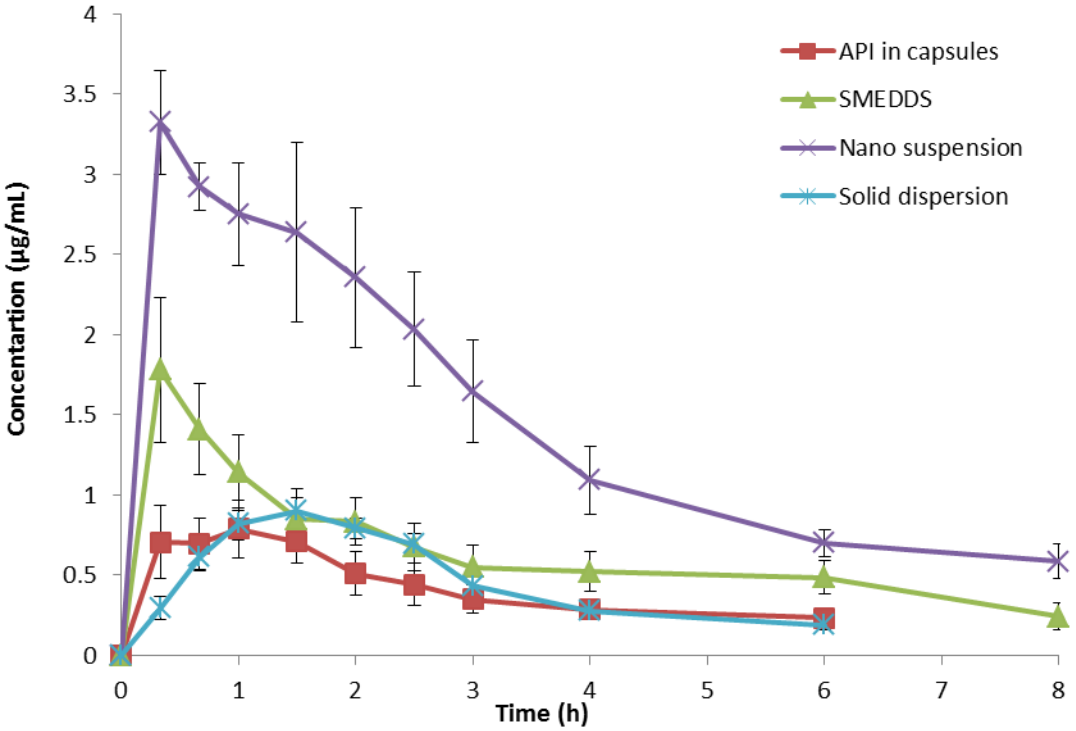


Figure 4.36: Plasma concentrations vs. time of API in capsules, SMEDDS, nano-suspension and solid dispersion formulations of furosemide

To eliminate the hydrophilic effect of excipients on furosemide absorption in the case of the nano-suspension, the same composition of the nano-suspension formulation was mixed with the un-milled API and administered at the same dose to the rats

Figure 4.37). The AUC_{0-8} of the excipients mixture with unmilled API increased as compared to the API alone, but was still lower than the nano suspension AUC_{0-8}. This implies that the drug exposure was not affected by the excipients alone, but that the reduction in particle size also contributed to an increase in drug absorption.

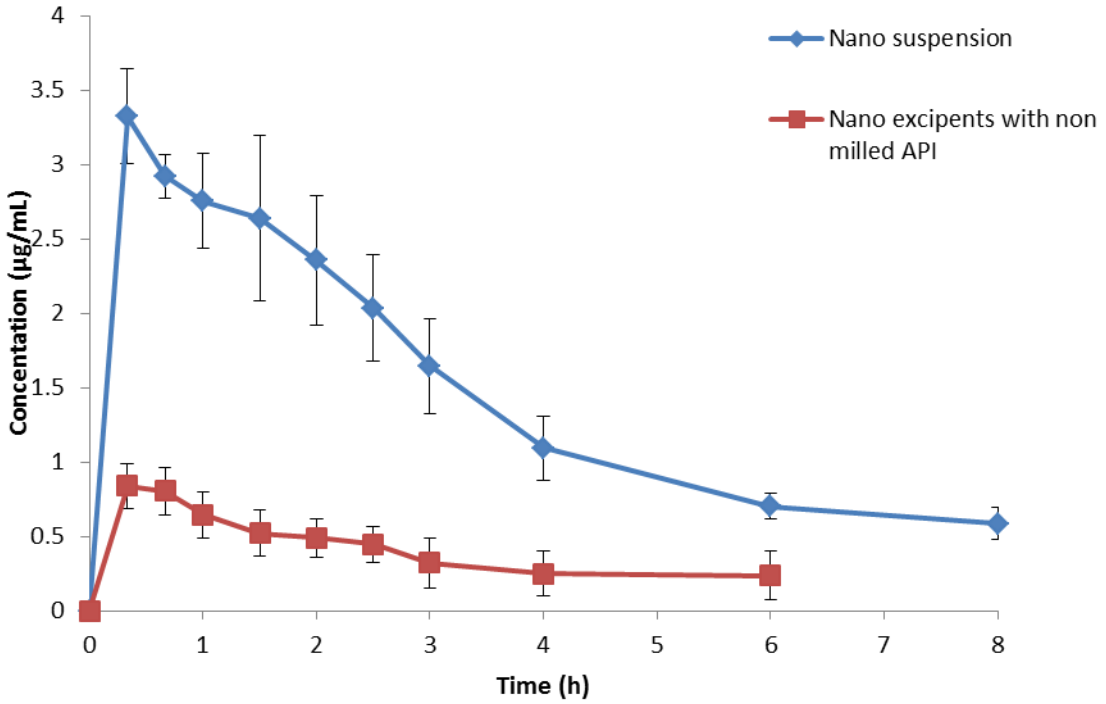


Figure 4.37: Plasma concentrations vs. time of nano-suspension and nano excipients with unmilled furosemide

Table 4.6: AUC_{0-8} and bioavailability values of the different furosemide dosage forms in rats

Dosage form	**AUC_{0-8} (µg*h/mL)**		**Bioavailability (%)**
	Mean ±SEM	CV %	
API in capsules	3.2±0.5 (n=6)	40.4	9.9
SMEDDS	4.5±0.8 (n=6)	45.0	14
Nano Suspension	10.4±1.4 (n=6)	32.5	32.5
Solid Dispersion	3.6±0.3 (n=6)	25.0	11
Oral suspension	1.7±0.1 (n=3)	9.3	5.3
Oral Solution	2.1±1.2 (n=3)	57.1	6.5

As described, furosemide administered as solution and as powder in capsules yielded approximately similar AUC_{0-8} values with high variability. In the first instance, this might imply that the solubility/dissolution of furosemide in rats at the administered dose is not a crucial factor in furosemide absorption. Similar results were also recorded in humans. Based on similar extents of bioavailability/AUC obtained following tablet and solution dosing, Kelly *et al*. (1974) concluded that solubility may not be the sole factor decreasing furosemide absorption. A similar extent of absorption from furosemide capsules and solution might be related to the fact that furosemide is a poorly permeable drug, and permeability is actually the limiting step in its absorption rather than solubility and dissolution. This was further confirmed by Waller *et al*. (1982).

The increase in the nano-suspension bioavailability in rats was fairly surprising considering that the *in vitro* results in FaSSGF /*m*Hanks and in the D/P system where no increase in drug release from the nano- suspension was observed. Moreover, it contradicts the assumption that it is not the dissolution rate which controls furosemide absorption, but the permeability based on the similar plasma profiles of oral solution, suspension and API powder. Suggested explanations for this include, firstly, that the oral solution results might be misleading in rats at this administered dose. Indeed, the pH of the oral solution was adjusted to 8 in order to completely dissolve furosemide in water (the decision not to add any dissolving agent was made based on the understanding that these agents might facilitate the drug absorption). Unpublished results from our group of *in vivo* gastric (fundus) pH in rats ranged from 2.9 to 5.6 (n=9). Considering the high variability obtained in the case of the oral solution, the differences in the gastric pH between rats might therefore contribute the high variability absorbed in the AUC_{0-8} in the case of the weak acid drug solution, and *in vivo* at low pH, precipitation cannot be ruled out. In which case, it may be that it is not permeability limited, and at the administered dose in rats, the dissolution has an effect on the drug absorption. The slight increase in the bioavailability of SMEDDS furosemide can be explained by additional absorption mechanisms such as direct partitioning of the drug from the micelle to the membrane, as mentioned before in the interpretation of the *in vitro* results from D/P system. The slight increase in SMEDDS formulation compared to the *in vitro* results might be related to the high levels of bile salts excreted in rats, making it difficult to observe significant differences in the absorption of lipophilic drugs.

The D/P system failed to predict the situation *in vivo* in rats. One explanation might be related to the higher dose administered to the rats compared to the dose tested in D/P system (which yields the situation where furosemide solubility is limited by dissolution and not permeability as the D/P system indicated). To further establish the *IVIVC* with the D/P system, a higher dose (1.4mg- comparable to the administered dose that was given to rats) was tested in the D/P system (Figure 4.38). Similar picture as was observed for the lower dose in the D/P system was obtained with the higher dose. High dissolution for all furosemide formulations and for the API was observed on the apical side (equivalent to 140mg in humans), which might imply that even at this high dose, furosemide absorption is not the solubility/dissolution rate limited in humans. On the apical side, the permeation percentage was still low in the case of all furosemide dosage forms (less than 0.1% at 2h), apart from the SMEDDS formulation, which increased to a 0.4% permeated amount at 2h. A possible explanation for this might be related to the different ratios of surface area available for the drug to permeate to the fluid volume. Due to obvious restrictions, the surface area of the Caco-2 layer membrane is limited as compared to the intestinal membrane in rats or in human. Therefore, the ability of the D/P system to capture the difference in permeation might be limited, and especially in the case of furosemide as a poorly permeable drug. Another possible explanation might be that the rat is not a good model to evaluate these formulations. Considering all the physiological differences in the gut from rat to human, it might be that the D/P will actually predict well the situation in humans as was published by Professor Yamashita but not in rats for some formulations or drugs. Moreover, in this study the predicted absorption from the D\P system was compared to the bioavailability values generated in rat model. It might be that a direct comparison to absorption values (by measuring the concentration of the drug in the portal vein of rats) in the rat model will yield a better correlation.

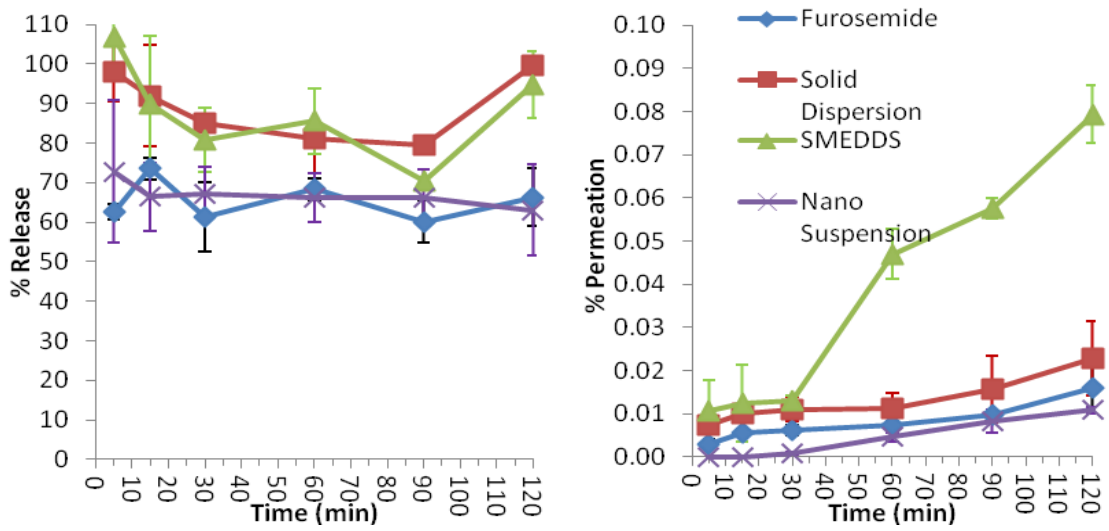


Figure 4.38: Dissolution and permeation of furosemide formulations-D/P system at higher dose

High variability in $AUC_{0\text{-}8}$ was observed across all formulations, with a relatively lower CV of oral suspension and solid dispersion. This might be due to the low number of rats analysed for each formulation. Moreover, Waller *et al.*(1988) suggested that the absorption was limited by occurring only from a specific site (absorption window) in the GI tract, and can explain the high inter-individual variability in man. However, this suggestion has not been verified in humans to date. Chungi *et al*. (1979) has confirmed that limited absorption might also occur in rats from the stomach, and slightly from the duodenum, yet since high variability was observed for all formulations, no definite conclusions can be drawn in terms of formulation efficacy decreasing variability in exposure.

4.3.5.4 Dipyridamole Formulations *In Vivo* in Rat Model

The pharmacokinetic and plasma concentrations vs. time of the different dipyridamole formulations were assessed in the rat model (Figure 4.39 and Table 4.7). Dipyridamole administered as API in capsules, solid dispersion and SMEDDS yielded similar plasma concentrations and AUC_{0-8} values around 150ng*h/mL ($p>0.05$). The dipyridamole nano-suspension, gave a higher plasma concentration and the AUC_{0-8} value was two-fold higher compared to that for the other formulations (320ng*h/mL). Bioavailability of the API, solid dispersion and SMEDDS formulations in rats were 55, 49 and 66% respectively, and did not significantly differ from each other ($p>0.5$). Nano suspension absorption was complete, and a bioavailability of 99% was calculated (Table 4.7).

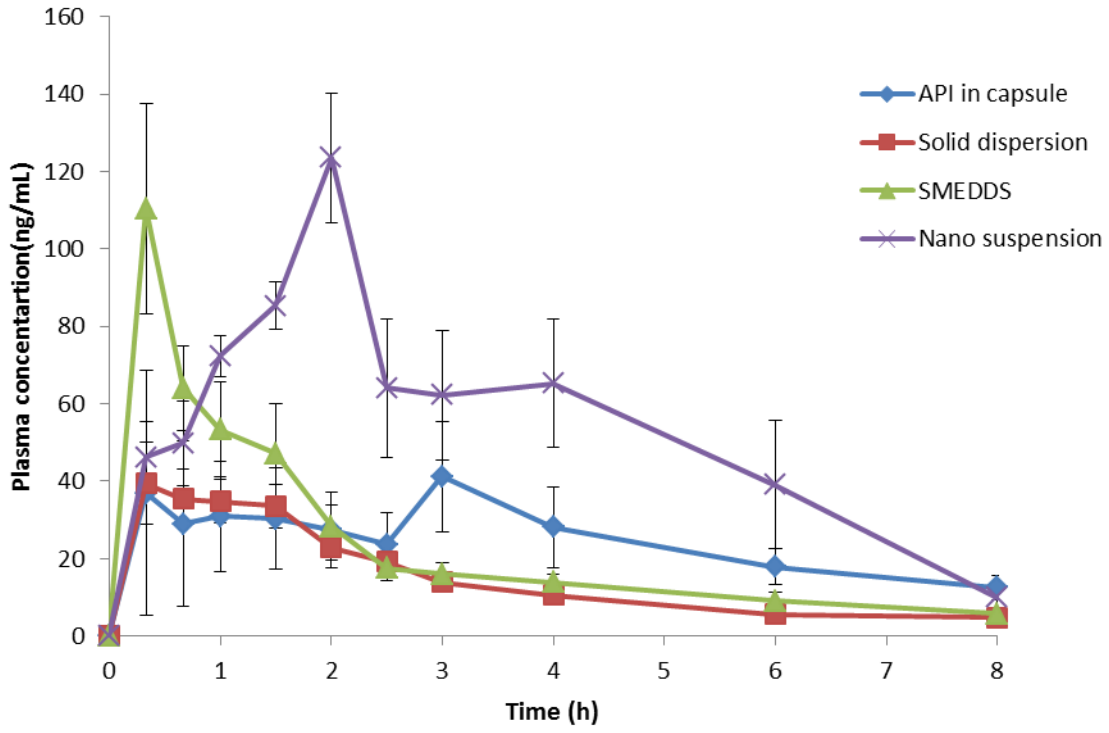


Figure 4.39: Plasma concentration vs. time of different dipyridamole formulations in rats.

Dosage form	AUC_{0-8} (ng*h/mL)		Bioavailability (%)
	Mean ±SEM	CV %	
API in capsules	155±42 (n=3)	41	53
SMEDDS	195±100 (n=4)	55	66
Nano Suspension	324±138 (n=3)	73	99.9
Solid Dispersion	145±12 (n=6)	12	49

Table 4.7: AUC_{0-8} and bioavailability values of the different dipyridamole dosage forms in rats

Again, in order to evaluate the effect of particle size reduction in the case of the dipyridamole nano-suspension, a similar formulation for the nano-suspension with the un-milled API was prepared and administered to rats (Figure 4.40). Unlike furosemide, the mean plasma concentrations were similar to the nano-suspension formulation, and gave complete absorption and 100% bioavailability. Variability for both formulations was high, indicating that in the case of dipyridamole; the excipients (polyvinyl alcohol and mannitol) in the nano-suspension formulation produced an increase in absorption more so than the particle reduction size.

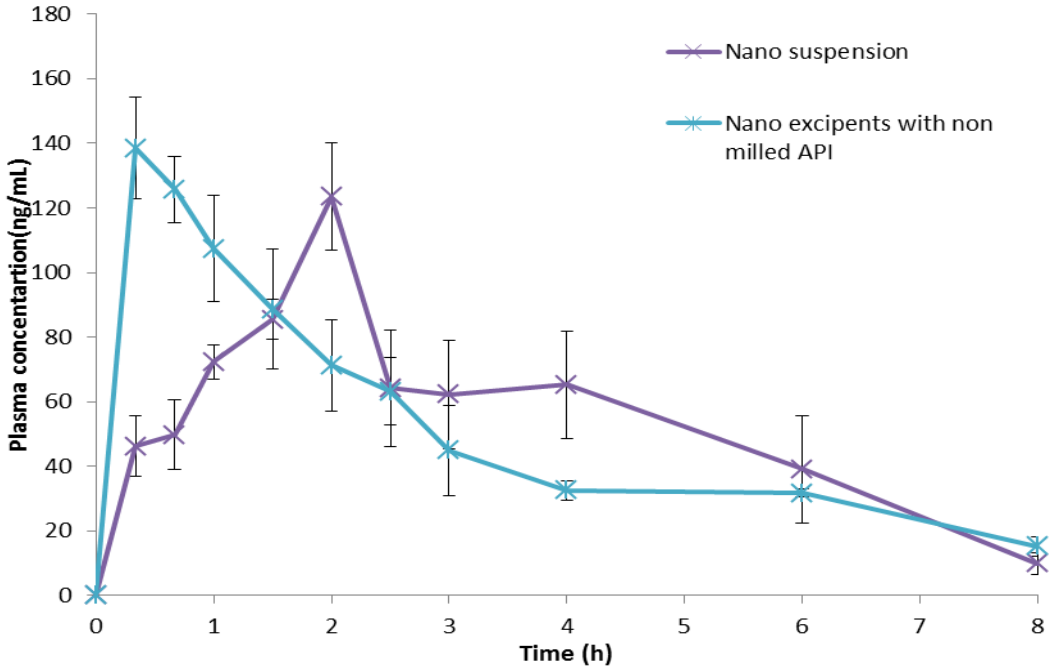


Figure 4.40: Plasma concentration vs. time of nano suspension and nano excipients with unmilled dipyridamole in rats.

In the case of dipyridamole, no increase in the drug exposure was absorbed *in vivo* as compared to the *in vitro* results in the D/P system. The low plasma concentration for the solid dispersion formulation can be attributed in the first instance to the fact that the dipyridamole solid dispersion was administered as a powder with no wetting agent or other excipients present in the formulation, whereas the nano-suspension and SMEDDS formulations were administered in their solution state in a similar way as for the furosemide solid dispersion. Thus, the wetting properties of the powder might affect its dissolution rate and extent of drug absorbed *in vivo* compared to the other formulations. Another possible explanation for the similarity in the absorption of dipyridamole solid dispersion and the API is the possibility that the drug dissolved from the amorphous form in the rat stomach. However, on reaching the higher pH environment in the duodenum, it precipitates back to its crystalline from, giving a similar plasma concentration. Interestingly, and similar to the furosemide nano-suspension, an increase in drug exposure of dipyridamole nano-formulation was observed which was not predicted form the D/P system. However in the case of dipyridamole, it was confirmed that this can be attributed to the hydrophilic properties of the excipients in the formulation, rather than the particle size reduction. Therefore, it might be that the differences in the rat GI fluid composition compared to the FaSSIF (bile salts) facilitated dipyridamole dissolution *in vivo* and not *in vitro*. Another possible explanation could be that the gastric environment affects the dispersion and dissolution of the nano suspension, which was simulated *in vitro* in the D/P system. It was suggested by Mackie *et al*. (2009) and Ouwerkerk-Mahadevan *et al*. (2011) that the rat was a better model to assess nano suspension based on comparison of rat, dog and human clinical data. This is further confirmed by this study where high exposure for both furosemide and dipyridamole nano-formulations was observed in the rat model but not when tested *in vitro*.

High variability in AUC_{0-8} values was observed for all formulations, which may be attributed to the use of small groups of rats (3-4) in each experiment. Although each formulation was administered to 6 rats, only 3-4 rats' samples could be analysed due to analytical problems. Therefore, variability could not been accurately assessed. For solid dispersions, where six rats were analysed, relatively low variability was observed (12%).

4.3.6 Summary

Based on the *in vitro* results from the D/P system and other tools investigated in this research, it seems that furosemide absorption is governed by low permeability of the drug in the dose range of 40-140mg in humans. In rats, the increase in exposure for the SMEDDS and nano-formulation might indicate that absorption is dissolution limited. For dipyridamole, BCS II compound, it is clear that its absorption is dissolution limited based on the *in vitro* and the *in vivo* results in rats. However, the formulations performances in rats were not predicted by the D/P system. High exposure for nano-formulations for furosemide and dipyridamole was observed in rats but not *in vitro,* whereas solid dispersion formulations for both drugs did not increase bioavailability in rats. Due to the lack of correlation between the rat studies to the D/P system results, it is difficult to conclude which of these models will predict better the situation in humans. The missing link is indeed the human data. In addition, based on the rat model, it was difficult to draw any conclusion regarding variability as variability was very high. As described, it might be that increasing the number of rats in each test will increase the ability to predict variability.

Chapter 5 -General Discussion & Future Work

5.1 General Discussion and Future Work

Oral drug absorption is a complex process which is affected by physiological, physicochemical and formulation factors, each of which is sensitive to inter- and intra-individual variability. As described in this thesis, there is great interest in the pharmaceutical community to understand this process and the underlines factors causing low and erratic absorption, in order to reduce drug development costs, efforts and most importantly to obtain a better therapeutic response in patients. As part of the research conducted in this PhD thesis, it was found that there is great confusion between bioavailability and absorption. Moreover, it is very difficult to obtain an accurate estimation of absorption and inter-subject variability in absorption from *in vivo* data from clinical studies in humans without making any assumptions. This is mainly related to the fact that there is no direct method to measure absorption *in vivo* (measuring drug concentration in the portal vein or in the gastrointestinal fluids in humans are rarely carried out due to ethical considerations). On the contrary, clinical trials are commonly carried out to evaluate drug pharmacokinetics and pharmacodynamics, hence, it is possible to estimate absorption values from bioavailability calculation based on different models. However, clinical trials involving a large number of subjects are not readily available in the public domain and some parameters which required estimating absorption and inter-subject variability are not routinely measured. In this investigation, 40 clinical trials with the required parameters to estimate absorption and inter-subject variability were utilised to correlate between absorption and inter-subject variability. However, due to limitations described previously, it was difficult to draw a definite conclusion. Moreover, in the attempt to explain inter-subject variability by correlating it to different physicochemical properties of the drugs, it was found that some of the physicochemical parameters may not reflect the *in vivo* situation reliably and there is a need to develop more robust *in vitro* methods to capture the complexity of the gut.

Many attempts have been made to predict the "average" absorption using different approaches. Some include quantitative structure-activity relationships based on physicochemical properties, others use animal models to extrapolate to absorption in humans and another approach which has been gaining great interest recently is the use of PKPB models. The later provides an approach utilising preclinical *in vitro* and *in vivo* data to predict the plasma concentration time profiles. With increasing knowledge on the GI tract

environment, most of these PBPK models were extensively developed to consider different physiological factors and how they account for variability. Population pharmacokinetics as represented by NONMEM, is successfully used to model drug pharmacokinetics and pharmacodynamics, especially when sparse data were collected in patients. Often NONMEM is used to estimate bioavailability and inter-subject variability to characterise drug absorption. Based on the difficulties of calculating f_a from available clinical trials (in the public domain), it was decided to utilise phase 1 clinical trials to estimate f_a using NONMEM to gain a better understanding of inter-subject variability in absorption instead of bioavailability. The well-stirred model was successfully implemented in NONMEM to estimate the typical population absorption profile. Estimations of f_a*f_g and inter variability were obtained for four compounds with different formulations. The rate limiting step solubility\dissolution was identified by comparing oral solution, IR tablet in the base form and IR tablet in the salt form. It was additionally found that variability in absorption did not differ between different formulations mainly because the increase in absorption was not significant. Formulation effects and food effect were investigated in the case of oral solution and ER formulation. Analysing the *in vitro* data enabled a better understanding of the drug behaviour in the GI tract based on the absorption estimations. The proposed work herein offers a more quantitative estimation of the absorption process and variability. Accurate estimation of absorption from phase 1 clinical studies using NONMEM would enable better understanding of the factors contributing to low and erratic absorption and therefore would promote the selection of the right formulation for further development. Moreover, understanding drug absorption variability will enable better planning and execution of phase 2 and 3 clinical trials (aiding improved selection of sample size and dosage regimen, etc.).

In recent years, more PBPK models are being implemented in NONMEM to evaluate drug performance in the population. The estimations for absorption in this research did not separate the fraction that escape gut wall metabolism to that absorbed. To our knowledge, there is no definite method to calculate f_g from plasma concentration vs. time data. However, it would be highly desirable to acquire a separate estimation of f_g either by using clinical trials that were conducted with co administration of P-gp or Cyps450 inhibitors, but it will then be necessary to consider the different intrinsic clearance of these subjects. Another possibility for future work to separate f_a from f_g is by incorporating the Q_{gut} model and the use of *in vitro* data.

For the purpose of this study, only parameters that were required to calculate the elimination from the liver and the kidneys were incorporated in the model (i.e. blood to plasma ratio, liver blood flow and renal clearance). However, there is no doubt that incorporating more physiochemical factors such as lipophilicity, ionization, solubility, protein binding, tissue drug concentration partition coefficients and physiological parameters such as gastric empting and transit time will increase the model accuracy and sensitivity to estimate absorption. Moreover, NONMEM's advantage in explaining the inter-subject variability by including covariates such as age, weight, and gender could be further investigated to increase the model fit.

Many compounds are being discovered and developed in the pharmaceutical industry, however, only a few have been successfully marketed. In the development of each compound great knowledge and experience is being generated, but due to the competitive industry environment, it is not always possible to define the factors which led to the compound failure. An initiative by the Orbito project (http://www.imi.europa.eu/content/orbito) aims to share the knowledge and experience to overcome the existing gaps in the biopharmaceutical and formulation science. One of the objectives of the Orbito project is, through the collaboration of pharmaceutical companies and academic groups, to construct a new database of poorly soluble drugs together with their clinical trials in humans and animals. This database will be characterised for its physicochemical properties (e.g. logP, pKa, solubility, permeability, etc.) and the resulting data will be used to develop new *in silico* models available to the common research community. The population pharmacokinetic approach presented herein can be further validated in such a data set once it is published.

In silico estimation of absorption and inter-subject variability is very important, however *in vitro* and *in vivo* tests are still required to identify factors contributing to inter-subject variability in drug solubility, dissolution and permeability. Two model drugs with reported with erratic bioavailability (attributed to absorption variability) in humans were chosen for this investigative purpose (dipyridamole and furosemide). Few *in vitro* tests to measure solubility, dissolution and permeability were utilised in this research to simplify our understanding as for the factors that cause variability, since there is no single *in vitro* tool that can capture the complexity of the GI tract.

Solubility measurements were carried out in pooled gastric, jejunum fluids and further in ileostomy fluids. Based on the results from this investigation, it appears that pH and buffer capacity vary considerably between individuals in ileostomy fluids, and this may be augmented in different disease states, further affecting drug solubility. The solubility measurements in human and simulated fluids showed that solubility-dependent pH and bile salt concentration may have a strong impact on dipyridamole solubility, and consequently its absorption, whereas furosemide solubility demonstrates high correlation with buffer capacity and pH. Based on these results alone, it might be that dipyridamole solubility *in vivo* will be controlled by bile salts concentration and variable pH between individuals. Furosemide solubility will vary between subjects with different buffer capacity and pH, especially in the lower parts of the gut where low volume of fluids is available for dissolution.

Our understanding of the GI environment has enormously increased, and significant progress has been made to create bio-relevant dissolution media to establish *IVIVC*. With regard to dipyridamole, the results from the dissolution tests confirmed the result from the solubility study and it is safe to conclude that dissolution and solubility are rate-limiting steps for its absorption, and are highly affected by pH and bile salts concentration in the intestine. Therefore, changes in pH and bile salts between individuals *in vivo* can help to explain the erratic absorption of dipyridamole. In the case of furosemide, although the saturated solubility was affected by bile salts and pH, it seems that dissolution tests alone cannot explain the variability *in vivo*. It can be concluded that dissolution is not the limiting step in furosemide absorption. These findings were striking, considering the fact that furosemide is classified as BCS IV and the results from the solubility study. Consequently, the classification of furosemide as BCS IV in the given dose (40mg) should ideally be reconsidered. The apparent contradiction in the results of the solubility study can be explained by the fact that in the solubility study the saturated solubility was measured. However, in the clinical dose, furosemide dissolved completely and did not reach its saturated solubility.

Permeation studies are routinely carried out to predict drug absorption through the gut. However, dissolution and permeation are continuous processes and the permeation of the drug is highly dependent on the amount dissolved in the GI lumen. The dissolution permeation system developed by Professor Yamashita was utilised in this investigation. Using the D\P system, it was found that bile salt concentration was irrelevant for the

dipyridamole permeation through the membrane. Moreover, it was found that pH plays an important role in the permeation of ionised drugs such as dipyridamole. This might suggest that *in vivo*, subjects with variations in pH along the GI tract will have considerable changes in the absorption of drugs with pka close to the GI fluids pH. For furosemide, a low permeable drug, no differences in the permeation profiles were observed, probably due to the lack of sensitivity of Caco-2 monolayer to detect small changes in the permeation of low permeable drugs. It is important to note that when interpreting the *in vitro* results from the D/P system to the *in vivo* situation there is a need to consider the small surface area available for permeation compared to the gut membrane surface area. It will be highly interesting to evaluate other cell monolayers such as MDCK in the case of low permeable drugs. In addition, investigation other physiological factors other than bile salt and pH in these systems will add more information on possible factors causing changes between individuals. For furosemide in particular, it may be that gastric emptying time and intestinal motility could shed further light on our understanding of variability in drug absorption.

The results presented herein, emphasise the complexity of the GI tract environment and the difficulties of capturing it based on only single *in vitro* method. It is highly important to attempt to simplify the situation *in vivo* and work stepwise in order to gain a better understanding of the gastrointestinal tract complexity by assessing each factor separately. Combining the results from different experiments will enable capturing of the gut complexity. With that, it is important to remember that the different stages in absorption are dependent on each-other and the extrapolation to the *in vivo* situation must be carried out based on this assumption, as it has been demonstrated herein by the use of the D\P system. Future work may include investigating *in vitro* GI models to predict inter-subject variability. This can be implemented by developing simulated gastric/intestinal fluids to mimic not only the average person, but also range of conditions of the fed and fasted gut. Moreover, the use of systems that can evaluate variation in gastric emptying or transit time like the TNO systems can be utilised.

Many attempts have been made by the pharmaceutical industry to overcome low and erratic absorption, in particular, by formulating the drug to increase the drug solubility and dissolution. Comparing three different formulation approaches to increase solubility and dissolution have shown that solid dispersion formulation results in the simplest design and evaluation followed by the reduction of the particle size to nano size and eventually SMEDD

formulation. There is no doubt that better understanding and evaluation of SMEDDS formulation *in vitro* will be needed in order to predict the formulation performance and mechanism *in vivo*. Further on, an early consideration of the API properties needs to be taken into account when choosing the excipients in the formulation. Solid dispersion and SMEDDS increased the drug dissolution to a similar extent and minimised the effect of bile salt or pH on the dissolution in the case of dipyridamole. On the contrary, when the formulations were tested in the *in vivo* in rat model, only nano-particles formulation has shown to increase dipyridamole bioavailability. Similarly, lack of correlation between the *in vitro* results of furosemide formulations in the D/P system to the *in vivo* results in rats was observed. It again emphasises the complexity of the GI tract and the difficulties in predicting from *in vitro* results or *in vivo* in animals to the *in vivo* in humans.

Future work of this thesis will include evaluation of different formulations of poorly soluble drugs using *in vitro* test (i.e. the D\P system) linking it to *in vivo* studies in animal models and *in vivo* clinical studies in humans. This can be implemented by establishing guidelines for applying different formulation approaches in the case of poorly soluble drugs. This could result in reducing development costs and efforts. To this purpose, it will be highly desirable to obtain a large dataset of poorly soluble/permeable compounds in different formulations with their physicochemical properties and *in vitro* evaluation. For the animal models, it will be preferable to generate absorption data instead of bioavailability (by measuring the drug concentration from the portal vein or in the animal gastrointestinal tract). Further on, comparing different formulation approaches in different animal models is needed, to better define the relationships between the physicochemical properties of a compound to formulation, and effectiveness as screening tool and predictability for humans. In human, utilising clinical trials from phase 1 using population approaches to calculate absorption as demonstrated in this research will enable an accurate measurement of absorption and inter-subject variability in absorption. Combining all these tools together will enable validation of existing *in vitro* and *in vivo* methods to understand absorption.

Appendix

1.1. Data for Compound AZD0865

Study objective(s)	Study design	Subjects	Dosing regimen
ADME of AZD0865	Phase I, randomized, open, crossover study	9 healthy males age 38-50 years	Single doses of 14C-labelled or non-labelled AZD0865 20 mg IV solution (as a single 60-minute infusion) 40 mg oral solution
Ascending dose- oral solution	Phase I study: Tolerability part with randomized, parallel, single-blind, placebo-controlled dose escalating design and efficacy part with non-randomized, single-blind design	27 healthy males age 22-39 years	Single doses of oral solution 0.08-4 mg/kg
Single oral doses given as mesylate salt tablets, a base form tablet and an oral solution	Phase I, randomized, open, crossover study	14 healthy males age 21-29 years	Single oral doses of 100 mg as micronised base tablet mesylate salt tablet micronised mesylate salt tablet oral solution
Single oral doses given mesylate salt tablets and a base form tablet at an elevated intragastric pH	Phase I, randomized, open, crossover, study The intragastric pH was raised by intravenous administration of omeprazole	14 healthy males age 21-37 years	Single oral doses of 100 mg as micronised base tablet mesylate salt tablet micronised mesylate salt tablet
effect of clarithromycin	Phase I, randomized, open, crossover, study	18 healthy males age 20-33 years	Single oral doses of an oral solution 40 mg alone and concomitantly with clarithromycin tablets 500 mg bid at steady state

Table A 1: AZD0865 phase 1 clinical trials included in the analysis

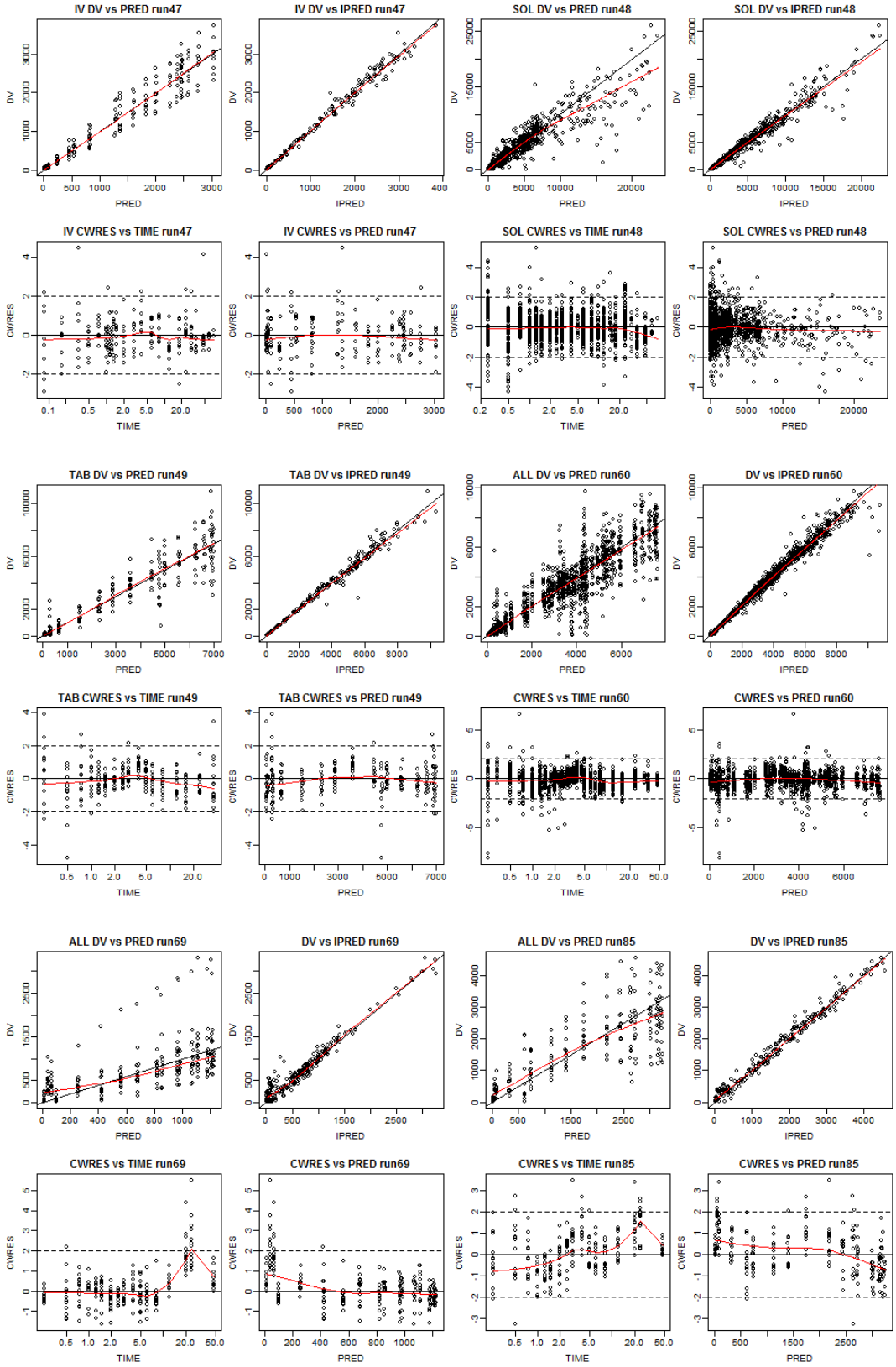


Figure A 1: Goodness of fit plots for compound AZD0865; Run 47 - IV model; Run 48 – oral solution; Run 49 -IR in the base form; Run 60– IR in the salt form; Run 69 – IR in the base form at elevated pH, Run 85 – IR in the salt form at elevated pH

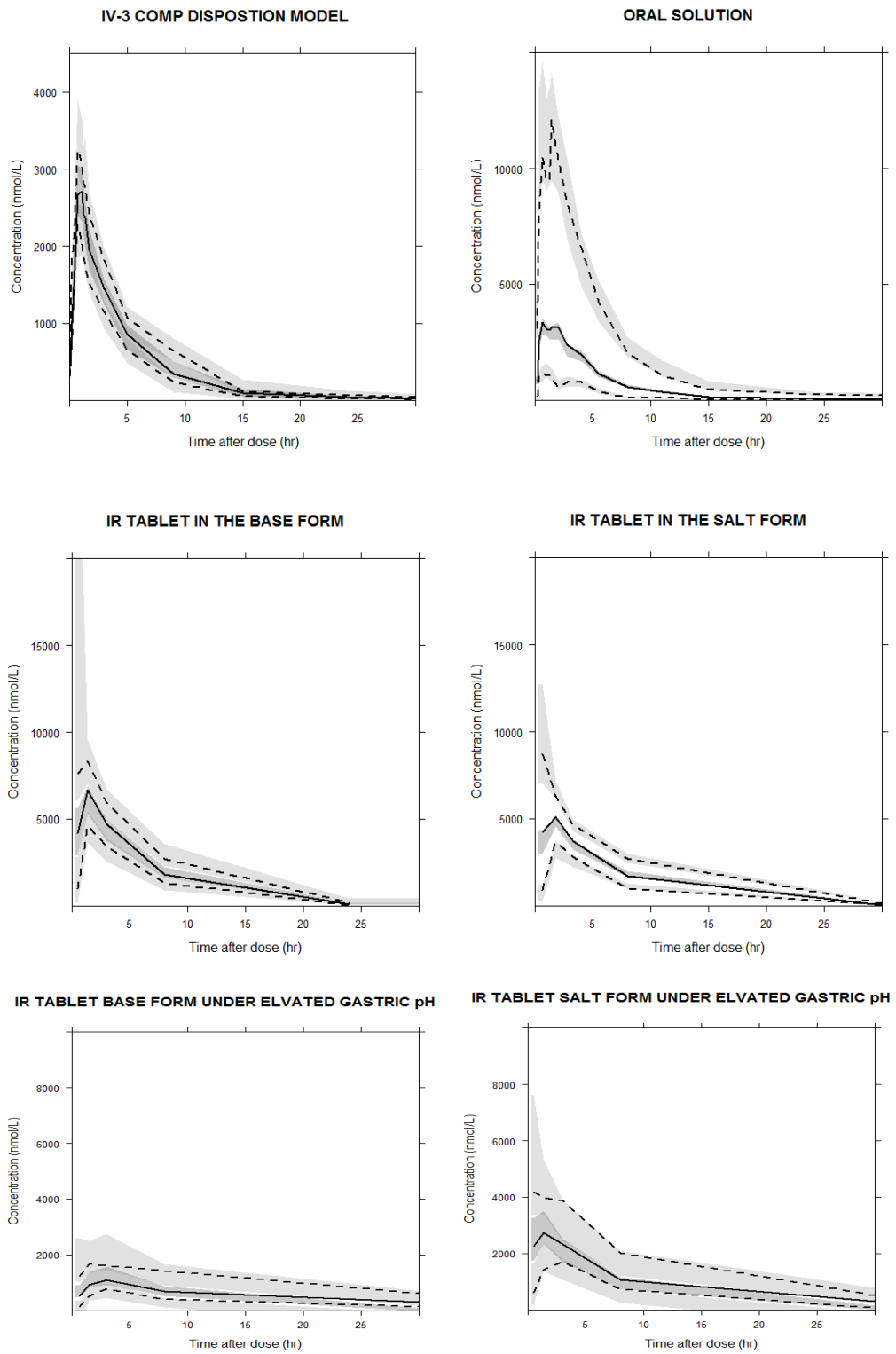


Figure A 2: Visual Predictive Checks for compound AZD0865; Run 47 - IV model; Run 48 – oral solution; Run 49 -IR in the base form; Run 60– IR in the salt form; Run 69 – IR in the base form at elevated pH, Run 85 – IR in the salt form at elevated pH

1.2. Data for Compound AZD242

Study objective(s)	Study design	Subjects	Dosing regimen
Single oral dose to healthy male subjects	Single dose, double blind, randomised, placebo-controlled, dose-escalation study	16 Healthy male subjects age 23-40 years	0.5 to 12 mg p.o.
ADME study	Single dose, open label, randomised, two-way crossover	8 Healthy male subjects age 30-50 years	1 mg p.o. 1 mg i.v.
Single oral dose	Single dose, single blind, randomised, placebo-controlled; Japanese bridging study	56 Healthy male Caucasians and Japanese subjects age 19-32 years	0.5, 2, 4, and 8 mg p.o.

Table A 2: AZD242 phase 1 clinical trials included in the analysis

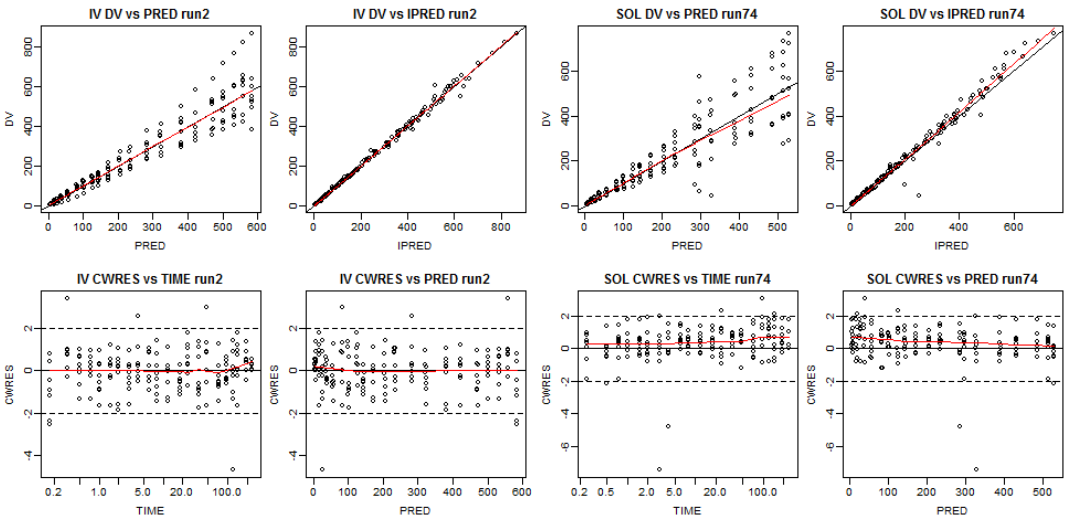


Figure A 3: Goodness of fit plots for compound AZD242; Run 2 - IV model; Run 74 – oral solution

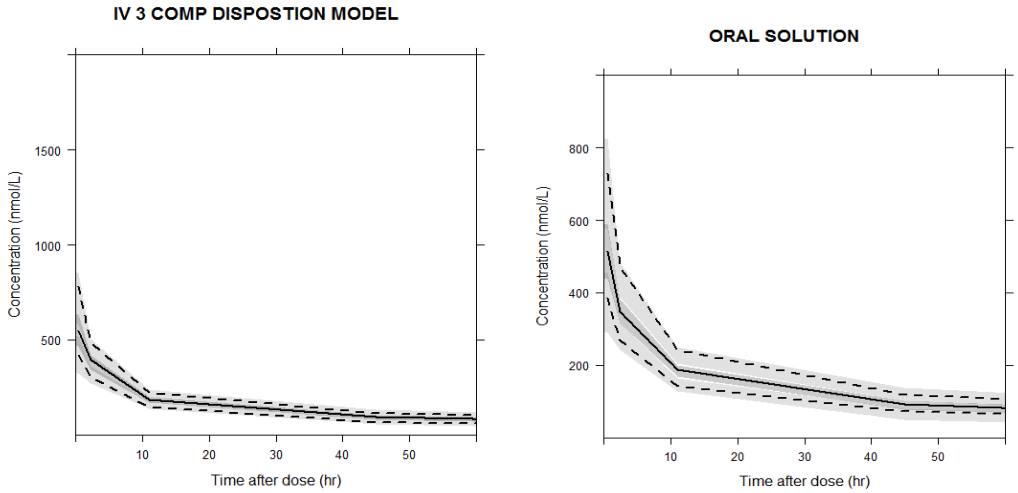


Figure A 4: Visual Predictive Checks for compound AZD242; Run 2 - IV model; Run 74 – oral solution;

1.3. Data for compound AZD1305

Study objective(s)	Study design	Subjects	Dosing regimen
ADME study	A Phase I, Open, Randomised, Single-Centre, Crossover	10 Healthy Male Volunteers 35 to 55years	Oral solution 180mg Iv infusion 70mg
Extended-release Formulations during fasting and fed condition.	A Phase I, Two-part, Randomised, Open, Single-Centre, Crossover Study	Healthy Male Volunteers 50 healthy male volunteers aged 20 to 45 years.	125 mg ER formulation and 125 mg oral solution
Single ascending oral and intravenous doses	A single-centre, single-blind, randomised, placebo-controlled, single-dose phase I study	30 healthy, male subjects Age 20 to 37 years	Oral solution of single ascending doses; 10 mg, 30 mg, 90 mg, 180 mg, 360 mg, 430 mg and 500 mg

Table A 3: AZD1305 phase 1 clinical trials included in the analysis

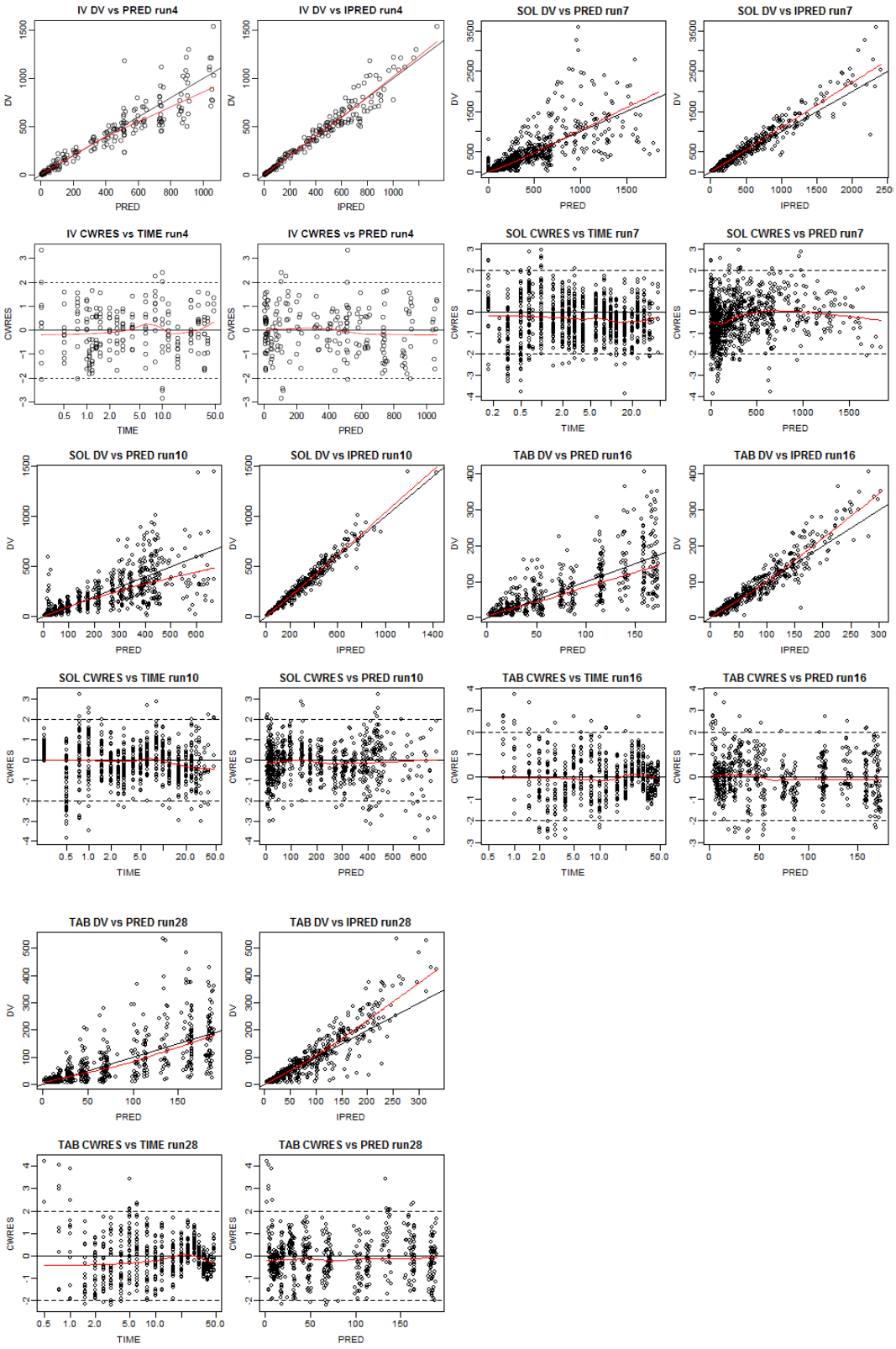


Figure A 5: Goodness of fit plots for compound azd1305; Run 4-IV model; Run 7 - oral solution fasted state; Run 10 -oral solution fed state; Run 16– ER in fasted state; Run 28– ER in fed state

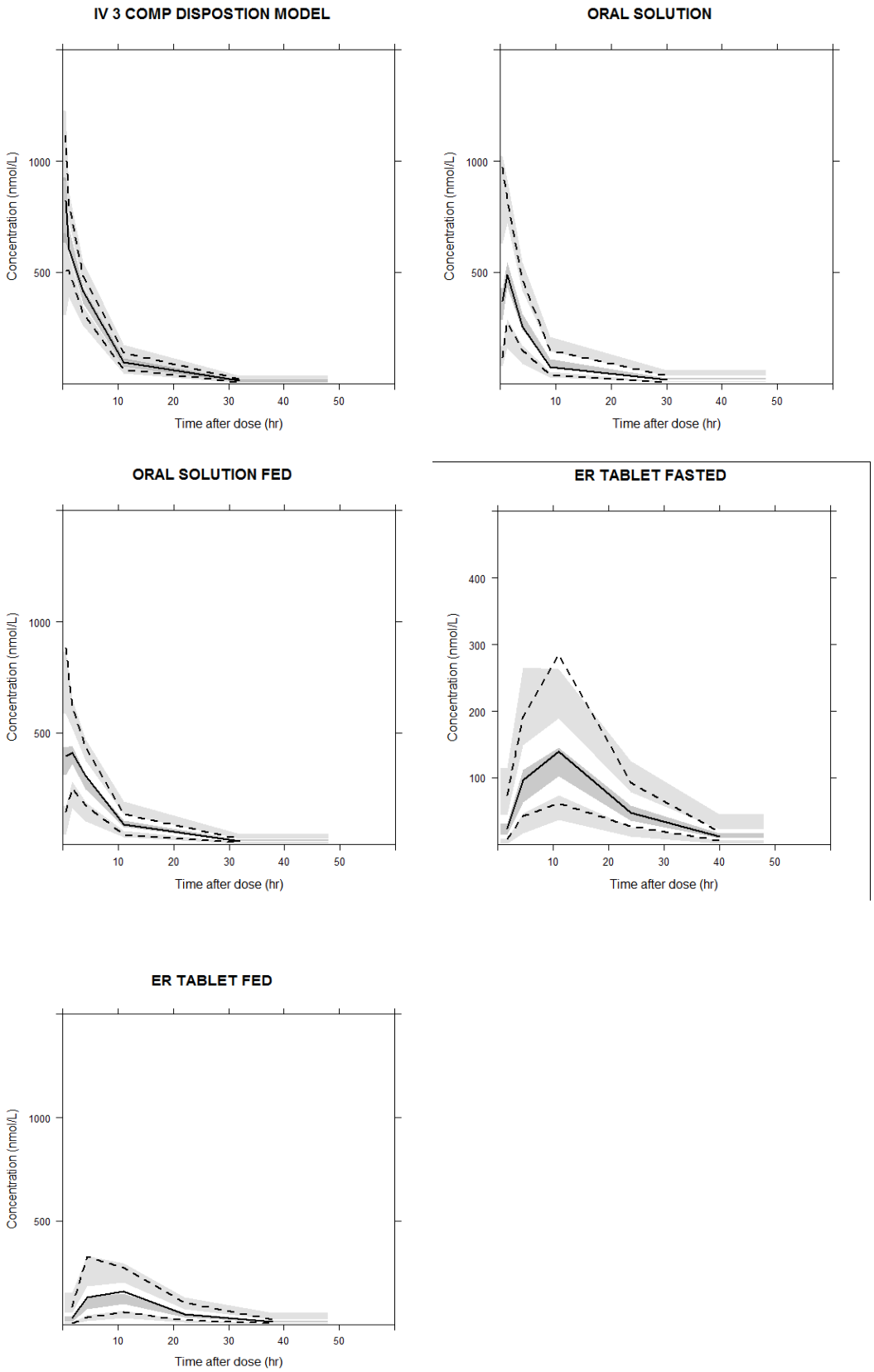


Figure A 6: Visual Predictive Checks for compound AZD1305; Run 4-IV model; Run 7 - oral solution fasted state; Run 10 - oral solution fed state; Run 16– ER in fasted state; Run 28– ER in fed state

1.4. Data for Compound AZD7009

Study objective(s)	Study design	Subjects	Dosing regimen
ADME study	A phase I, randomised, open, single-centre study	10 healthy male subjects aged between 35 and 55 years.	Single doses of oral solution (500mg) and iv infusion for 60 min (100mg)
Ascending single oral doses	A randomised, single-blind, placebo-controlled, single centre Phase I study	19 healthy, Japanese male subjects Age 20 to 40 years	Single oral solutions (50 – 600 mg)
Oral solution	A single-centre, single-blind, randomised, placebo-controlled	45 healthy male subjects aged between 20 and 40 years	Solution in escalating doses (5-1000 mg)
Prolonged-release formulations and an immediate-release formulation	A phase I, randomised, open, single-centre study	36 healthy male volunteers	250 mg prolonged-release formulations or as one immediate-release (IR) formulation.
Prolonged release formulations	An open, randomised, single-centre study (phase I)	30 healthy male subjects, aged between 20 and 45 years	Prolonged-release tablet, 250 mg

Table A 4: AZD7009 phase 1 clinical trials included in the analysis

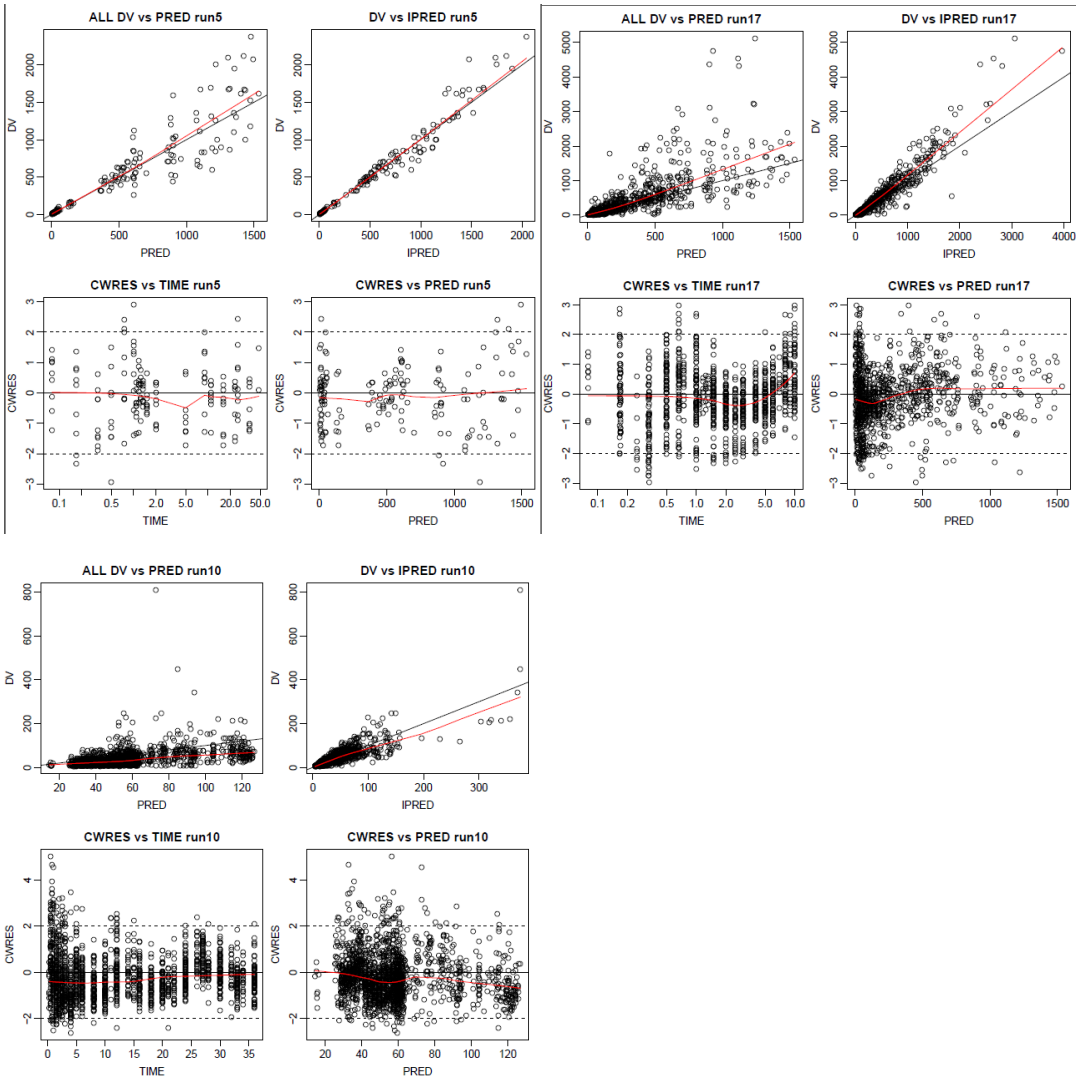


Figure A 7: Goodness of fit plots for compound AZD7009; Run 5- IV model; Run 17-oral solution; Run 10 – PR tablet

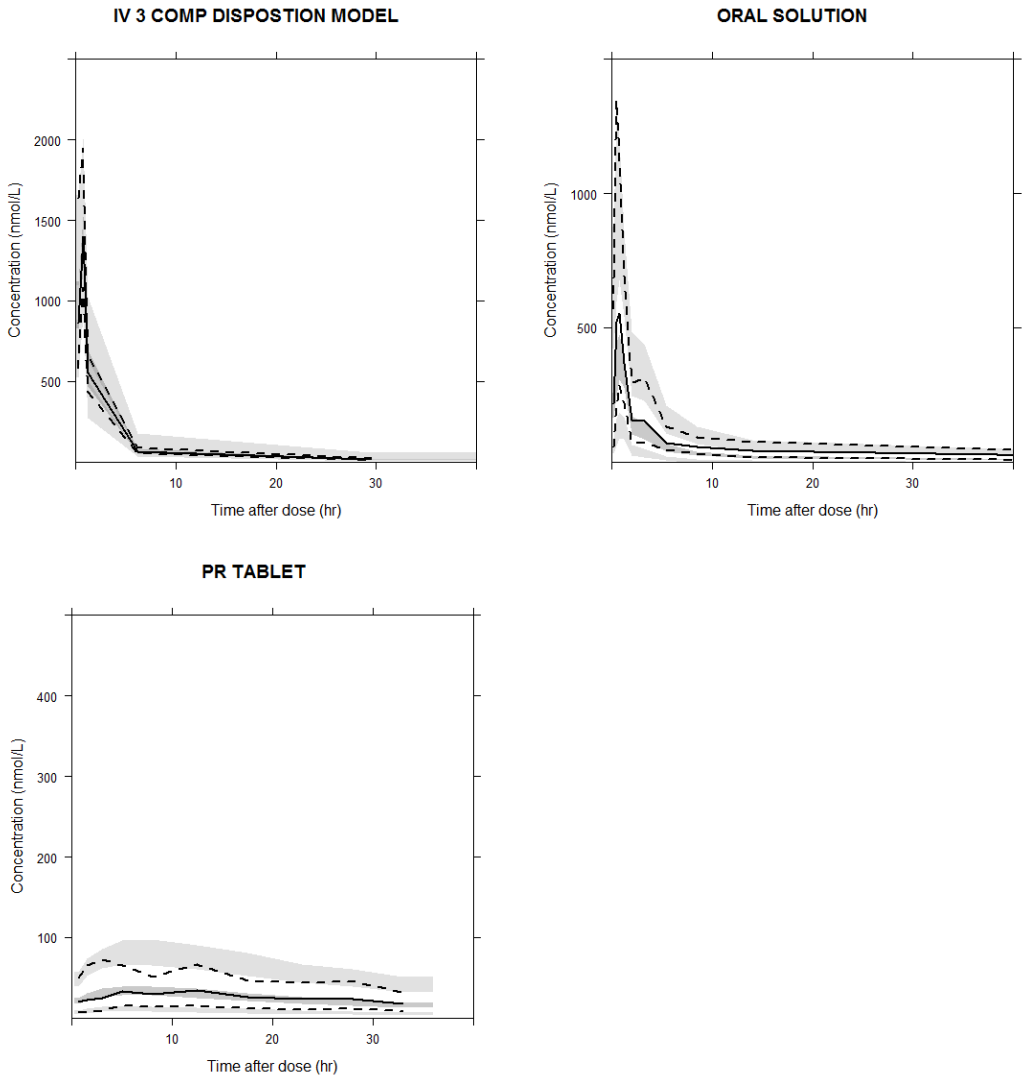


Figure A 8: Visual Predictive Checks for compound AZD7009; Run 5- IV model; Run 17-oral solution; Run 10 – PR tablet

```
$PROBLEM AZD0865 IV & sol
$INPUT ID IDORIG TIME AMT RATE WT DV EVID AGE HGT BMI SEX BOQ CMT DOSEID FORM
$DATA C:\course\PKFiles\PKDatafiles\Sarit\AZD0865_IV_PO_dataIV&SOLUTION_140110.csv IGNORE=@
$DATA IGNORE=(DV<0)
$SUBROUTINE  ADVAN5 TRANS1
$MODEL
 COMP = (DEPOT)
 COMP = (CENTRAL)
 COMP = (PERI1)
 COMP = (PERI2)
$PK
TVCLI = THETA(1) ; typical value of CLI
TVV   = THETA(2)
TVQ1  = THETA(3)
TVV1  = THETA(4)
TVQ2  = THETA(5)
TVV2  = THETA(6)
TVKA  = THETA(7)   ;absorption rate constant
TVFA  = THETA(8)
TVALAG1 =THETA(9)  ;lag time
;
CLI  = TVCLI*EXP(ETA(1)) ; individual value of CLI
V    = TVV*EXP(ETA(2))
Q1   = TVQ1*EXP(ETA(3))
V1   = TVV1*EXP(ETA(4))
Q2   = TVQ2*EXP(ETA(5))
V2   = TVV2*EXP(ETA(6))
KA   = TVKA*EXP(ETA(7))
FA   = 1/(1+EXP(-(TVFA+ETA(8))))
ALAG1 = TVALAG1*EXP(ETA(9))
;
LV  = 0.0512*WT**0.75          ; Price liver vol
BPR = 0.625 ; CB/CP=0.625         ; blood to plasma ratio
FQ  = 50.4*LV*BPR              ; Price liver blood flow males
CLR = 0.11*(WT/70)**0.75       ; renal cl in L/h normolized to mean weight
;
CLH = FQ*CLI/(FQ+CLI)
ER  = CLI/(FQ+CLI)
CL  = CLH + CLR
F1 = FA*(1-ER)
;
;RATE CONSTANTS
K12 = KA
K20 = CL/V
K23 = Q1/V
K32 = Q1/V1
K24 = Q2/V
K42 = Q2/V2
;
$ERROR
IPRED  = A(2)/V
Y      = IPRED + IPRED*EPS(1) + EPS(2)
```

```
;
$THETA  3.94711 FIX; 1. TVCLI
$THETA  8.5878 FIX; 2. TVV
$THETA  28.8593 FIX; 3. TVQ1
$THETA  9.34582 FIX; 4. TVV1
$THETA  0.437166 FIX ; 5. TVQ2
$THETA  9.65122 FIX; 6. TVV2
$THETA  (0,2.46362) ; 7. TVKA
$THETA  0.321801 ; 8. TVFA
$THETA  (0,0.19473) ; 9. LAG TIME
;
$OMEGA  0.0447735 FIX ;     1. CLI
$OMEGA  0.0747225 FIX ;       2. V
$OMEGA  0.142219 FIX ;      3. Q1
$OMEGA  0.0688518 FIX ;      4. V1
$OMEGA  0.203194 FIX ;      5. Q2
$OMEGA  0.50476 FIX ;      6. V2
$OMEGA  0.2  ;      7. KA
$OMEGA  0.1  ;      8. FA
$OMEGA  0.05  ;   9. ALAG1
;
$SIGMA  0.00667009  ; variance PROP res error
$SIGMA  104.923  ; additive residual error
;
$ESTIMATION METHOD=1 INTER MAXEVAL=9999 PRINT=1
$COVARIANCE MATRIX=R
;
$TABLE ID TIME IPRED CWRES EVID                      NOPRINT ONEHEADER FILE=sdtab91
$TABLE ID CL V Q1 V1 Q2 V2 KA FA ALAG1 ETA(1) ETA(2) ETA(3) ETA(4)
       ETA(5) ETA(6) ETA(7) ETA(8) ETA(9)            NOPRINT NOAPPEND ONEHEADER FILE=patab91
$TABLE ID AGE HGT BMI SEX BOQ CMT DOSEID FORM        NOPRINT NOAPPEND ONEHEADER FILE=cotab91
```

Figure A 9: NONMEM script

Publications

Following manuscript was published as a result from this thesis work:

1. Rabbie, Sarit; Flanagan, Talia; Martin, Paul and Basit, Abdul: "**Inter-subject variability in drug solubility"** International Journal of Pharmaceutics

Under preparation:

2. Rabbie, Sarit; Flanagan, Talia; Martin, Paul; Basit, Abdul and Standing, Joseph: "**Estimating the variability in fraction absorbed as a paradigm for informing formulation development in early clinical drug development"**

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