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Sex differences in the gastrointestinal tract of rodents and humans

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Declaration

This thesis describes the research conducted in the School of Pharmacy, University College London between 2015 and 2019 under the supervision of Professor 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 publications.

Signature: _____ Date: _____

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Abstract

The rat is one of the most commonly used animals in pre-clinical studies, however, there is a lack of knowledge on the potential sex differences in rat gastrointestinal (GI) tract physiology. Consequently, key sex differences towards the translation into humans in the drug development process may be obscured. This research project aimed to uncover potential sex differences in male and female rats by the characterisation of GI tract physiology. The evaluation of luminal fluids (pH, buffer capacity, osmolality and surface tension) and the membrane efflux transporter P-glycoprotein (P-gp) were conducted in two species of rats, namely the Wistar rat and the Sprague Dawley (SD) rat. P-gp expression was also characterised in male and female human intestinal tissue to assess its correlation against rats. No distinct sex differences were observed in the characterisation of luminal fluids in Wistar and SD rats. With respect to P-gp, however, male Wistar rats expressed a statistically higher ($p < 0.05$) level in the jejunum and ileum when compared to female Wistar rats. The involvement of food and the factor of time were also evaluated in subsequent investigations. In the fed-state, P-gp expression decreased in the male small intestinal segments of Wistar rats. In females, however, the reverse was observed where P-gp expression significantly increased when compared to the fasted-state which returned back to control levels after 4 hours. No sex differences in intestinal P-gp expression were found in the SD rat model. Ex vivo studies using ganciclovir (a P-gp substrate) showed significant sex-dependent effects on intestinal permeation albeit specific to only Wistar rats. In addition, unlike SD rats, the intestinal expression of P-gp in Wistar rats highly correlated to that in humans. Jejunal and ileal P-gp expression in human male subjects were statistically higher ($p < 0.05$) than human female subjects which was similarly identified in Wistar rats. This project is the first to report a distinct sex difference in P-gp expression in Wistar rats and humans. As such, the appropriate animal model should be considered during pre-clinical drug development, especially for the oral administration of P-gp substrates. This research project also highlights that the rat is not just a rat; distinct strain differences should be considered and specifically chosen for the optimisation of pre-clinical studies, and ease of translation into human subjects.

Impact Statement

In the pharmaceutical industry, rats are one of the most common animal models used in pre-clinical studies. Such animal models are used to elucidate drug performance and guide the translation of a drug in humans in early stage clinical trials. However, unlike in human clinical trials where the effect of drugs are assessed in both sexes, pre-clinical studies often impose a male-bias in its research. The findings in this project has demonstrated that the gap in our knowledge on sex differences may obscure key findings in early drug development, intended to guide and minimise potentially major side effects in both male and female subjects. The current study firstly identified a significant sex difference ($p < 0.05$) in duodenal buffer capacity and in the expression of the intestinal efflux membrane transporter P-glycoprotein (P-gp) in Wistar rats. With a focus on P-gp, subsequent studies revealed that both food consumption and the factor of time directly altered P-gp expression. As such, a sex difference in the intestinal permeation of the P-gp substrates ranitidine and ganciclovir were found via an Ussing chamber study. This study further explored P-gp expression in male and female Sprague Dawley (SD) rats which is another commonly used rat strain. Interestingly, the Wistar and SD rat are often interchangeable used in pre-clinical drug development. However, no significant sex differences were found in P-gp expression in the SD rat, although a higher stomach fluid value was found in male rats when compared to females. Moreover, this report is the first to identify a significant differential expression of P-gp in the male and female human small intestine ($p < 0.05$) which highly correlated to that of the Wistar rat. This thesis highlights i) a distinct sex difference in rat gastrointestinal (GI) properties and ii) the most commonly used rat strains are innately different in GI characteristics and advises that the Wistar rat is the superior rat model for the translation of P-gp drug substrates into humans. In summary, this work outlines that the rat displays a sex and strain difference in GI physiology. As such, the data herein should be taken into consideration for an optimised pre-clinical study design in both industry and academic research. This work further aims to encourage researchers to pursue investigations into sex differences and to contribute towards narrowing the knowledge gap between animal pre-clinical trials and first-in-humans.

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Abbreviations

GI tract	Gastrointestinal tract
P-gp	P-glycoprotein
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
CVD	Cardiovascular drug
MI	Myocardial infarction
ACEIs	Angiotensin-converting-enzyme inhibitors
GIST	Gastrointestinal stromal tumour
CML	Chronic myelogenous leukemia
ADME	Absorption, distribution, metabolism, and elimination process
CYPs	Cytochrome P450
GFR	Glomerular filtration rate
MS	Multiple sclerosis
OATP	Organic-anion-transporting polypeptide
PEPTs	Peptide transporter
OCT	Organic cation transporter
ENT	Equilibrative nucleoside transporter
ASBT	Apical sodium–bile acid transporter
MRPs	Multidrug resistance-associated protein
BCRP	Breast cancer resistance protein
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
HTA	Hydroxytyrosol acetate

ATP	Adenosine triphosphate
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
PCR	Reverse-transcription polymerase chain reaction
KBR	Krebs-Bicarbonate Ringer's solution
mRNA	Messenger ribonucleic acid
BSA	Bovine serum albumin
TBS-T	Tris-buffered saline with tween
IgG	Immunoglobulin G
SD rat	Sprague Dawley rat
CRL	Charles River laboratories
HAR	Harlan laboratories
SD	Standard deviation
AUC	Area under curve
TMD	Transmembrane domain
NBD	Nucleotide binding domain
MDR/mdr	Multi-drug resistance gene
BDDCS	Biopharmaceutical drug disposition classification system
P_{app}	Apparent permeability coefficients
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
NaCl	Sodium chloride
EDTA	Ethylenediaminetetraacetic acid
Na_3VO_4	Sodium orthovanadate
PMSF	Phenylmethylsulfonyl fluoride

MRM	Multiple reaction monitoring
Arg	Arginine
HSA	Human serum albumin
STDs	Standard peptide solutions
IS	Internal standard peptide solution
QCs	Quality control samples
RSD	Relative standard deviation
CCK	Cholecystokinin
GLP-1	Glucagon-like peptide-1
TEER	Tissue transepithelial electrical resistance
UGTs	UDP-glucuronosyltransferases
NaOH	Sodium hydroxide
HCl	Hydrogen chloride
$CaCl_2$	Calcium chloride
$MgCl_2$	Magnesium Chloride
$NaHCO_3$	Sodium bicarbonate
KH_2PO_4	Monopotassium phosphate
K_2HPO_4	Dipotassium phosphate
PBS	Phosphate-buffered saline
CO_2	Carbon dioxide
PXR	Pregnane X receptor
BBB	Blood brain barrier

CHAPTER 1:

Introduction

1.1 Overview

With the understanding of how different the sexes are in innate physiology (Freire et al., 2011), it is well recognized that men and women behave differently in clinical medicine treatment (Lind et al., 2017). For example, women have been found to be 1.5-1.7 times more likely to develop a drug side effect when compared with their male counterparts (Rademaker, 2001). Moreover, according to a five-year review study from 1997 to 2001, the U.S. General Accounting Office (GAO) reported that eight out of the ten drugs withdrawn from the market were due to greater risks of adverse effects in women (GAO, 2001). The main reason for the observed sex difference in drug performance was believed to be the sex difference in physiology features, such as metabolism (phase I and II) enzymes, transporter proteins, cardiac output as well as renal clearance rate, which contributed to differential drug pharmacokinetics between men and women (Soldin, 2009). As such, pharmaceutical research is required to retract from a 'one size fits all' approach and instead, evaluate the impact of sex-related drug effects via better understanding of the physiological differences between the sexes in the drug development.

However, to this day, early phase drug development traditionally fails to evaluate the differences between the sexes (Downing et al., 2014). Pre-clinical research has demonstrated a tendency to focus on males in both cell and animal studies, which may be obscuring key sex differences. In turn, this leads to a lack of evidence-based information for females in healthcare (Nature, 2010; Clayton et al., 2014). To reduce the male orientated bias in research, in December 2013, the European Commission demanded applicants of the research program, Horizons 2020, to include sex analyses in their projects (European Commission, 2016). Furthermore, the US National Institute of Health (NIH) requires applicants to incorporate a sex-balanced group of participants in the pre-clinical research (Downing et al., 2014; Clayton

et al., 2014). However, despite these multiple calls to action, no resounding guideline or standardisation practice have been proposed to consider the variations between male and female animals (Clayton et al., 2014). To date, publications often continue to neglect the need for sex-based evaluation in pre-clinical studies (Mogil et al., 2005; Beery et al., 2011). In order to advance pharmaceutical research, it is crucial to establish a better understanding of the innate differences between the sexes during the early drug development phase.

Laboratory animals are routinely used during pre-clinical research to act as intermediary models for the evaluation of potential compounds and to better guide drug development for human medication. The majority of oral medicines are tested pre-clinically on rats due to their inexpensiveness, ease of handling and, more importantly, due to their similarities to the human gastrointestinal (GI) tract (Hatton et al., 2015). Therefore, determining the physiological differences between male and female rats is of utmost importance. However, the information about their GI physiology, especially between the sexes, is not completely understood. In addition, the knowledge regarding the effect of external factors such as food consumption and circadian rhythm on GI physiology between sexes is also missing. Because drug bioavailability is impacted by a certain number of physicochemical factors such as GI fluid pH, buffer capacity, surface tension, osmolality, and P-glycoprotein (P-gp), a biological membrane efflux transporter expressed throughout the GI tract (Horter et al., 2001), the lack of knowledge on GI physiology between the sexes may have great consequences for oral drug delivery.

Given the above consideration, better understanding of the sex differences in GI tract of laboratory rats will benefit the drug design and formulation strategy during the early drug development and provide fundamental knowledge in sex differences research and

furthermore contribute the personalized medicine development.

1.2 Sex differences in clinical medicine treatment

1.2.1 Sex differences in drug performance

Even though it has been well-understood on a biological level that every cell contains sex-specific deoxyribonucleic acid (DNA) and contribute to our organ operation, metabolism works, consumption activity as well as cognitive function, the data generated from male subjects has been considered one-size-fits-all in biomedical research. In the 1970s, one of the world's longest running studies of age, Baltimore Longitudinal Study of Aging (BLAS), investigated more than 1000 men but zero women, even though women account for a large proportion of the elderly population (National Institute on Aging, 2019). Until 1993, the U.S Food and Drug Administration (FDA) issued the guideline "Guideline for the study and evaluation of gender differences in the clinical evaluation of drugs", which firstly introduced sex as a subgroup population and required that both sexes need to be represented in all phases of clinical trials to avoid sex differences in drug efficacy and side effect (Liu et al., 2016). Later on, in 2001, the Institute of Medicine published "Exploring the Biological Contributions to Human Health: Does Sex Matter?" and concluded that sex (being male or female) should be recognized as an important variable in research and increased knowledge in this area should be cultivated (Wizemann et al., 2001). Since then, the growth of sex-based biological knowledge has become a branch of science and led to the term-differentiation between the "sex" and "gender" (Pinn, 2003). "Sex" refers to the biological origin of male and female based on chromosomal differences, which determines the physiological processes and organs of the body beyond reproductive ability. "Gender" describes the self-representation, social, and cultural views of sex.

Nowadays, it is widely recognised that sex plays an important role in drug performance and result in a different clinical treatment between males and females. One example is cardiovascular drug (CVD) treatment (Rathore et al., 2002; Tamargo et al., 2017). A post hoc subgroup analysis including 6800 patients demonstrated that digoxin therapy was related to an increased risk of death among women and the absolute difference of digoxin related death was 5.8 between male and female patients. The potential reason was due to the lower volume distribution and clearance rate in female patients, which resulted in a higher serum digoxin concentration (Tamargo et al., 2017). Moreover, it has been reported that aspirin treatment lowered the risk of stroke but not of myocardial infarction (MI) or cardiovascular death in female subjects whereas aspirin reduced the risk of MI but not the risk of stroke in male subjects. In addition, it has been reported that women are less likely to adhere to statin treatment compared to their counterparts which is partially due to the higher risk of statin intolerance among women (Goldstein et al., 2016). Similar to statins, angiotensin-converting-enzyme inhibitors (ACEIs) have been found to display a higher risk of adverse effects in female subjects compared to that of in male subjects (Hudson et al., 2007), such as torsades de pointes (2–2.3-fold), skin diseases (up to 2-fold) and cough (2-fold). Apart from CVD, the sex difference in medicine performance can also be found in anti-cancer drugs (Nicolas et al., 2009; Anderson, 2008). Female patents with imatinib-resistant gastrointestinal stromal tumor (GIST) can suffer more adverse reactions from the sunitinib treatment compared to male subjects. In addition, sorafenib treatment for liver cancer achieved better complete remission among female patients but induced more side effects in the clinical practice as well. Imatinib, used for multiple cancers like chronic myelogenous leukemia (CML), was better tolerated and more effective in men than in women. Another study conducted by Seaber demonstrated that female patients experienced a higher bioavailability of zolmitriptan after 5mg or above oral administration than that of in male patients (Seaber et al., 1997). With the progress of the

sex-based research, an increasing number of sex differences had been observed in the clinical treatment and Table 1.1 is a summary of the sex difference observations.

Table 1.1 Summary of sex differences in clinical medicine treatment

Drugs	Results	References
Cardiovascular drugs		
Digoxin	The rate of death increased 4.2% from placebo to digoxin treatment in females whereas decreased 1.6% in males	Rathore et al., 2002
Aspirin	Aspirin treatment lowered the risk of stroke but not of myocardial infarction (MI) in females whereas reduced the risk of MI but not the risk of stroke in males	Tamargo et al., 2017
Statins	Greater risk of adverse reactions, lower tolerance and adherence in females	Goldstein et al., 2016
ACEIs	Higher risk torsades de pointes, 2–2.3-fold, skin diseases, up to 2-fold, and cough, 2-fold, among female patients	Hudson et al., 2007
Anticancer drugs		
Imatinib	Better tolerated and more effective in males than in females	Nicolas et al., 2009
Sunitinib	Induce more side effects in female patients	Anderson, 2008
Sorafenib	Achieved complete remission among female patients but accompanied with the higher rate of side effects	Li et al., 2015

Antiretroviral drugs		
Zidovudine	Incidence of adverse effects higher among female patients	Squires et al., 2000
Didanosine	Nearly 3-fold increase in the risk of an adverse event in females	Moore et al., 1996
nRTI-containing regimens	83% cases of lactic acidosis and 85% of the 20 fatal cases happened to female patients	Brinkman et al., 1999
Others		
Zolmitriptan	A significant higher bioavailability in female subjects with 5mg or above administration	Seaber et al., 1997
Cephradine	Slower rate of absorption and lower bioavailability in the female	Aichhorn et al., 2007
Naratriptan	Oral bioavailability being greater in women results in peak concentration is higher in women than men	Lutz et al., 2010
olanzapine	Significantly higher plasma levels in female	Johnson et al., 1997
clozapine	significantly higher plasma levels in female	Choi et al., 2007

1.2.2 Sex differences in Physiology

Clinical treatment for patients depends on the interaction between the administrated medicine and individual's physiological features. The reason for the observed sex differences in drug performance therefore is mainly due to the physiological differences between males and females, which results in differences in drug absorption, distribution, metabolism, and elimination (ADME), as shown in Figure 1.1.

For example, it was reported that the activity of gastric alcohol dehydrogenase was lower in women compared to men, which resulted in the increased bioavailability of ethanol after oral administration in women (Fletcher et al., 1994). In addition, as the different gastric emptying time between men and women under fed state, a shorter absorption time for an enteric coated aspirin was observed in women (Mojaverian et al., 1988). Apart from absorption, one cytochrome P450 (CYPs) catalyst of oxidative metabolism in human liver, CYP3A4, has been reported that displayed a higher expression of both protein and mRNA level in women compared to men (Wolbold et al., 2003; Diczfalusy et al., 2008). It provides potential explanation to the fact that cyclosporine (Kahan et al., 1986) and nifedipine (Krecic-Shepard et al., 2000) displayed a higher clearance rate in women. Moreover, because of the higher enzyme activity of CYP1A2, CYP2E1 and CYP2D6 in men's liver, the clearance rate of these enzyme substrates, including caffeine, olanzapine, clozapine, chlorzoxazone, propranolol as well as metoprolol are higher in men (Cesena et al., 2007; Franconi et al., 2007); as a result women are more likely to suffer side effects of these drugs when taking the same dosage. Futhermore, the higher renal blood flow and glomerular filtration rate (GFR) in men also attributes to quicker clearance of drug that are actively eliminated via the kidney like methotrexate and gabapentin compared to that of in women (Soldin et al., 2009; Anderson, 2008).

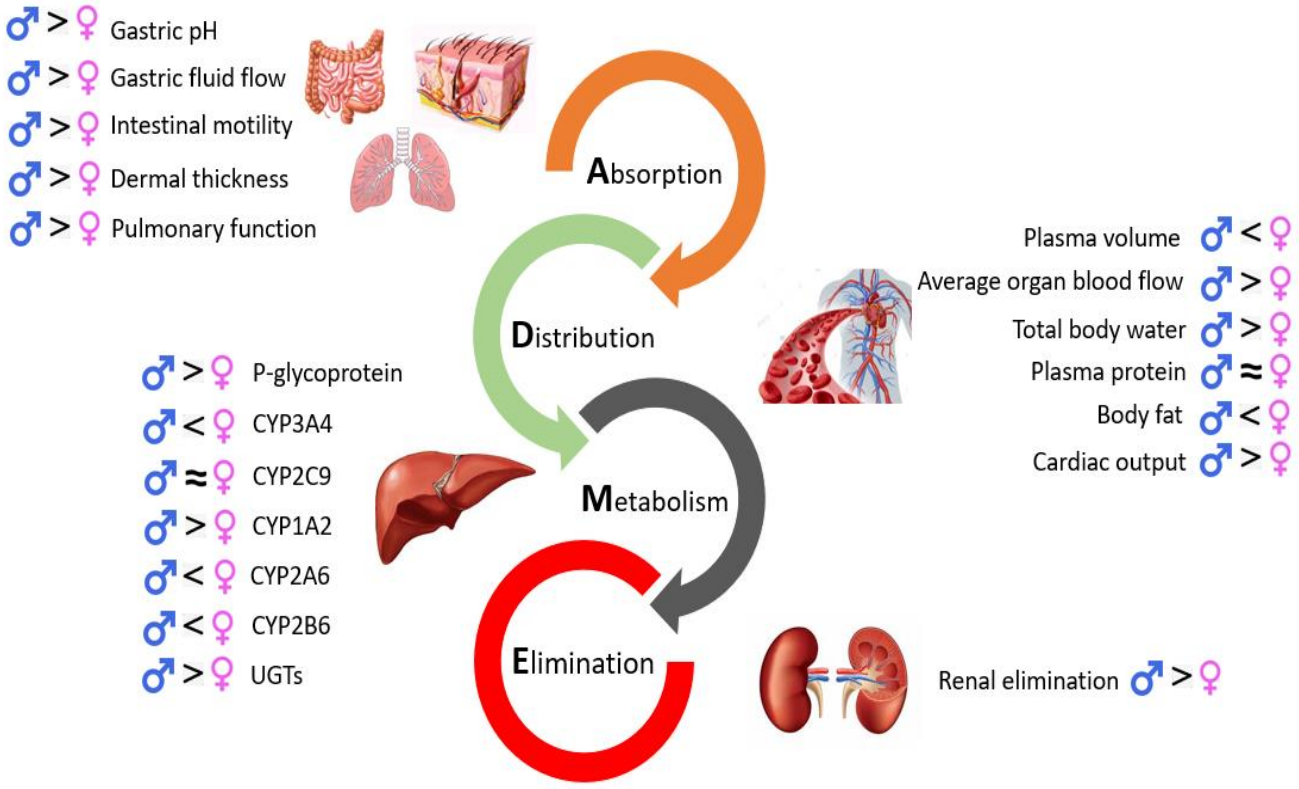


Figure 1.1 Sex differences in physiological features which influence drug absorption, distribution, metabolism and elimination (Soldin et al., 2009; Soldin et al., 2011)

1.3 Pre-clinical drug development and sex difference research

1.3.1 Drug development process and pre-clinical studies

Drug development is a time-consuming and costly process involving multiple steps as shown in Figure 1.2. It has been estimated that the average time for a novel compound to reach the market is normally 12 years with the cost exceeding US $1 billion (Ng, 2015). Among the multiple steps, the pre-clinical stage is of vital importance as it's the final process before new compounds move into clinical trials and into human. Based on both in vitro and in vivo pre-clinical experiments using laboratory animals, researchers can understand the pharmacokinetics, pharmacodynamics, and toxicology properties of the compound. Pharmacodynamics normally provides the information about the dose causing toxicity and the dose eliciting a therapeutic effect, which establishing the therapeutic index of a compound.

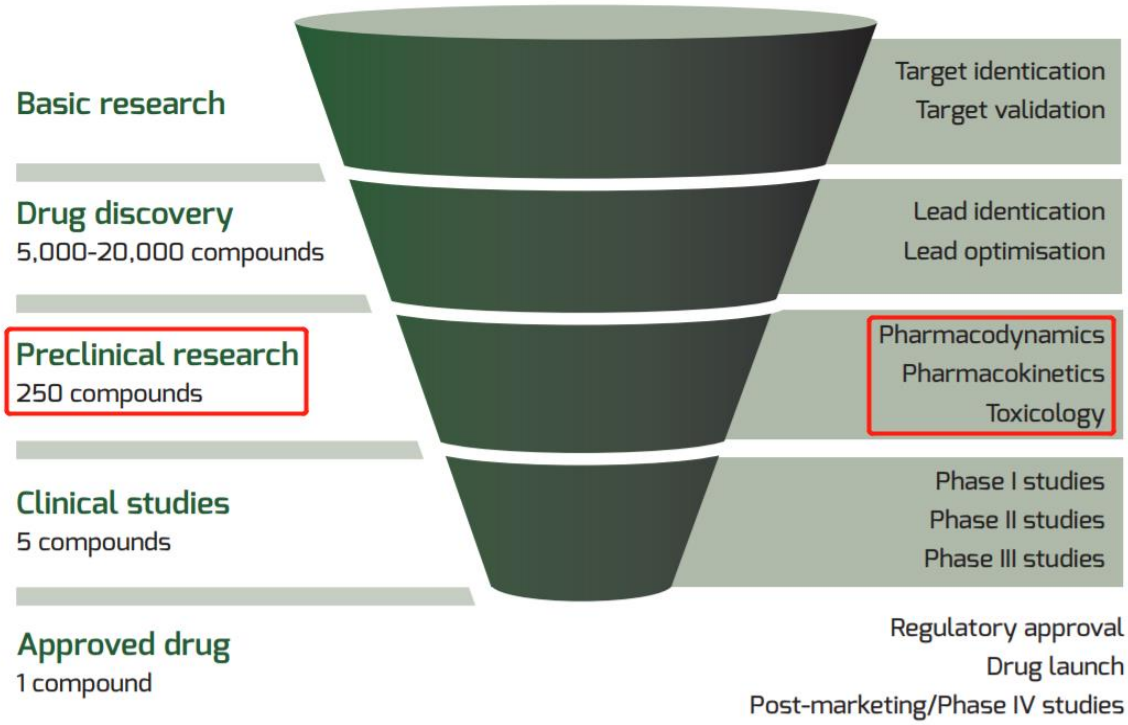


Figure 1.2 The process of a new drug development from basic research to final approval, adapted from Honek, 2017.

Researchers are able to evaluate how potent and efficacious the drug is with the consideration of its desired pharmacological effect including safety and adverse reactions. For pharmacokinetics study, it describes the change of plasma concentration of the compound with time as a result of ADME, which is critical for establishing dose range and administration schedule in the subsequent phases of the clinical trial (Faqi, 2013). The safety issue regarding a new compound will be assessed by the preclinical toxicology study. The study is conducted to determine a suitable and safe starting dose for the human testing and identify the potential biomarkers for adverse reactions monitoring in further steps.

With the above evidence for the compound's biological effect, researchers will know its dosing and toxicity levels which are important to determine whether it is justified and

reasonably safe to proceed with clinical studies (FDA, 2017). All pre-clinical studies are required to comply with the guidelines dictated by Good Laboratory Practice to ensure reliable results and the generated data will be checked by authorities such as the FDA as well (FDA, 2017). Therefore, pre-clinical studies are vital to protect the human subjects in the clinical trials and can be translated into applicability in the further clinical trials, which can facilitate the clinical study completion and contribute to the final authorities approving of the new drug candidate.

1.3.2 The presence of 'sex' in animal pre-clinical research

When Erasistratus (304-258 BC) first performed experiments on living animals, the story of laboratory non-human creatures was started (Cohen and Loew, 1984). In pre-clinical research, the commonly used species include small rodents (rat, mouse, guinea pig and rabbit) and larger mammals (dog, monkey, pig and non-human primate) (Hatton et al., 2015). To this day, laboratory animal ranging from zebrafish to non-human primates plays a vital and indispensable role in the development of human medical science with more than 100 million of laboratory animals used in experiments annually (Meigs et al., 2018). The number for UK in 2011 was over three million which mainly included mice (71%), fish (15%), rats (7%) and birds (4%) (Home office, 2012).

Today, because of sex-related research and regulatory requirements in drug clinical studies, the knowledge of the role of sex in medicine clinical treatment has been well-accumulated and the awareness of the importance of sex differences in clinical treatment has been built-up among researchers. However, in the pre-clinical study, there has not been a corresponding revolution and a significant sex bias exists in the animal experiments as researchers continue to neglect sex-based considerations and analyses in preclinical studies (Beery et al., 2011).

According to Figure 1.3, the number of both sexes-included articles in human study rapidly increased after 1989 and consisted of over 60% among the study, whereas male-based articles dominated the articles in the animal study (Beery et al., 2011). Moreover, a survey of approximately 2000 animal studies conducted by Irving and Annaliese found that male bias was pronounced in pharmacology (5 males to 1 female) and physiology (3.7 males to 1 female) study (Zucker et al, 2010). More recently, a systematic assessment of leading cardiovascular journals from 2006 to 2016 revealed that sex bias was increasingly prevalent in pre-clinical cardiovascular research (Ramirez et al., 2017). Full articles with the original in vivo data from laboratory animals in cardiovascular-related research have been investigated. Among these 3396 articles, 20% studies did not specify the sex of the animals they used. In the rest studies with reported animal sex, 71.6% articles were exclusively used male subjects while 12.9% studies used female and 15.5% studies used both sexes. Moreover, the percentage of male-only studies increased from approximately 55% in 2006 to 60% in 2016. Another recent study reported the sex-bias in pre-clinical research on age-related hearing loss (Villavisanis et al., 2018). In the study, 231 relevant articles from 2006 to 2015 were investigated and only two thirds of articles stated the sex of animal. The sex of animal reported in the papers 67% were males and 33% were females, and only 15% of investigated articles discussed sex-based results.

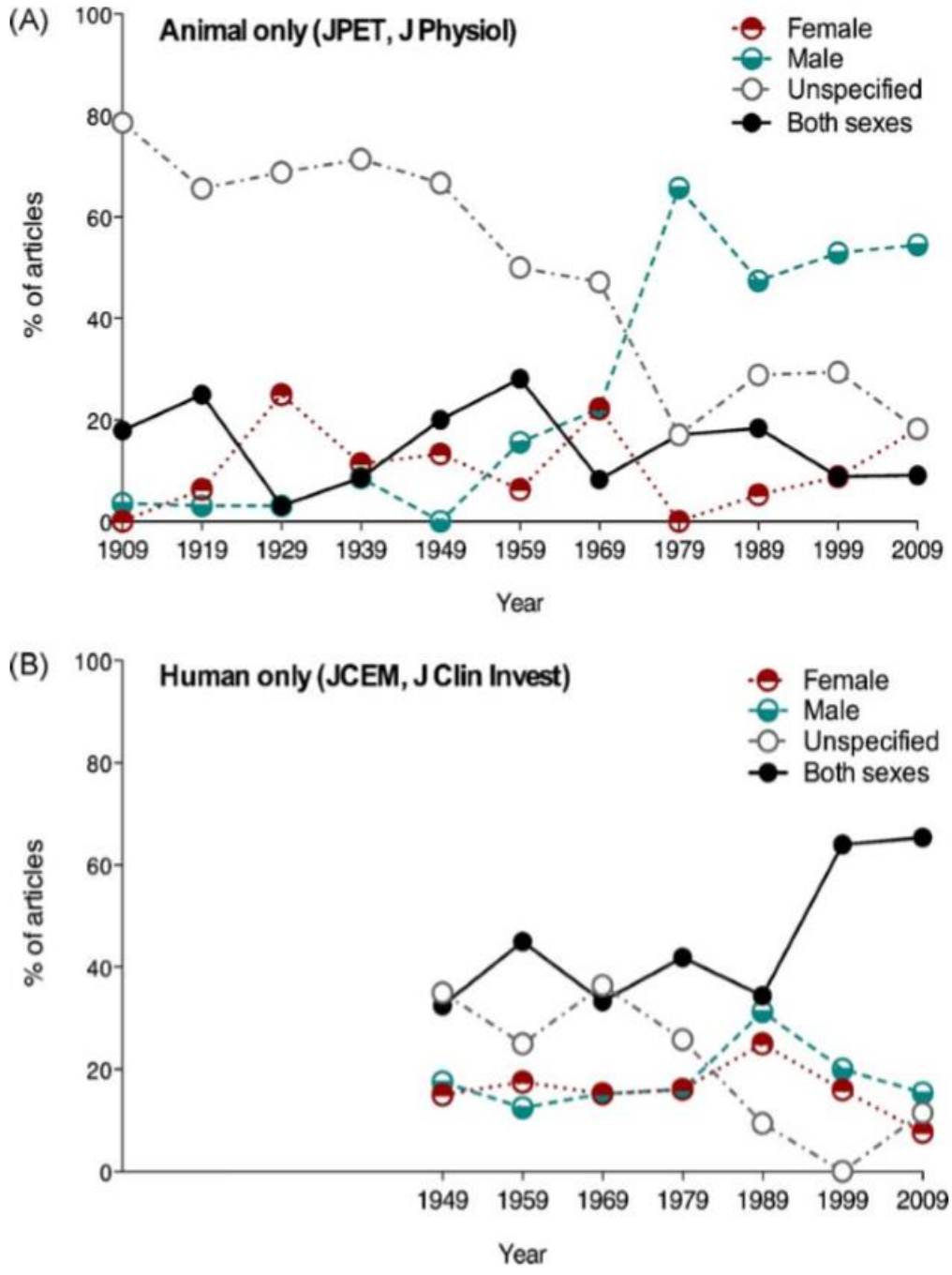


Figure 1.3 The change of sex involvement in animal and human literatures over years, adapted from Beery et al., 2011. (A) Combined data from two journals publishing primarily non-human animal research: JPET and J Physiol. Human studies were excluded from consideration for this graph. (B) Combined data from two clinical journals: JCEM and J Clin Invest.

Considering the extensive use of laboratory animals and the importance of pre-clinical studies,

the over-reliance on male animals will lead to a serious lack of female information in pre-clinical database. As a result, the obscured sex-related issues such as over-effect, less-effect or other unexpected effects may affect the results of the clinical trials and even worse, cause health impairment on the participants. What makes it even worse, women were reported to experience higher rates of adverse drug reactions than men do in clinical practice (Franconi et al., 2007). Therefore, it is important to involve both sexes in pre-clinical research and it has been demonstrated that the participation of female animals can be effective in bridging the gap between animal and human work. One example was multiple sclerosis (MS). The sex difference in MS, which women are more susceptible to MS but less rate of severe forms than men, was elaborated by the MS rodent model with both sexes. According to Voskuhl et al and Wisdom et al studies, both reproductive and nonreproductive factors were responsible for the sex differences and the successful oestrogen therapy on the rodent model resulted in the use of an oestrogenic ligand as a candidate neuro protective agent for MS (Voskuhl et al., 2001; Wisdom et al., 2013). In addition, the better understanding of the animal sex-related physiological features may contribute to the treatment of neurological disease. It was demonstrated that mice with XY chromosomes in the central nervous system showed greater neurodegeneration compared to those with XX chromosomes, which linked the inherited effects to imprinting of genes on sex and non-sex chromosomes (autosomes) and resulted in a new research direction for the sex-skewed neurological conditions, including Parkinson's disease, schizophrenia and stroke (Du et al., 2014). In light of the significance of sex involvement in animal research, multiple calls to action have been made in recent years including the European Commission Horizons 2020 and the US NIH (Downing et al., 2014; Clayton et al., 2014; European Commission, 2016).

1.4 Sex differences in the gastrointestinal tract system

As a nutrition absorptive system, the gastrointestinal tract is comprised of the stomach and intestines. The stomach is a digestive organ which can secrete digestive enzymes and gastric acids. For the intestine, it can be further divided into small intestine (including duodenum, jejunum, and ileum) and large intestine. The small intestine is the main absorption site for the intake substances such as food and drug, whereas the large intestine mainly contributes to water re-absorption and excretion of waste materials. Figure 1.4 was the schematic for the gastrointestinal tract of rodent versus human. Because of such important absorption function, small intestine shared 70%~80% of total gut length in both human and rodent (Hatton et al., 2015). In addition, stomach pH value is normally lower than intestine due to its initial digestion and sterilization in both human and rodents (Evans et al., 1988; Afonso-Pereira et al., 2018; Christfort et al., 2018). It was reported in human that the range of stomach pH was 0.4~4 while the intestine pH was start at around 5 in duodenum and gradually increased to approximately 7.5 in ileum and then kept in the range of 6.4~7 in colon (Evans et al., 1988). Moreover, due to the different function, the mucus layers are also varied from stomach to intestine (Johansson et al., 2013). For both stomach and large intestine, which absorption is not the main function, there are two mucus layers, outer unattached layer and inner attached layer. In terms of small intestine, due to absorption activity, the mucus layer is single unattached layer which can benefit the absorption process.

Given the complexity and importance of GI system, the sex variations on the system such as luminal environment as well as membrane transporters are needed to be considered in oral drug development.

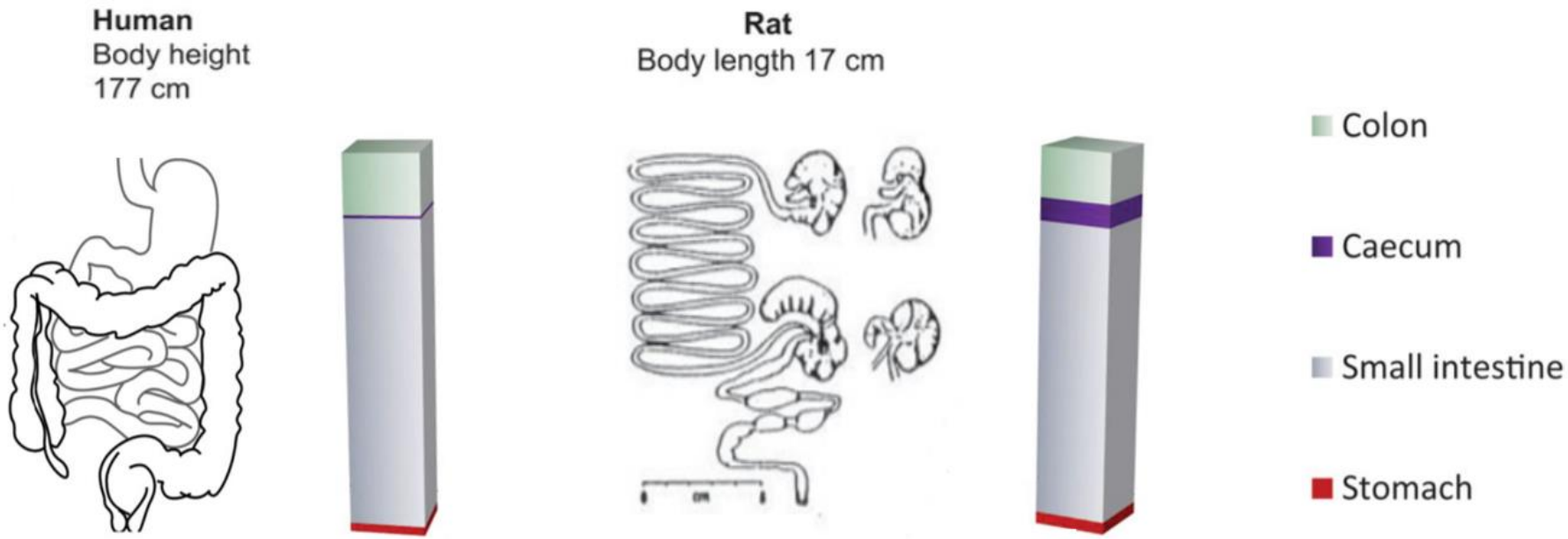


Figure 1.4 Schematic for the gastrointestinal tract of rodent versus human and the proportion of the total gastrointestinal length represented by the stomach, small intestine, cecum, and colon, adapted from Hatton et al., 2015

1.4.1 Luminal environment

After oral administration, the drug substance comes into contact with the luminal environment and is absorbed into the blood circulation via the following processes: disintegration, dissolution, diffusion and membrane permeation (Schanker, 1971). The sex-related variation on physicochemical properties of the luminal environment, therefore, is a necessary consideration in clinical studies. A study involving 113 women and 252 men demonstrated that women under fasted state displayed a higher gastric pH (2.79 ± 0.18) compared to their counterparts (2.16 ± 0.09) and the reason was partially due to the fact that the basal acid output in men was almost twice as high in men than in women, 4.0 ± 0.2 and 2.1 ± 0.2 mmol/h respectively (Feldman and Barnett, 1991). In terms of small intestine pH, no sex variation was found in the duodenum and jejunum according to the human studies with a small amount of experiment subjects (Lindahl et al., 1997; Perez et al., 2006). However, the basal secretion of mucosal bicarbonate in duodenum was significantly higher in women than in men, 189.5 ± 23.5 vs. 120.7 ± 16.2 mol/cm h, respectively (Tuo et al., 2008). In addition, a sex-related differences in faecal pH was reported in the study with 26 subjects, 15 men and 11 women. In the study, under a controlled diet, there was a significant sex difference where women faecal pH was 7.18 ± 0.08 and men faecal pH was 6.51 ± 0.07 (Stephen et al., 1986). Apart from the luminal pH, the fluids volume in the gut is another critical concern. A study conducted by Gotch et al measured the fluid volume of stomach, small intestine and proximal colon from *post-mortem* subjects (8 males and 5 females). It was found that male subjects possessed more volume of fluids in stomach and small intestine than female subjects after the body mass normalization (Gotch et al., 1957). More recently. a similar conclusion was also drawn from the Bouras et al study, which demonstrated that post-prandial gastric volumes were much higher in men than women (Bouras et al., 2002). The luminal content can directly contact and interact with drug substance and therefore affect the

absorption process as well. Hence, there were several investigations on the composition of luminal content between sexes. One study evaluated the osmolality and ionic strength of the luminal fluids and found that both stomach and jejunum showed no sex difference (Lindahl et al., 1997). One well-documented sex difference was luminal bile acids according to the previous studies. It has been demonstrated that the ratio of primary to secondary bile acids in the gallbladder was lower in young adult women than in men and the composition of the bile changed during the menstrual cycle and pregnancy (Fisher et al., 1973; McMichael et al., 1980). For the other sex-related investigations, faecal materials have been the commonly used subject of sex-related studies because of its quantifiable and easy-to-access properties. One observed sex difference was the gut microbiota. Women appeared to have significantly higher levels of faecal bifidobacterial than men whereas the faecal concentration of *Bacteroides prevotella* was significantly higher in males than in female subjects (Whelan et al., 2009; Mueller et al., 2006). Considering the complexity and difficult-operation of human studies, especially for the characterization of distal small intestine and proximal colon, the corresponding experiment in pre-clinical studies, as aforementioned, might help understanding the potential sex differences in luminal environment and bridging the pre-clinical and clinical data in oral drug development.

However, there was less known about the sex-related luminal psychology among the pre-clinical animals. Due to its inexpensiveness, ease of handling and 90% similarity to human being at genetic level, rats have been widely used in pre-clinical studies for more than 200 years (Shanks et al., 2009). More interestingly, a recent study reported that the ranitidine bioavailability displayed a sex-related change in rats under PEG 400 co-administration, which had been observed in human studies previously (Afonso-Pereira et al., 2016). In addition, it has been demonstrated that certain drugs including ivermectin, Rho 123 and

verapamil experienced sex-related intestinal permeation in rats (Mariana et al., 2011; Oltra-Noguera et al., 2015).

As contrasted to the extensive application and observed sex-related drug behaviour, the literature source of rat's sex-related luminal physiology was limited. One latest study published in 2017 from our group characterized the luminal environment between sexes (Afonso-Pereira et al., 2018). It was reported that female rats under fed state displayed a lower stomach pH than male rats. Moreover, the buffer capacity of luminal fluid in caecum and colon was higher in female rats, while male rats exhibited higher osmolality in duodenum, ileum and colon. Another newly released study investigated the gastric mucus thickness and accumulation rate among fasting rats. It was demonstrated that the blood flow in the gastric corpus mucosa was approximately twice as high in male rats compared to females and the permeability of the gastric mucosa performed a higher level in females than in males after taurocholate experiment (Shore et al., 2017). Table 1.2 summarised the current knowledge of sex differences in luminal environment in both human and rats.

Table 1.2 The summary of sex differences in luminal environment properties in human and rat.

	Human	Rat
Stomach		
pH	Fasting [1]: *M* 2.16 ± 0.09 *F* 2.79 ± 0.18	Fed [10]: Wistar rat *M* 4.6 *F* 3.6
Basal acid output (mmol/h)	Fasting [1]: *M* 4.0 ± 0.2 *F* 2.1 ± 0.2	
Fluid volume	Post-mortem (ml/kg) [2]: *M* 2.3 ± 1.5 *F* 1.4 ± 1.4 Fed (ml) [3]: *M* 744 ± 20 *F* 675 ± 14	
Osmolality (mOsm/kg)	Fasting [4]: *M* 195± 41.0 *F* 186 ± 30.4	
Ionic strength	Fasting [4]: *M* 0.100± 0.027 *F* 0.101 ± 0.023	
Mucosa blood flow (mL/min•g)		Fasting [11]: Sprague Dawley rats *M* 1.12 ± 0.12 *F* 0.51 ± 0.03
Duodenum		
pH	Fasting [5]: *M* 6.75 ± 0.63 *F* 7.16 ± 0.29	
Mucosal bicarbonate secretion (mol/cm h)	Unknown [6]: *M* 120.7 ± 16.2 *F* 189.5 ± 23.5	
Fluid volume (ml/kg)	Post-mortem [2]: *M* 4.2 ± 1.6 *F* 2.2 ± 1.3	
Jejunum		
pH	Fasting [4]: *M* 7.2 ± 0.56 *F* 7.1 ± 0.68	

Osmolality (mOsm/kg)	Fasting [4]: *M* 270± 11 *F* 271 ± 19.1	
Ionic strength	Fasting [4]: *M* 0.140± 0.011 *F* 0.137 ± 0.017	
Caecum		
Buffer capacity (mmol·$L^{-1}\Delta pH^{-1}$)		Fed [10]: Wistar rat *M* 39 *F* 83 ± 29
Colon		
Faecal pH	Fed [7]: *M* 7.18 ± 0.08 *F* 6.51 ± 0.07	
Fluid volume (ml/kg)	Post-mortem [2]: *M* 1.3 ± 2.1 *F* 1.4 ± 1.3	
Faecal bifidobacterial (log10 g−1)	Fed [8]: *M* 8.6 ± 1.1 *F* 9.2 ± 0.7	
Bacteroides prevotella	Fed [9]: *M* > *F* （P=0.036）	
Buffer capacity (mmol·$L^{-1}\Delta pH^{-1}$)		Fed [10]: Wistar rat *M* 42 ± 6 *F* 78 ± 3

- M means data male subjects and F stands for female subjects

- [1] Feldman and Barnett (1991); [2] Gotch et al. (1957); [3] Bouras et al. (2002); [4] Lindhal et al. (1997); [5] Perez et al. (2006); [6] Tuo et al. (2008); [7] Stephen et al. (1986); [8] Whelan et al. (2009); [9] Mueller et al. (2006); [10] Afonso-Pereira et al. (2018); [11] Shore et al. (2017).

1.4.2 Membrane transporters

Drug intestinal permeation is the last but the vital step in drug absorption process. After disintegration and dissolution, the drug substance finally reaches and crosses the GI mucosal membrane to get into the circulation. As shown in Figure 1.5, the permeation process involves many membrane transporters located either on the apical side of the membrane or the basal side of the membrane. Based on the function, these transporters can be classified into two groups: uptake transporters and efflux transporters. The uptake transporters including OATP, PEPT1, PEPT2, OCT1, ENT1, ENT2, ASBT, function as absorption assistance transferring the drug substance from luminal side to the blood side and helping absorption. On the contrary, efflux transporters (P-gp, MRPs and BCRP) perform as protective epithelium guard pumping any potential toxic or allogeneic substance out of intestinal cells and limiting the absorption (Estudante et al., 2013). Different transporters target certain range of substances known as their substrates. For example, OATP uptakes the chemicals contained steroidal or peptide structural backbones and/or are anionic, OCT prefers the hydrophilic organic cation with relatively low molecular weight, such as cimetidine and ranitidine, peptide bonds and free terminal carboxyl groups are normally the substrates of PEPTs (Estudante et al., 2013; Klaassen et al., 2010). In terms of efflux transporters, P-gp, as the first identified and well-documented transporter, can efflux an extensively wide range of substrates outside of enterocytes and has been regard as the major efflux pump conferring multidrug resistance in human tissues. FDA highlighted its importance by suggesting the P-gp assessment on the potential drug candidate on both pre-clinical and clinical levels (FDA, 2017). As a result, the sex variation on these membrane transporters, in particular P-gp, need to be considered during the drug development.

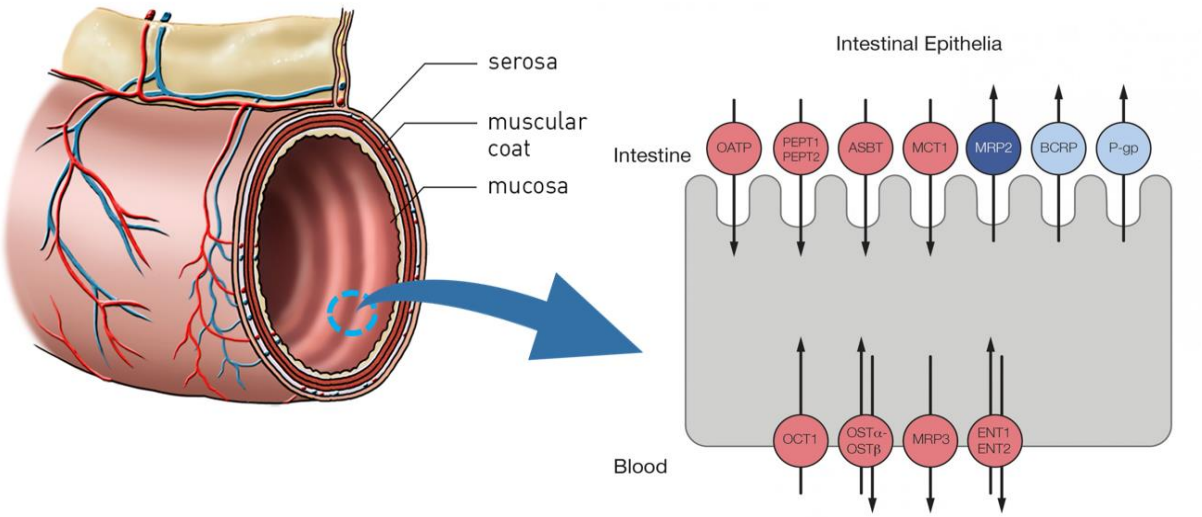


Figure 1.5 Membrane transporters which can modulate the absorption of drug substances.

A study conducted by Mouly et al in 2003 investigated the P-gp expression level in duodenum, jejunum and ileum part of intestine tissues obtained from four people and revealed that there was an increasing trend of P-gp expression from proximal intestine to distal intestine but no mention about sex difference (Mouly et al., 2003). The reason for the missing discussion about sex difference may because the numbers of people in this study was too small to analyse the sex factor. Later on, by re-analysing the data from Schuetz et al work (Schuetz, et al., 1995), Potter et al mentioned in their work that P-gp quantified in 25 people intestine samples showed higher value in males group compared to that of females group. This work proved that there is a sex difference in intestinal P-gp but it was unclear about the specific part of intestine because there was no mention about the intestine samples type. And then, in a short time, two studies were published and claimed that there was no sex difference in P-gp intestinal expression. In one study, Paine and her team investigated the P-gp expression in proximal intestine (duodenum) between men and women from ninety-three intestine biopsy samples including forty-five from females. The results revealed that there was no significantly sex difference as the mean relative P-gp expression was 0.66 in male

subjects and 0.73 in female samples (Paine et al., 2005). Another study explored the P-gp expression in stomach, jejunum and ileum intestine (Canaparo et al., 2007). The tissue samples of stomach were obtained from three men and two women with an average age at 73, jejunum samples were from five men and two women with age of 64 and for ileum samples, there were six men and two women donors with an average age of 69. In all three parts of intestine, there was no sex difference in P-gp expression. However, the samples involved in this study were too small to obtain a convincing answer about the sex difference in intestinal P-gp. In a nutshell, although Paine's work confirmed that there was no sex difference in proximal intestine, there was still demand for a reliable investigation on P-gp expression along the intestinal tract between men and women. A summary of the sex difference studies in P-gp intestinal expression was listed in Table 1.3.

In pre-clinical study, rats are commonly used as intestinal animal model for the drug permeation study and exhibited a high correlation (R^2=0.7) with human in drug intestinal permeation as shown in Figure 1.6. In the study, the permeabilities of 17 drugs were evaluated in both rat and human jejunum (Cao et al., 2006).

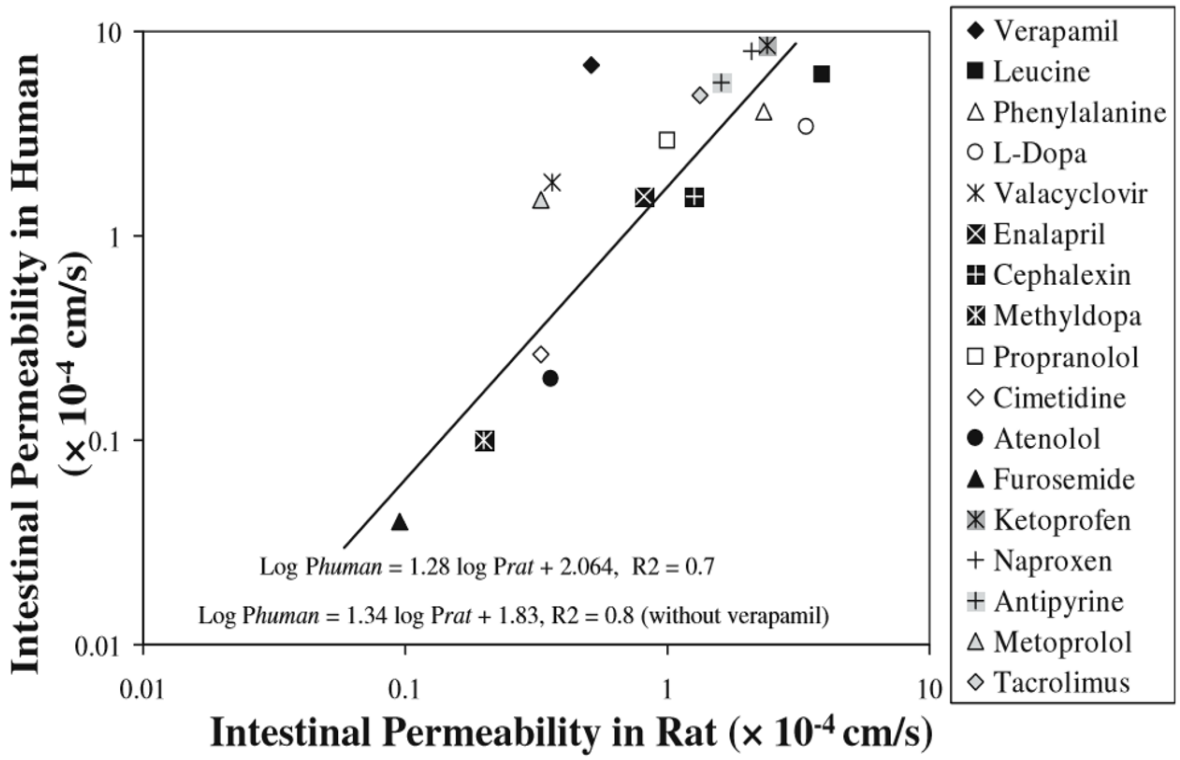


Figure 1.6 The intestinal permeability of 17 drugs in rat and human jejunum, adapted from Cao et al., 2006.

There are several studies reported that P-gp substrate performed sex difference in rat intestinal permeation. A recent study conducted by Mariana et al demonstrated that the accumulation rate of ivermectin, a P-gp substrate, in everted guts sacs (jejunum) was 4.16+0.31 nmol/min/g in female rats and 2.88+0.21 nmol/min/g in male rats (Mariana et al., 2011). However, similar to the luminal environment research, the study concerning the sex difference in P-gp expression is missing. There was only one published work investigated the sex difference in P-gp which was conducted by MacLean et al in 2008 (MacLean et al., 2008). In the study, it was demonstrated that there was a similar increasing trend on P-gp expression to that of humans but no sex-related variation along the intestinal tract.

Table 1.3 The summary of sex difference in P-gp expression in both human and rat studies

Species	Tissues	Sex differences in P-glycoprotein expression	References
Human	Duodenum, jejunum, ileum from 4 individuals	P-gp expression increased from proximal to distal regions, no sex difference claimed in the research	Mouly et al., 2003
Human	Stomach, jejunum, ileum tissues from 20 individuals	P-gp expression were similar in stomach, jejunum and ileum tissues and there was no sex difference in P-gp expression among these tissues	Canaparo et al., 2007
Human	Tissues from 25 patient's intestinal biopsy	P-gp expression level in control female group lower than controlled male group.	Kenneth et al., 1997 Potter et al., 2004
Human	Duodenal biopsy tissues from 45 females and 46 males	There was no sex difference in P-gp expression in proximal intestinal tissues between males and females	Paine et al., 2005
Human	Gastric, duodenal, colonic and rectal tissues from 27 individuals including 15 females	MDR1 mRNA was checked and results showed an increasing trend from stomach and duodenum to colon. No claim the sex difference in terms of MDR1 mRNA expression	Thorn et al., 2005
Rat	Duodenum, jejunum, ileum, colon tissues (n=5)	In male rats, P-gp expression increased from duodenum to colon tissues. There was no sex difference in P-pg expression along intestinal track.	MacLean et al., 2008

1.5 Rationale for this study

Variabilities in drug performance between the sexes have been reported and well-considered in human clinical studies. Despite this, potential sex differences are traditionally under-evaluated in the complete pharmaceutical research and pipeline. Sex differences in human intestinal physiology such as the expression of protein membrane transporters are still limited with large gaps of knowledge unknown. Pre-clinical research has also demonstrated a tendency to focus on healthy male rats during early drug development studies which may consequently conceal profound sex differences in drug effects. As the most commonly used animal model in pre-clinical research, further knowledge and understanding in the rat physiology is paramount to translate pre-clinical data to clinical research. As such, the aim of this research project was to investigate potential sex differences in the GI of both rats and humans.

As the luminal fluid is the main place for drug disintegration, dissolution and diffusion in the GI, the physicochemical properties of luminal fluids consequently play an important role in oral drug absorption (Schanker, 1971). In addition, as highlighted by FDA, the expression of intestinal P-gp can modulate the permeation of the drug in the intestine. Therefore, any variation in the expression of P-gp expression can elicit differences in oral bioavailability (FDA, 2017). As such, the exploration of sex differences firstly began with the characterisation of the physicochemical properties of luminal liquids including surface tension, pH, osmolality, buffer capacity and the expression of the intestinal membrane transporter, P-gp.

The current thesis can be summarised into the following parts:

- Chapter 2 – Characterisation of luminal fluids along the GI tract including surface tension, pH, osmolality and buffer capacity, and relative quantification of intestinal P-gp expression in male and female Wistar rat under the fasted state.

- Chapter 3 (Phase I) – Development of an LC-MS/MS method aimed to quantify absolute intestinal expression levels of P-gp in male and female Wistar rats to assess the influence of sex and food consumption.

  Chapter 3 (Phase II) – Assessment of the effect of time on intestinal P-gp expression in male and female Wistar rats in the fasted and fed state.

- Chapter 4 – Characterisation of GI physiology including luminal fluid properties and P-gp expression with respect to potential sex differences in the Sprague Dawley rat.

- Chapter 5 – Quantification of P-gp expression in male and female human small intestinal tissues and the comparison of P-gp expression between human and rats.

CHAPTER 2:

Sex differences in the rat gastrointestinal tract

2.1 Introduction

2.1.1 Pre-clinical study in oral drug development

Elucidating the underlying mechanism of the gastrointestinal (GI) absorption process after oral administration is essential for the assessment of safety, pharmacokinetics and pharmacodynamics of the potential drug candidate. The complexities of human GI physiology, however, make it difficult to simulate GI absorption activity. In addition, *in vitro* studies remain largely inadequate before human clinical trials. Alternatively, pre-clinical studies can provide an effective way to evaluate the potential compounds through *in vitro-in vivo* correlations. Animal pre-clinical studies have been widely applied in the oral drug development arena which includes the assessment of certain GI physiological features influencing drug absorption, toxicological assessment of xenobiotic and vaccines, and more importantly, the dose estimation when extrapolating animal data to humans (Hatton et al., 2015).

Various animal species including rodents, guinea pigs, rabbits, dogs, monkeys and pigs are now commonly used during pre-clinical studies. The matters of cost and the correlation between animal and human tissue with respect to parameters such as intestinal absorption have resulted to the varying frequency of use among different species (Figure 2.1) (Kararli, 1995). The majority of oral drug pre-clinical studies are tested on rodents due to their inexpensiveness, ease of use and the ability to identify compounds with promising or toxic biopharmaceutical properties (Hatton et al., 2015; Iannaccone and Jacob, 2009).

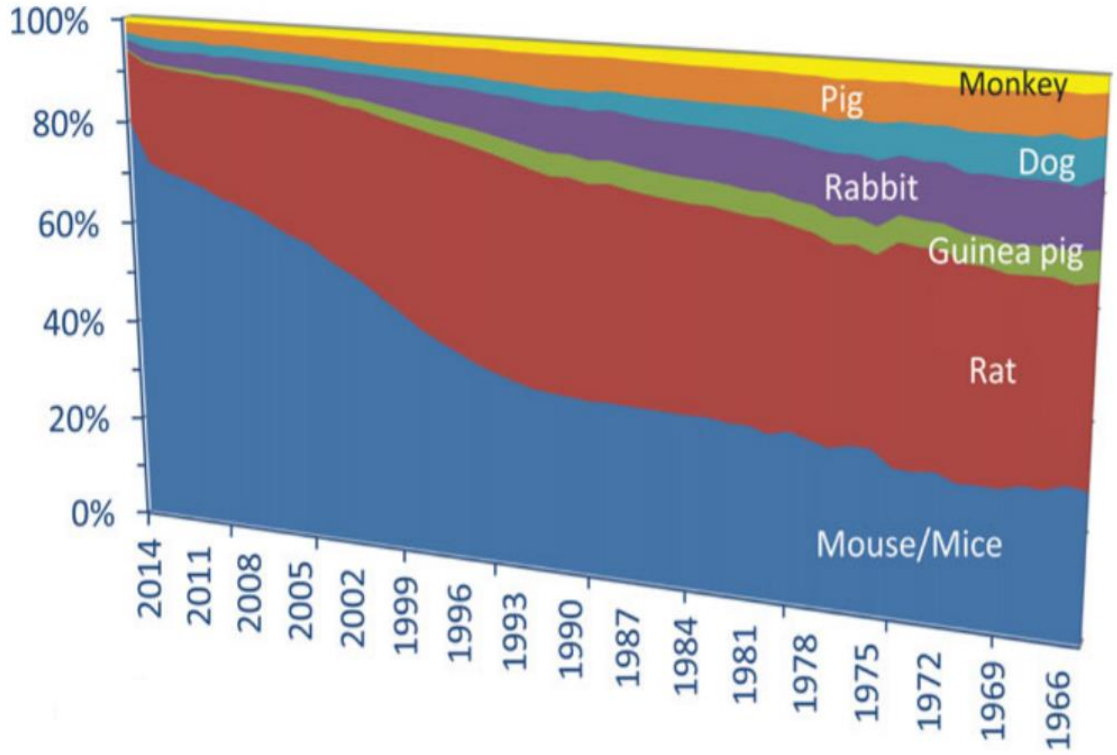


Figure 2.1 Number of publications in thousands (line plots, bottom) and percentage distribution (area plot, top) indexed in PubMed since 1965 employing use of various animal species in research, adapted from Hatton et al., 2015.

2.1.1.1 Rat

The rat was the mammalian species specifically domesticated for use in the laboratory and has been utilised for more than 200 years in the medical sciences (Shanks et al., 2009). All the laboratory rats currently used globally are originally bred from the wild brown rat known as Rattus norvegicus. In 1906, a colony of Rattus norvegicus with an albino mutation was established at the Wistar Institute in the United States of America. The species was specifically intended for medical research and coined as the Wistar rat. Based on Wistar albino rats, there are currently 117 albino strains of laboratory rats which have been widely used in pre-clinical development as a multipurpose model to assess safety, efficacy, ageing, nutrition, diet-induced obesity, oncology and surgical models (Oltra-Noguera et al., 2015).

With the rat being extensively used in oral drug pre-clinical studies, a sex difference phenomenon regarding the absorption of orally administered drugs in the GI tract has been observed in rats (Table 2.1). A study investigated the pharmacokinetics and pharmacodynamics of azosemide in rats and demonstrated that the 8-h urinary excretion of potassium was 0.395 mmol/g in males but 0.766 mmol/g in females after the oral administration of 10ml/kg azosemide (Lee et al., 1999). Moreover, a similar sex difference was observed on the absorption of schizandrin; female rats were reported to have an approximately 20-times higher area under the curve (AUC) (0-tn) following 10 mg/kg schizandrin oral administration compared with their male counterparts (67.8 ± 23.0 vs. 3.0 ± 1.3 mg/L.min) (Xu et al., 2008). In addition, there was a higher success rate following the oral administration of 10 micromol/kg/day oleoyl-oestrone in males in terms of inducing fat loss compared to females after 10 days (Grasa et al., 2001). Furthermore, a differential response was observed in the oral administration of 5 mg/kg hydroxytyrosol acetate (HTA) in males and females; plasma concentration increased by 1.6-fold in males albeit only a 0.9-fold increase was exhibited in females (Dominguez-Perles et al., 2017). Interestingly, a recent study reported a similar sex difference phenomenon of one drug bioavailability in both humans and rats. According to the study by Ashiru et al., PEG 400 can significantly ($p < 0.05$) increase the oral bioavailability of ranitidine in men but not in women (Ashiru et al., 2008). In response to this, a study conducted by Afonso-Pereira et al. further demonstrated that following the co-administration of PEG 400 and ranitidine, rats exhibited the same sex-specific phenomena (Afonso-Pereira et al., 2016). Furthermore, another investigation reported a sex-related difference in the GI absorption of ranitidine and ampicillin potentially due to the innate differences in GI physiology between the sexes (Mai et al., 2017).

Table 2.1 The identified sex difference in drug performance after oral administration in rats.

Drugs	Sex differences behaviour	References
Verapamil	100 μM verapamil *in situ* loop perfusion exhibited higher permeability in female rats	Oltra-Noguera et al., 2015
Azosemide	With 10 mg/kg oral administration of azosemide, the 8-h urinary excretion of potassium and 8-h kaluretic efficiency was significantly decreased in male rats compared to female.	Lee et al., 1999
Dietary ethanol	Female rats performed a better capacity to convert ethanol-derived carbons to lipid and store in adipose tissue than male rats	Cornier et al., 2000
Oleoyl-oestrone	10 micromol/kg/day oleoyl-oestrone administrations produced more significant slimming effect in male rat.	Grasa et al., 2001
Schizandrin	Intragastric (i.g) administration of 10 mg/kg in female enjoyed roughly 20 times of AUC (0-t_n) of that in male	Xu et al., 2008
Ivermectin	Higher ivermectin systemic availability was observed in female rats	Lifschitz et al., 2006
Ranitidine ***Ampicillin***	PEG 400 significantly increased the bioavailability of ampicillin and ranitidine in male rats, but not in female ones	Mai et al., 2017 Afonso-Pereira et al., 2016
Hydroxytyrosol acetate (HTA)	Higher orally dose in male resulted in 1.6-fold higher plasma levels but 0.9-fold in female rats	Dominguez-Perles et al., 2017

2.1.2 Influence of GI tract on drug oral absorption

Following oral administration, compounds will enter the GI tract and go through the absorption process including dosage form disintegration, dissolution, diffusion and permeation. The absorption process is important as it determines the actual concentration of the active pharmaceutical ingredients taken from the GI tract into systemic circulation which is known as bioavailability. Therefore, variation in the GI tract in terms of physiology and function can result in differing drug GI absorption and consequently bioavailability.

2.1.2.1 GI luminal fluids

The luminal fluids contained in the GI tract is often the starting point to initiate the absorption processes of drug substances. Firstly, the solid-state dosage form is needed to dissolve in the GI fluids. The unionised dissolved drug molecule can then permeate through the intestinal membrane and be absorbed into circulatory system. The physicochemical properties of the GI fluids, therefore, play an important role in drug luminal dissolution. For example, due to the different pH environments in the stomach and intestine, enteric-coated drug formulations can avoid drug dissolution in the stomach but can begin to disintegrate in the intestinal lumen which can lead to an improved therapeutic effect. According to Fadda *et al.*, the saturation solubility (mg/mL) of mesalamine was subject to change in the fluid of different segments along the GI tract; mesalamine solubility was reported to be 1.97 ± 0.25 in jejunum, 3.26 ± 0.08 in ileum, 6.24 ± 1.13 in ascending colon and 7.95 ± 0.21 in transverse/descending colon. This also highly correlated to the pH value (standardised coefficient $\beta = 0.219$, $p < 0.05$) and buffer capacity ($\beta = 0.849$, $p < 0.0001$) of the GI fluids in intestinal segments (Fadda et al., 2010). Interestingly, a similar result was reported in a rat study; the solubility of mesalamine achieved a high correlation with the luminal pH and buffer capacity of R^2 0.59 and 0.69 respectively (Merchant et al., 2015). Another study involving 24 model drugs demonstrated

that lecithin and bile acids contained in the intestinal fluids are also key factors for the drug intestinal solubility (Soderlind et al., 2010).

2.1.2.2 Intestinal membrane transporters

There are many types of membrane proteins located in intestine which served as uptake or bi-directional transporters as aforementioned in Chapter 1.4.2. The importance of these membrane transporters as one of the determinants of oral drug pharmacokinetics has become increasingly studied (Giacomini et al., 2010). Among these transporters, P-glycoprotein (P-gp) has been regarded as the one of most important transporters and needs to be comprehensively investigated during drug development (Sharom et al., 2011; Zakeri-Milani et al., 2014; Mai et al., 2018; Murakami and Takano, 2008; FDA, 2017).

P-gp is a cross-membrane protein and functions as a primary active transporter that move substrates across the cell membrane (Figure 2.2). As a transmembrane protein, the structural topology of P-gp consists of two distinct regions with each region containing six putative transmembrane domains (TMD) and one nucleotide binding domain (NBD). The function of P-gp is achieved by the combination of ATP and hydrolysis (Zinzi et al., 2014; Johnstone et al., 2000; Linton, 2007).

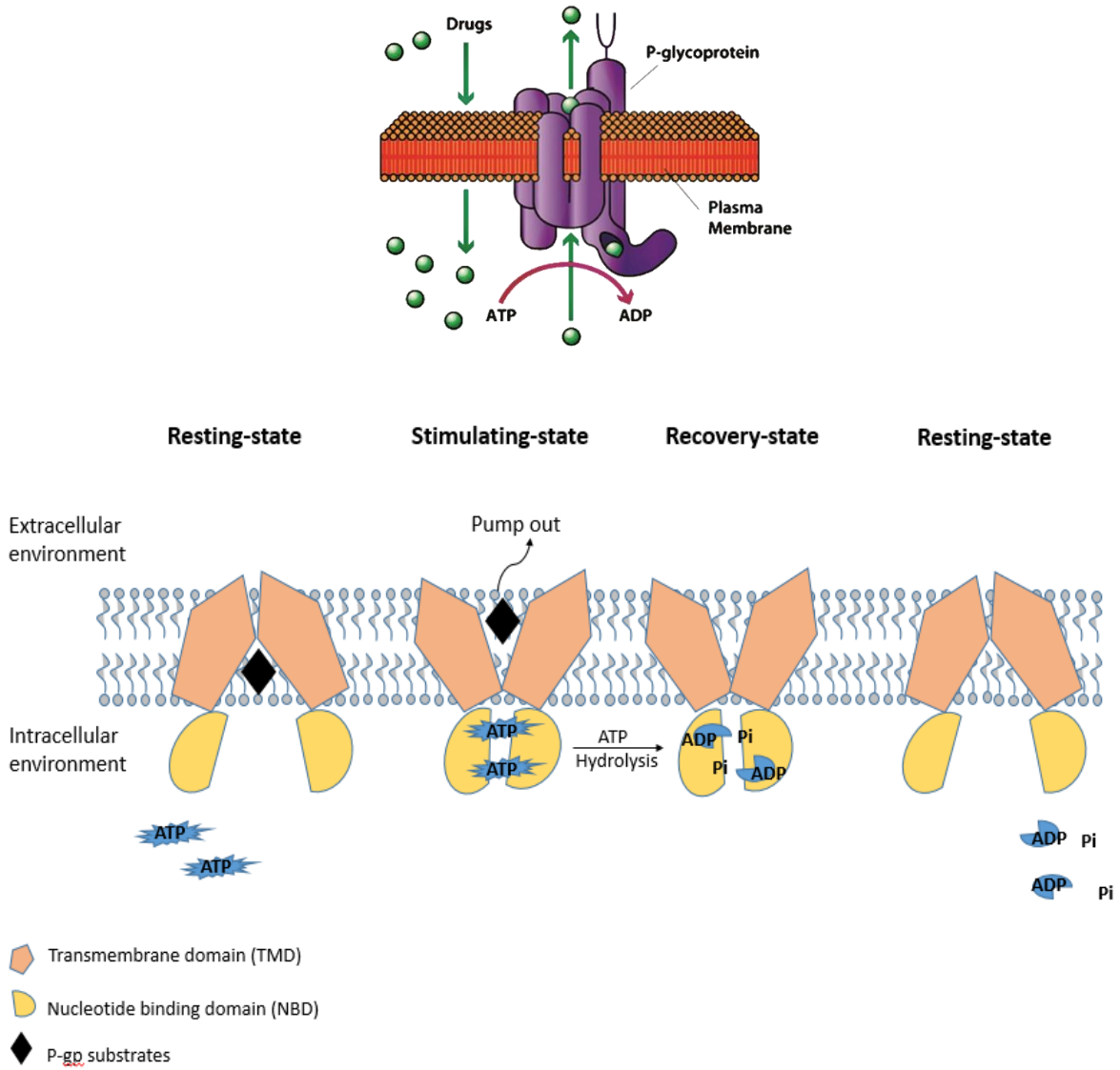


Figure 2.2 Structure and molecular mechanism of P-glycoprotein in intestinal membrane.

P-gp belongs to the ATP-binding cassette (ABC) superfamily. In humans, P-gp is encoded by the multi-drug resistance gene 1 (MDR1), whereas for rodents, multi-drug resistance gene 1a and 1b (mdr1a/1b) are responsible for the expression of P-gp (Klaassen et al., 2010). It has been identified that P-gp is ubiquitously expressed in several, albeit specific, normal body tissues including the intestinal tract, liver, kidneys and the blood brain barrier.

In the intestine, the major function of P-gp is to limit the absorption of xenobiotics and increase the elimination of these substances from the intracellular to extracellular environment (Fortuna et al., 2011). In this way, P-gp is also capable of limiting various drug compounds from intestinal absorption and oral bioavailability. Research conducted by Kim et al. found that the oral bioavailability of three poorly absorbed drugs, indinavir, nelfinavir and saquinavir, increased two to five-times in knockout mdr1a (-/-) mice when compared with wild-type mice (Kim et al., 1998). Another study showed that a 72% absolute bioavailability of tacrolimus was achieved in mdr1a (-/-) mice following oral administration, whereas only a 22% absolute bioavailable was reported in normal mice (Chiou et al., 2000). According to the biopharmaceutical drug disposition classification system (BDDCS) system, the intestinal absorption of drugs in Class II, III and IV can be affected by P-gp. This is especially significant for Class III and IV drugs of which P-gp plays the dominant role in their net absorption (Wu and Benet, 2005). Table 2.2 shows a list of drugs which had been identified as P-gp substrates.

Table 2.2 Summary of identified therapeutic drugs which are substrates of P-glycoprotein.

Substrates	**Measured in vitro values / in vivo effects**	***In vitro*[1]**	***In vivo*[2]**
Digoxin	Caco-2 cell experiment: P_{app} (A-B) – 1.1 and P_{app} (B-A) – 8.5 Clinical study: increase of plasma levels with P-gp inhibitor, quinidine	√	√
Acebutolol	Caco-2 cell experiment: P_{app} (A-B) – 1.1 and P_{app} (B-A) – 4.1	√	
Paclitaxel	Caco-2 cell experiment: P_{app} (A-B) – 0.8 and P_{app} (B-A) – 8.4 Clinical study: increase of apparent bioavailability with P-gp inhibitor, valspodar (PSC-833)	√	√
Docetaxel	Clinical study: increase of bioavailability with P-gp inhibitor, cyclosporin A		√
Etoposide	Caco-2 cell experiment: P_{app} (A-B) – 0.7 and P_{app} (B-A) – 4.1	√	
Labetalol	Caco-2 cell experiment: P_{app} (A-B) – 0.8 and P_{app} (B-A) – 9.6	√	
Talinolol	Clinical study: lower AUC with P-gp inducer, rifampin Caco-2 cell: R value (P_{app} B-A/ P_{app} A-B) decreased from 9.6 to 1.41 with the addition of verapamil, a P-gp inhibitor (0.5 mM)	√ [3]	√
Cyclosporin A	Clinical study: lower plasma levels with P-gp inducer, St John's wort	√	√

Table 2.2 Continued

Substrates	Measured in vitro values / in vivo effects	*In vitro*[1]	*In vivo*[2]
Methotrexate	Clinical study: increase of AUC and lower clearance with addition of P-gp inhibitor, omeprazole/pantoprazole		√
Indinavir	Caco-2 cell experiment: P_{app} (A-B) – 2.4 and P_{app} (B-A) – 22.8 Clinical study: lower plasma levels with addition of P-gp inducer, St John's wort	√	√
Rhodamine 123	Caco-2 cell: R-value (Papp B-A/Papp A-B) decreased by 88%, 82%, 70% and 82% with the addition of P-gp inhibitors PSC833, cyclosporin A, verapamil, quinine, respectively Rats *in vivo*: Pre-treatment with cysclosporin A increases the C_{max} of Rhodamine 123 by 64.7%	√[4]	√[5]
Domperidone	Caco-2 cell experiment: P_{app} (A-B) – 5.7 and P_{app} (B-A) – 16.1	√	
Loperamide	Caco-2 cell experiment: P_{app} (A-B) – 5.8 and P_{app} (B-A) – 7.6 Clinical study: CNS adverse effects when co-administration with P-gp inhibitor, quinidine.	√	√

[1] Lin *et al.,* 2011**; [2]** Marchetti *et al.,* 2007; [**3]** Fortuna *et al.,* 2011; **[4]** Van *et al.,* 2000; **[5]** Dorababu *et al.,* 2009

Currently, the most commonly used method for P-gp quantification is Western-blotting technique. In 1979, Harry Towbin and his colleagues from the Friedrich Miescher Institute in Switzerland developed a new method for the separation and detection of proteins, now known as the Western blot technique (Towbin et al., 1979). To this day, Western blot analyses have been widely used as a protein and RNA analytical technique in all biological-related disciplines. The Western blot experiment consists of two steps; separation and detection. In protein separation, the target protein is firstly separated from the matrix by gel electrophoresis. The denatured protein mixture is then loaded into agarose gel for electrophoresis which are separated based on molecular weight. For example, P-gp is a 170 kDa transmembrane glycoprotein and can be separated from other small proteins such as beta-actin, a 42 kDa protein (Linardi and Natalini, 2006). After separation, the target protein is then detected through antibody binding shown in Figure 2.3. The target protein is initially bound to a primary antibody which is the specific antibody to the target protein – for example, the mouse monoclonal anti-P-gp is specific to bind with P-gp. The secondary antibody with an enzyme-conjugated tail will then combine with the primary antibody. Finally, the enzyme-conjugated tail can generate a signal resulting to the target protein being detected and quantified.

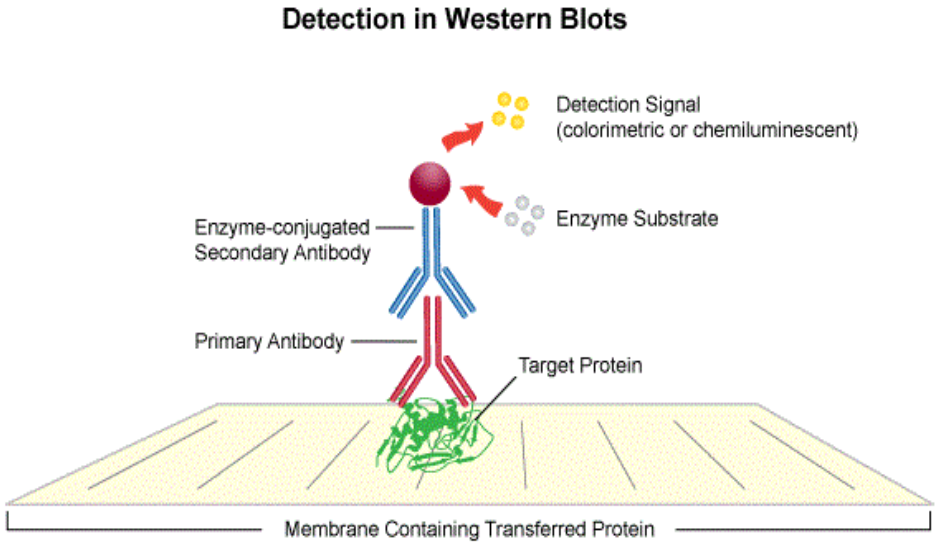


Figure 2.3 The principle of protein detection in Western-blotting technique

2.1.3 Current knowledge of sex differences in rat GI physiology

In light of the observations of sex difference in GI drug absorption as well as the potential implications from differences in physiology, the comprehensive understanding of rat GI physiological features, therefore, is essential to elucidate to avoid the observed sex difference in pre-clinical rat studies. However, with the prevalence of male-bias experiments, little known on the potential sex differences in rat GI physiology. A study conducted in 2008 investigated the intestinal P-gp in male and female rats which reported no sex differences in rat P-gp expression along the GI tract (Merchant et al., 2008). However, according the previous studies aforementioned, verapamil, ivermectin, ampicillin and ranitidine are all P-gp substrates and display a distinct sex difference in intestinal absorption. The lack of knowledge and the contradiction between the previous studies, therefore, result in the need for a comprehensive understanding of the sex difference in rat GI physiological features.

2.2 Aims

- Characterise the physicochemical properties of luminal liquids including pH, osmolality, surface tension and buffer capacity in both male and female Wistar rats

- Determine the relative expression of P-gp along the intestinal tract in both male and female Wistar rats via Western-blotting experiment

- Quantify the messenger ribonucleic acid (mRNA) coding for the intestinal P-gp, mrb1a and mrb1b, along the intestinal tract in both male and female Wistar rats via real time Reverse-Transcription Polymerase Chain Reaction (PCR) experiment

2.3 Materials

HPLC-grade water, methanol, peroxide-free tetrahydrofuran and trifluoroacetic acid were purchased from Fisher Scientific (Loughborough, UK) for luminal fluid characterization. NaOH and HCl (0.1 M standards) were used for buffer capacity determinations and were procured from Sigma Aldrich (Dorset, UK). NuPAGE LDS Sample Buffer, Tris Buffered Saline, 10 X Solution, NuPAGE MOPS SDS Running Buffer (20X), NuPAGE Transfer Buffer (20X) and SuperSignal West Pico Chemiluminescent Substrate were purchased from Thermo Scientific (Paisley, UK). Tween 20, Bovine Serum Albumin and Monoclonal Anti-β-Actin were obtained from Sigma Aldrich (Dorset, UK). TBE Running Buffer (5X) and 10X TBE Electrophoresis Buffer were bought from Thermo Scientific (Paisley, UK). All other chemicals and kits are noted individually in the following methods.

2.4 Methods

2.4.1 Experimental animals

Male and female Wistar rats (8 weeks old), purchased from Harlan UK Ltd (Oxfordshire, UK). Male and female Wistar rats weighed approximately 250g and 200g, respectively. All these rats were housed at room temperature (25℃) and in a light-dark cycle of 12h. All housed rats were provided with food (EURodent Diet 22%) and water ad libitum and allowed to acclimatise for at least 7 days prior to experiment. All procedures were approved by the Home Office (PPL No.70/6421) and were conducted in accordance with the Animals (Scientific Procedures) Act 1986, UK.

2.4.2 Characterization of luminal fluids in the GI tract

All characterizations were performed on supernatant obtained from the gastrointestinal fluids from the laboratory animals. 6 male and 6 female rats were fasted overnight and then sacrificed by CO_2 asphyxiation in the following morning at around 8:30 am. The pH of

gastrointestinal tract was then measured *in situ* using a pH meter (HI99161) equipped with an FC202 electrode designed for measurements in viscous and semi-solid materials (Hannah Instruments, Bedfordshire, UK). The pH of the GI tract section was determined by introducing the pH probe into the opening created by sectioning parts of the GI tract. For each GI segment two *in situ* measurements were taken, one at the proximal opening (A) and the second at the distal one (B).

After that, the whole GI tract was promptly extracted and divided into stomach, duodenum, jejunum, ileum, caecum and colon. The gastrointestinal sections were emptied into 1.5 mL Eppendorf tubes and centrifuged (Centrifuge 5415D, Eppendorf AG, Hamburg, Germany) at 13000 rpm for 20 minutes. The supernatant obtained was kept at -80°C until analysed as follows:

- Osmolality was measured with a Digital Micro-Osmometer (Type 5R), Hermann Roebling MESSTECHNIK, Berlin, Germany.
- Surface tension was measured using a Delta 8 Tensiometer (Kibron Inc) controlled by Delta-8 manager software (version 3.8). The measurement was performed using a DynePlates (96-well plate designed for tensiometer), with 50 µL of sample in each well.
- Buffer capacity was measured at pH changes of 0.5 and 1.0 units by adding aliquots (10 µL) of 0.1 M HCl (for intestinal fluids) or 0.1 M NaOH (for gastric fluids) to a 300 µL supernatant pooled sample from GI fluid to achieve the desired pH change. Buffer capacity was then calculated using following equation 1:

$$\beta\ (\mathrm{mmol/L/\Delta pH}) = (\mathrm{M\_a} \times \mathrm{V\_a})/\Delta\mathrm{pH}\ \times\ 1000/\mathrm{V\_b}$$

Where β is the buffer capacity M_a is the molarity of the acid, V_a is the volume of acid in mL, V_b is the volume of buffer in mL, ΔpH is the change in pH unit.

Due to the small amount of fluids available from some of the intestinal segments, some tests were run in pooled samples, in which fluids from the same segment of different animals were mixed to increase the available volume to perform the tests.

2.4.3 Tissue preparation

6 male and 6 female rats were subject to an overnight fasting of 12 h prior to the experiment. On the day of experiment at approximately 8:30 am, rats were sacrificed by a CO2 euthanasia chamber. The whole intestinal tract was then rapidly removed and kept in an ice-bath filled with Krebs-Bicarbonate Ringer's solution (KBR) at pH 7.4. 1 litre of KBR solution was prepared freshly before the experiment at room temperature (Table 2.3). The intestine was then cut into four segments; the duodenum (1 cm from the ligament of Treitz); jejunum (10 cm from the ligament of Treitz); ileum (1 cm from the cecum) and colon. Tissue pieces from the mid part of the duodenum, the proximal part of the jejunum, the distal to mid part of ileum and the descending colon were separated. 1 cm of tissue was used for the P-gp quantification and 2 cm for mRNA determination. The separated tissue was then opened along the mesenteric border and the mucosal layer was obtained by gently squeezing the serosal-side of tissue with a cover slip on ice-cold glass plate. The prepared tissue with the mucosal layer was then freshly used for the following studies.

Table 2.3 Preparation of Krebs-Bicarbonate Ringer's solution, pH 7.4

Components	**mM**	**Concentration (g/l)**	**Components**	**mM**	**Concentration (g/l)**
D-glucose	10	1.8	**$NaHCO_3$**	25	2.1
$MgCl_2$	1.2	0.114	**KH_2PO_4**	0.4	0.054
$CaCl_2$	1.2	0.133	**K_2HPO_4**	2.4	0.418
NaCl	115	6.7			

2.4.4 Western-blotting experiment

2.4.4.1 Total protein extraction

The prepared tissue from section 2.4.3 was placed into a glass vial containing 3 ml of freshly prepared lysis buffer (50 mM Tris, 250 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 1% Nonidet P40 and a protease inhibitor cocktail) and homogenised for 20 s at 10,000 rpm with a T18 digital ULTRA-TURRAX® (IKA). The homogenised tissue solution was then incubated in a 4°C fridge for 2 h for protein extraction. Two hours later, the solution was transferred to a 1.5 ml Eppendorf tube and centrifuged with 10,000 rpm at 4°C for 10 min. The supernatant was transferred to micro-tubes and stored at –20°C until used for analysis (stable for 6 months).

2.4.4.2 Total protein quantification

The total extracted protein was quantified according to the instruction adapted from the Pierce BCA Protein Assay Kit (ThermoFisher, UK). Firstly, a calibration curve was determined by the dilution of one albumin standard (BSA) ampule with lysis buffer. 8 standards were prepared with a working range of 20 – 2000 ug/ml conducted in triplicate as followed from the Pierce BCA Protein Assay Kit (ThermoFisher, UK). The following was used to determine the volume of working reagent (WR) required:

Equation 2 $$(\#\text{ standards}+\#\text{ unknowns}) \times (\#\text{ replicated}) \times (\text{volume of WR per sample}) = \text{total volume of WR required}$$

The WR was prepared by mixing 50 parts of BCA Reagent A with 1 part BCA Reagent B following the instructions from the Pierce BCA Protein Assay Kit (ThermoFisher, UK).

2.4.4.3 P-gp quantification via Western-blotting

25 μg protein sample calculated from the total protein concentration from 2.4.4.2 was

suspended in NuPAGE® LDS Sample Buffer (Invitrogen, Carlsbad, CA) and kept in a 70°C incubator for 10 min to induce denaturation. The denatured protein sample was then loaded on a NuPAGETM NovexTM 4 – 12% Bis-Tris gel (Invitrogen). 5 µl Sharp Pre-Stained protein standard (Invitrogen) was also loaded as molecular weight marker. Gel electrophoresis was then applied according to the instructions from the manufacturer. The separated protein samples in the gel were then transferred to a nitrocellulose membrane with an XCell SureLock™ Mini-Cell Electrophoresis System (Invitrogen). Membranes were blocked with 3% bovine serum albumin (BSA) in tris-buffered saline with tween (TBS-T) and incubated for 1 h at room temperature (25°C). For the detection of P-gp and reference protein (β-actin) blots were incubated for 1 h at room temperature with the respective primary antibodies, diluted in a 3% bovine serum albumin (BSA) in TBS-T: mouse monoclonal anti-P-gp (C-219 3:200; Enzo Life Science, Exeter, UK) and anti-β-actin mouse monoclonal antibody (1:2000; Sigma-Aldrich, Poole, UK). The detection of bound antibodies was completed with affinity-purified rabbit anti-mouse immunoglobulin G (IgG) coupled to peroxidase (secondary antibody; Sigma) and diluted to 1:5000 in 3% BSA in TBS-T. After 1 h incubation with the secondary antibody conjugated with horseradish peroxidase, P-gp and β-actin protein bands were visualised by a chem-iluminescence detection method with Pierce™ ECL Western Blotting Substrate (ThermoFisher). The blots were then photographed with a ChemiDoc XRS camera (Bio-Rad). The relative expression of P-gp was calculated using the Image Lab™ software linked to the camera (Bio-Rad).

2.4.5 Reverse-Transcription Polymerase Chain Reaction experiment

2.4.5.1 mRNA extraction and evaluation

The prepared tissues from section 2.4.3 were kept in an RNAlater buffer (Thermo Scientific). The total mRNA from the tissues were then extracted following the instruction from Pure Link RNA Mini Kit and On-column PureLink® DNase Treatment protocol. The extracted

mRNA samples were stored in a –80°C freezer until analysis. The frozen mRNA samples were firstly thawed on ice and 2 µl mRNA solution was then transferred to a NanoDrop 2000c Spectrophotometer (Therma Scientific) for the evaluation of the purification and quantification of the extracted mRNA prior to experiment.

2.4.5.2 mRNA quantification via real time PCR

A Two-step real time PCR was applied for mRNA quantification and the sequences of designed primer were listed in Table 2.4. The first step was the preparation of cDNA. 1 µg of extracted mRNA from each sample was reverse transcribed to cDNA by following the instruction from iScriptTM cDNA Sythesis Kit (Bio-Rad). The second step was Real Time-qPCR (RT-qPCR). A 7500 Real Time PCR System (Thermofisher) was applied and the method was adapted from the user guide of SYBR® Green PCR Master Mix and SYBR® Green RT-PCR Reagents Kit. The experiment was conducted in a microAmp optical 96-well reaction plate with each well containing a 50 µl reaction system. This included a 1 ng transcribed cDNA, 25 µl SYBR Mix solution, 5 µl forward primer, 5 µl reverse primer and 10 µl RNAse-free water. The sequences of the primers were shown in Table 1. The relative quantification of mRNA, mdr1a, mdr1b and β-actin (internal standard) was carried out with the programme as follows; The amplification program consisted of one pre-incubation cycle at 95°C with a 12 min hold, followed by 40 amplification cycles with denaturation at 95°C with a 15 s hold, an annealing temperature of 60°C with a 10 s hold and an extension at 60°C and a 1 min hold. Amplification was followed by a melting curve analysis. This ran for one cycle with denaturation at 95°C with a 15 s hold, annealing at 60°C with a 1 min hold and melting at 95°C with a 30 s hold. A negative control was included for each analysed sample by adding deionised water instead of primers. The control group which contained deionised water instead of cDNA was also included in each run. The relative expression of mdr1a and mdr1b mRNA in different samples were obtained by designing the programme on 7500 Real.

Time PCR System based on the principal of previous studies (Merchant et al., 2014). The relative expression of P-gp mRNA, mdr1a and mdr1b were calculated using a 7500 software (version 2.0.6, Thermofisher).

Table 2.4 Sequences of designed primers used in the real-time qPCR experiment

Primers	**Sense and Antisense**	**PCR product (*bq*)**	**Reference**
mdr1a	Forward 5'-CACCATCCAGAACGCAGACT -3'	159	This paper
	Reverse 5'-ACATCTCGCATGGTCACAGTT-3'		
mdr1b	Forward 5'-AACGCAGACTTGATCGTGGT-3'	144	This paper
	Reverse 5'-AGCACCTCAAATACTCCCAGC-3'		
β-actin	Forward 5'-GCAGGAGTACGATGAGTCCG-3'	74	This paper
	Reverse 5'-ACGCAGCTCAGTAACAGTCC-3'		

2.4.6 Statistics analysis

All results are expressed as mean ± SD (n = 6) and were analysed by one-way ANOVA, followed by post-hoc Tukey analysis with a 95 % confidence interval using IBM SPSS Statistics 16 (SPSS Inc., Illinois, USA).

2.5 Results and discussion

2.5.1 Sex differences in luminal liquid physicochemical properties

2.5.1.1 pH

Figure 2.4 represents how the pH measured in situ changes along the GI tract in both male and female Wistar rats. For both sexes, the GI fluid pH profile followed a similar trend. In both males and females, pH was lowest in the stomach, with the antrum having a lower pH value than the fundus. This result was expected due to the active secretion of hydrochloric acid, with the pH increasing in the small intestine due to the presence of bicarbonate ions, bile and other species that neutralise the stomach acid.

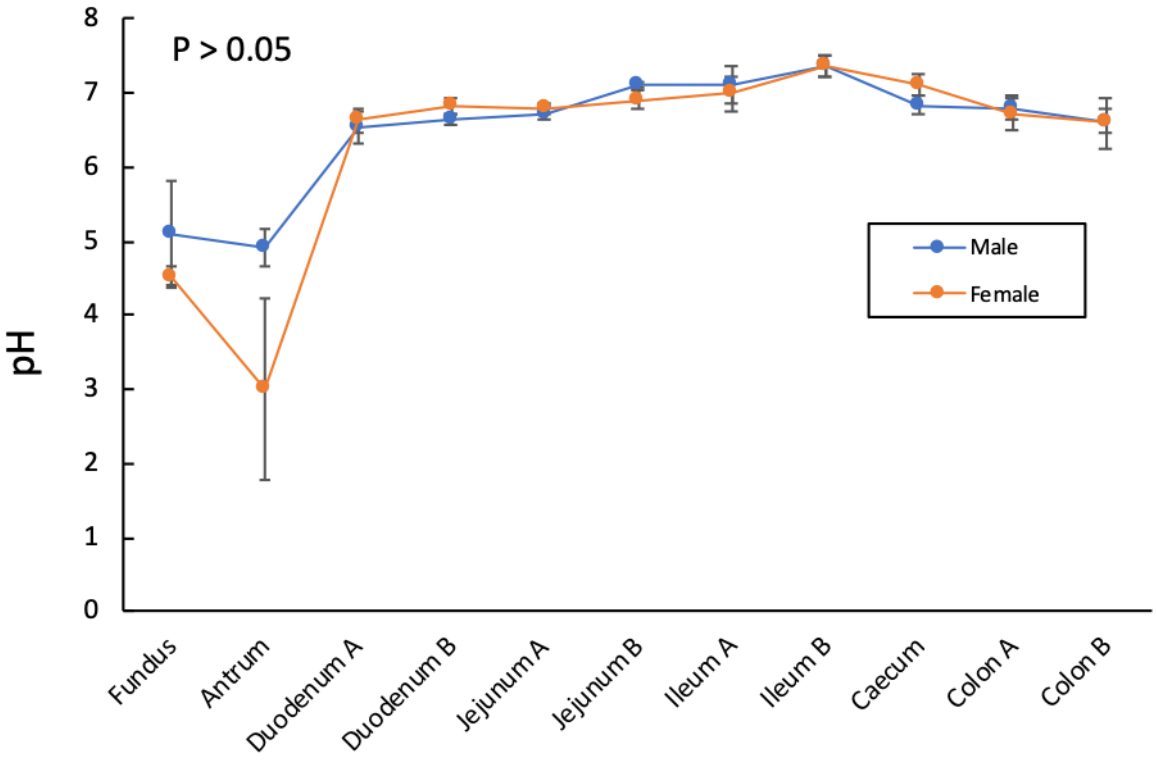


Figure 2.4 pH of the luminal environment of sections of the GI tract of male and female Wistar rats. A – Denotes proximal portion of the segment; B – denotes distal portion of the segment. The results are measured *in situ*, n=6.

Overall, there was no sex difference in pH along the GI tract ($p>0.05$). In both sexes, a sharp

rise in pH was observed from the antrum to the duodenum, which remained stable until the distal ileum where a small pH increase was observed. The pH then reduced slightly in the caecum and the colon, which was expected as it is a common site for fermentation and acid species production (Barnes, 1962). A similar pH profile trend was also found in human (Evans et al., 1988). The standard deviation was highest in the stomach (both fundus and antrum), whilst remaining quite low throughout the rest of GI tract. This suggests that inter-individual variability was higher in stomach, which may be due to the animals coprophagy (Barnes et al., 1957). Moreover, following rodent sacrifice, it was observed the rats had different volumes of gastric contents, and thus, may have contributed to the variability observed.

With the highest variation, stomach pH values still displayed a slight difference between male and female rats, female exhibited a relative lower pH in fundus and antrum compared to male. There are conflicting literature views on this subject however; previous research has shown that oestrogens are inhibitors of the gastric acid secretion whilst testosterone is an inducer (Amure et al., 1970; Maitrya et al., 1979). Hence, it is commonly thought that a higher pH would be exhibited in females due to lower acid secretions. However, in a recent study, it was found that males have a higher gastric blood flow than their female counterparts (Shore et al., 2017). Oestrogen administration was able to reduce the mean blood flow in the gastric mucosa by 31% in males, however, remained largely unchanged in females. The thickening of the mucus layer was also demonstrated at a faster rate in females than males. This suggests that females may be more “resistant” to feminine hormones and may be more effective in repairing damage to the gastric wall. If the mucus-producing rate of females is higher, it may suggest an evolutionary biological adaptation to higher stomach acidity. GI fluid pH is widely known to affect drug ionisation by influencing drug solubility, stability, absorption and,

ultimately, bioavailability. As such, the observed differences in GI fluid pH between male and female rats may have implications for the in vivo testing of oral dosage forms. For example, the differences in pH between the sexes could affect the behaviour of pH responsive formulations, potentially leading to incorrect pharmacokinetic extrapolation in humans (McConnell et al., 2008; Merchant et al., 2014).

2.5.1.2 Buffer capacity

The overall trend of buffer capacity was found to be relatively different between male and female Wistar rats (Figure 2.5).

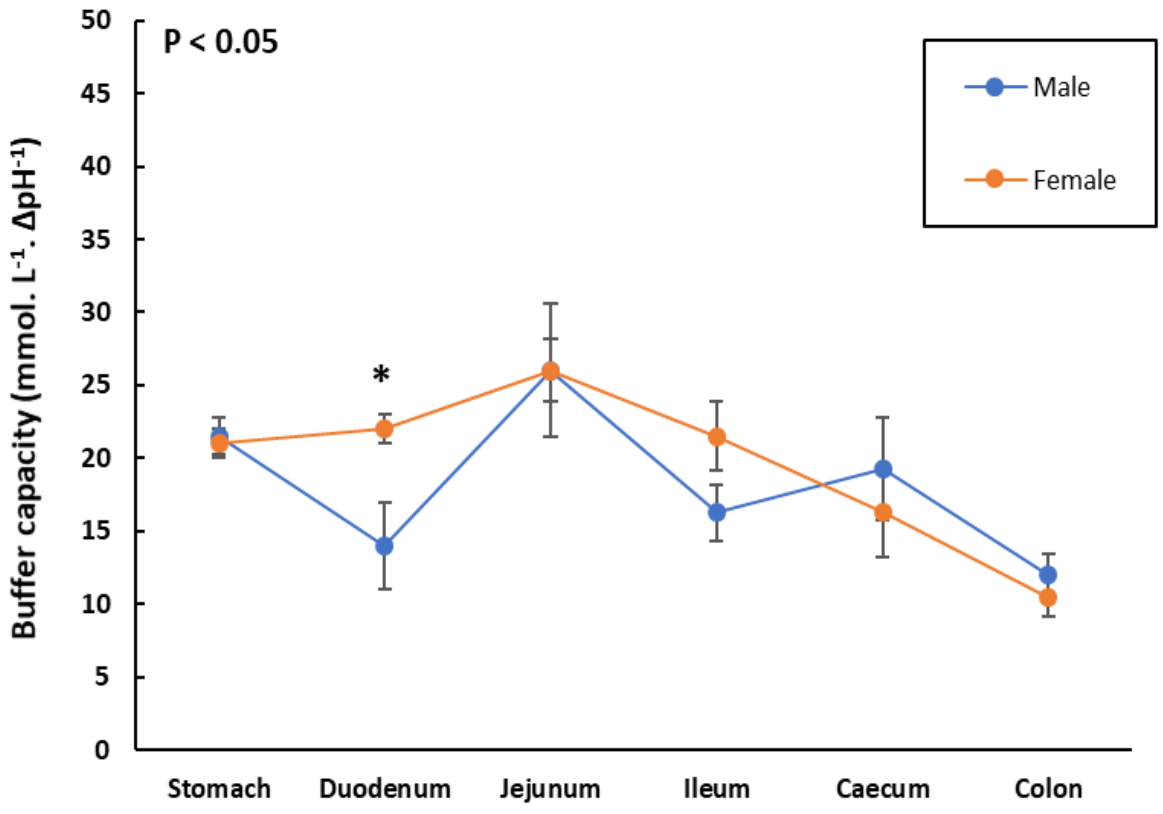


Figure 2.5 Buffer capacity (ΔpH=1.0) of pooled fluids of sections of the GI tract of male and female Wistar rats, n=6. The values are the mean of several pooled fluids measurements. *denotes a statistical significance ($p<0.05$) between males and females.

In female rats, the buffer capacity was found to increase from stomach to small intestine. After reaching the highest point in jejunum, the buffer capacity displayed a continued downward trend and reached the lowest point in colon. On the contrary, male rats displayed a fluctuating buffer capacity along the GI tract. It displayed higher values in stomach and jejunum but lower values in duodenum, ileum and caecum. Similar to that of in female, buffer capacity met the lowest value in colon. Females were found to have a higher buffer capacity than males in the duodenum, where 14 ± 3 mmol. L^{-1}. ΔpH^{-1} in male and 22 ± 1 mmol. L^{-1}. ΔpH^{-1} in female. This could be partially due to the bile salts. It has been known that rats do not possess gall bladder in the enterohepatic circulation and therefore are physiologically compensated by the enlargement of the duct system (Oldham-Ott et al., 1997). Without the collection process, the produced bile acids continually excrete into duodenum via duodenal papilla. A study collected the bile from 6 male and 6 female rats and observed a sex difference in the bile acids (Yousef et al., 1972). Specifically, even though the total bile acid outputs were similar in rats of both sexes, significant sex differences were found in the amounts of individual bile acids secreted. The female rat secretes considerably more cholic and lithocholic acids, and considerably less β-muricholic, deoxycholic, and hyodeoxycholic acids. The female rat also secretes less glycine-conjugated bile acids and in contrast to the male the bulk of this is glycocholic acid. The bile acids with the sex-related difference in composition consist of the luminal liquid in duodenum which can directly affect the buffer capacity.

The buffer capacity of the GI luminal fluids plays a major role in the dissolution of ionisable drugs. In particular, buffer capacity determines the microclimate pH in the diffusion boundary layer adjacent to the dissolving surface (Horter et al., 2001). As such, the differences between male and female rats buffer capacity may again pose implications for the in vivo testing of

oral dosage forms.

2.5.1.3 Osmolality

The osmolality of GI fluids in male and female rats showed similar profiles across the whole GI tract (Figure 2.6). In both cases, osmolality was found to increase from stomach to proximal small intestine and reduced distally. A distinct variation can be observed in duodenum segment in both male and female rat.

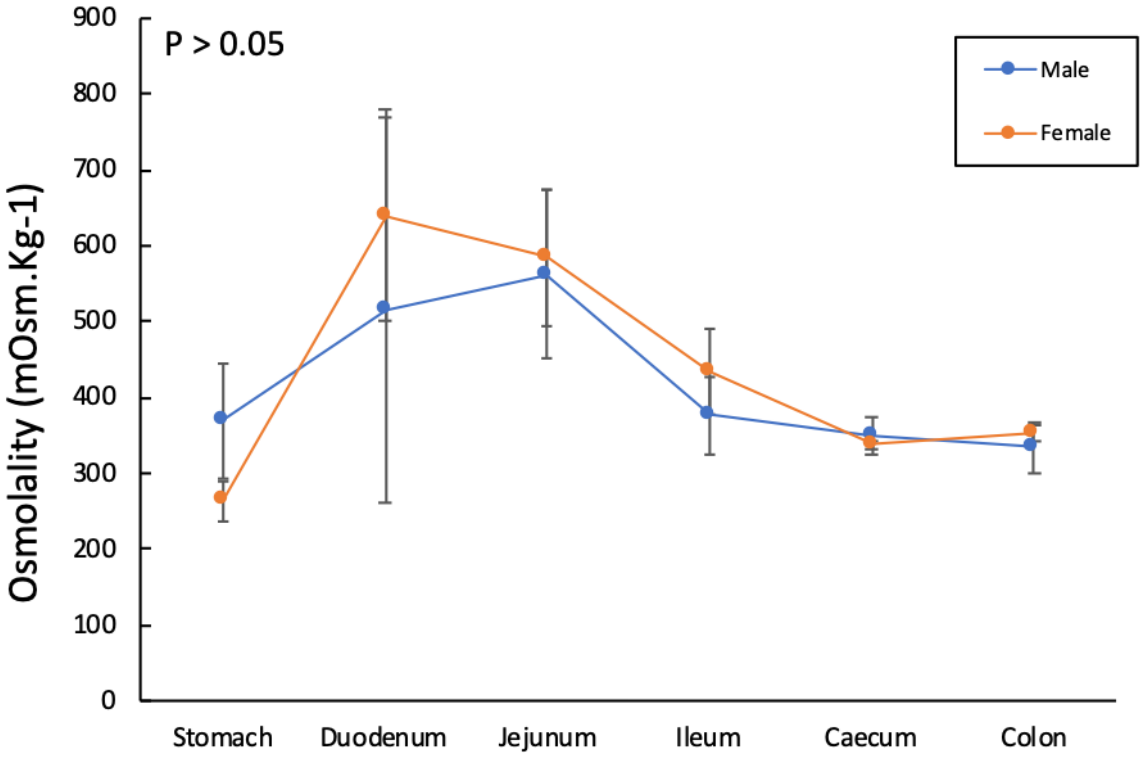


Figure 2.6 Osmolality of the GI fluids in male and female Wistar rats, n=6.

The highest value and obvious variation of osmolality in duodenum may due to bile salts secretion. The osmolality of luminal liquids mainly affected by the food consumption because the chyme that floods into the intestine from the stomach typically contains macromolecular components which will be digested and therefore increase the osmolality dramatically such as

starch. In current study, however, after overnight fasting, the main components contained in luminal fluids will be own organ secretions such as bile salts. As rodents do not have gall baller, duodenum is the place initially collect the secretions and achieve the high concentration (Oldham-Ott et al., 1997). In addition, it was reported that the variation of bile secretion pattern existed in both fasting and fed rats, while the only the general level of bile flow decreased in fasting state (Vonk et al., 1978). Such phenomenon was also reported in other mammals. It was demonstrated that the bile salt independent flow was extremely variable during fasting but was increased and stabilized by feeding in primates (Strasberg et al., 1974). A study collecting human duodenal fluids found that the phospholipids concentration displayed a higher variation in fasted human subjects when compared to human subjects under fed state (Riethorst et al., 2016).

GI fluid osmolality may contribute to oral drug absorption by affecting drug solubility; it has been reported that the “salting-out” and “salting-in” effect can change solubility (Pegram et al., 2008). Osmolality and fluid volume have been investigated in relation to buffer capacity and pH, given that the alteration of salt concentration and ingested fluids can affect both parameters through stimulating the secretion of gastric acid, bile and pancreatic juices (Horter et al., 2001; Fordtran et al., 1966). The continuous digestion and absorption of osmotically-active species may contribute to a distal reduction of the osmolality of the luminal environment. This finding is important due to the alteration of ionic content may further influence drug ionisation, and hence, limit drug absorption.

2.5.1.4 Surface tension

As shown in Figure 2.7, the surface tension values of GI fluids were similar amongst male and female rats. In both cases, the highest surface tension was recorded in stomach and then experienced a dramatic decrease in duodenum and then maintained at the low level in the rest of the GI tract. The surface tension of gastric acid in humans was previously reported to lie in the range of 35-45mNm^{-1} (Finholt and Solvang 1968), which is also reflected in the results here. No significant sex differences were observed along the intestinal lumen ($p>0.05$).

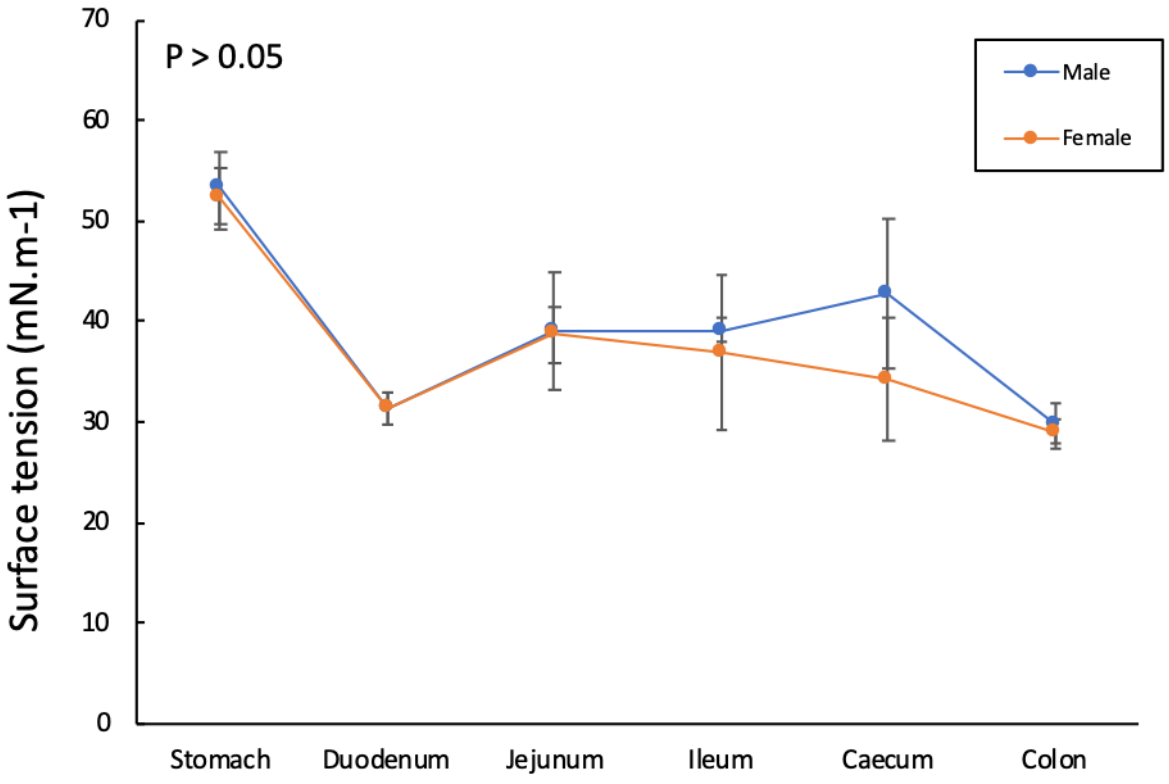


Figure 2.7 Surface tension of the fluids along the GI tract in male and female Wistar rats, n=6.

It can be noticed that the surface tension of the GI fluids determined here was significantly lower than that of water which is approximately 72mN.m^{-1} (Vargaftik et al., 1983). This can be easily understood by the presence of a myriad of compounds that act as surfactants in the

GI tract, of which bile salts are the most widely known (Dressman et al., 1998). Because the bile salts are released in the upper duodenum, the surface tension of the chyme is reduced. As discussed above, due to the lack of gall bladder rat bile is not concentrated and stored but released continuously in the small intestine. As a result, the presence of bile salts along the intestine lumen reduces the surface tension luminal liquids. In stomach, however, the mainly components of the fluids were water and hydrochloric acid in the fasting rat, which contribute to the higher surface tension compared to the rest of GI tract (McConnell et al., 2008).

The standard deviation of these mean surface tension values was relative low in the duodenum and jejunum, suggesting that the luminal environments in the upper GI tract were homogeneous with little inter-subject variability. However, in the ileum and caecum, the standard deviation was high, suggesting that the mechanism behind the distal increase of the surface tension is more variable. One possible mechanism is the variation on the retrieving bile salts. After releasing into duodenum, the bile salts were diluted and retrieved along the intestine and more factors involved in the distal segments.

For oral drug delivery, surface tension of GI fluids may impact drug absorption. Surface tension has been found to contribute to the degree of solvation of drug particles and respective wettability (Overhoff et al., 2008), as well as being inversely related to the dissolution rate of some active substances (Finholt et al., 1968).

2.5.2 Sex differences in P-gp protein expression along the GI tract

2.5.2.1 Calibration curve for total protein quantification

A calibration curve was used for the determination of the concentration of an unknown solution from a generated regression equation from standard known compounds. Figure 2.8

shows that net absorbance at 562 nm is linearly proportional to the known concentration of the standard albumin protein in lysis buffer using a plate reader. The coefficient of determination was 0.99 which indicated that the calibration curve was accurate by virtue of the 8 data points. In addition, no systemic error was observed due to the linear curve and an intercept identified at 0. The regression equation (Equation 3) can be rearranged to determine unknown protein concentrations of a solution for total protein quantification (Equation 4); Table 2.5 listed the protein concentrations determined in the samples for the Western-blotting experiment.

Equation 3 $y = 0.0012x + 0.1455$

Equation 4 $x = \frac{(y - 0.1455)}{0.0012}$

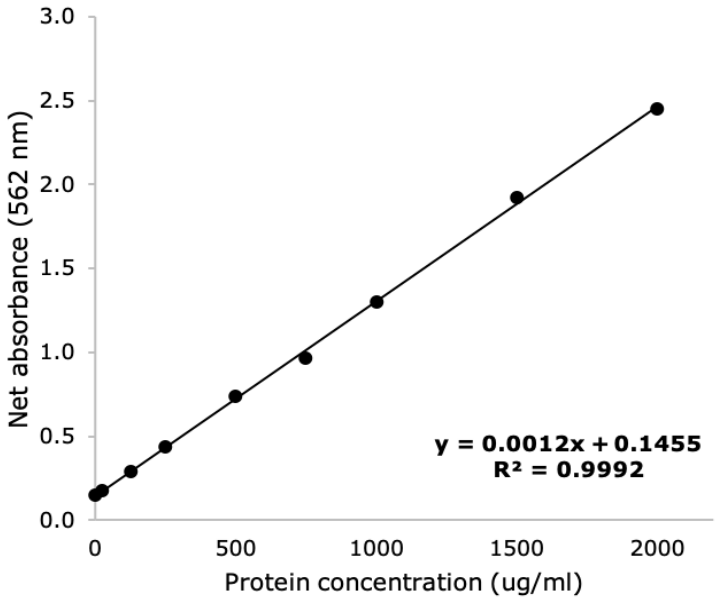


Figure 2.8 Calibration graph determined for the calculation of unknown protein concentration.

Table 2.5 The protein concentrations in the samples extracted from different segments of GI tract in male and female rats.

Sex	Animal	Protein concentration (μg/ml)			
		Intestinal segment			
		Duodenum	**Jejunum**	**Ileum**	**Colon**
Male	1	1240	1140	1016	1611
	2	1385	1097	1102	1681
	3	1278	1444	1277	1419
	4	1460	1106	1082	1649
	5	1045	1139	1083	1533
	6	2016	976	1095	1249
Female	1	1029	1073	1278	1540
	2	1085	1086	1213	2110
	3	1356	1144	1229	1932
	4	1384	896	1050	1640
	5	1031	815	1183	1486
	6	962	1108	933	1386

2.5.2.2 P-glycoprotein expression

Figure 2.9 and Figure 2.10 shows that there was a significant sex difference ($p<0.05$) in the P-gp expression of rats under the fasted state conditions. It is shown that the P-gp along the male rat's GI tract gradually increased from duodenum to colon, while maintained at a low expression level along the GI tract in female rats.

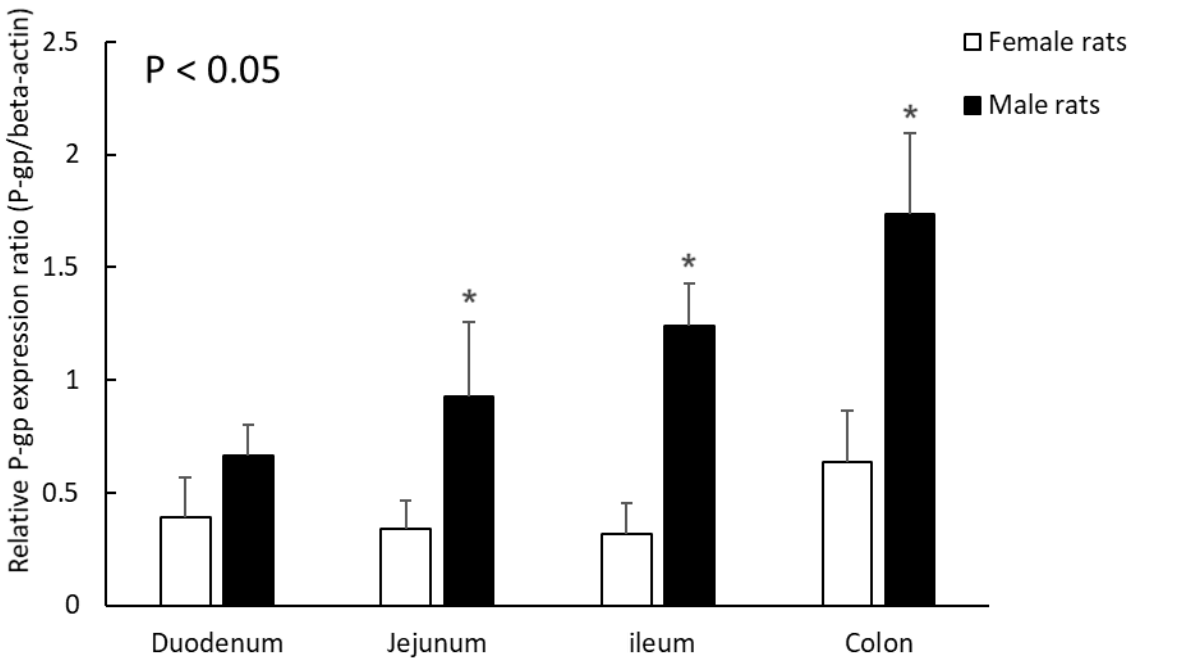


Figure 2.9 P-gp relative expression along the intestine tract of male and female rats in fasted state, n=6. *denotes a statistically significant ($p < 0.05$) between males and females.

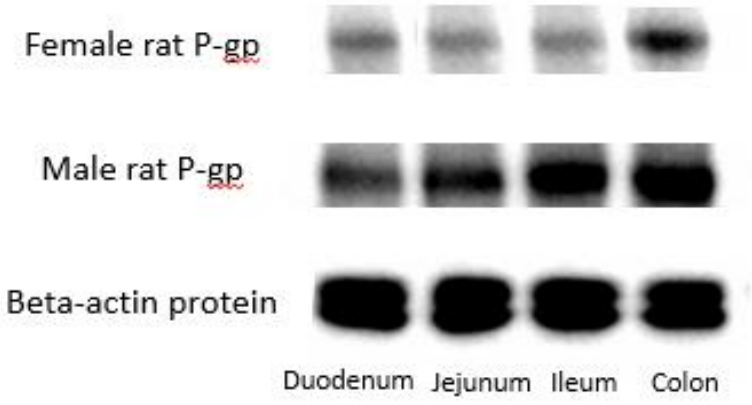


Figure 2.10 Western-blotting results of intestinal P-gp expression in fasted male and female rats.

As shown in Table 2.6, the relative expression of P-gp increased from the proximal to distal intestine in male rats were 0.66 ± 0.14 in the duodenum, 0.93 ± 0.33 in the jejunum, 1.24 ± 0.18 in the ileum and 1.73 ± 0.36 in the colon. Contrastingly, female rats displayed a relatively lower P-gp expression level and exhibited a significant ($p < 0.05$) sex difference,

especially in jejunum, ileum and colon (0.34 ± 0.13, 0.32 ± 0.14 and 0.63 ± 0.23 respectively).

Table 2.6 The relative expression of P-gp along the intestinal tract in male and female rats (n = 6, mean ± SD).

	Duodenum	**Jejunum**	**Ileum**	**Colon**
Female rats	0.39 ± 0.18	0.34 ± 0.13	0.32 ± 0.14	0.63 ± 0.23
Male rats	0.66 ± 0.14	0.93 ± 0.33	1.24 ± 0.18	1.73 ± 0.36

Interestingly, our results reported here contrast to the work of previous studies that have evaluated P-gp expression in rats. A study conducted by MacLean et al. (McConnell et al., 2008) demonstrated that there were no sex differences in P-gp expression along the intestine. In their study, the relative expression of P-gp in both male and female rats increased up to 5-fold from the proximal to distal intestine. The rat strains may contribute to this difference. In the current study, Wistar rats were bought from Harlan UK ltd. while rats used in Maclean et al study were Han-Wistar bought from Charles River. In a study, Sprague Dawley (SD) rat, another commonly used strain of rat, from Charles River laboratories (CRL) and Harlan Laboratories (HAR) were investigated and found that CRL rats showed higher body fat mass (49.6%), higher gross liver weights (22.2%), lower testicular weights (30.8%) and lower cholesterol levels (25.4%) than HAR rats (Brower et al., 2015). Moreover, the limitation of Western-blotting technique may be another reason. The relative expression of P-gp was determined using internal standard, housekeeping protein. In the current study, beta-actin was used as the internal standard, whereas villin protein was used to normalise the P-gp expression in the previous study. Apart from the study conducted by MacLean et al., the current work is different from another recent published work from our group. In the study, a

marked sex differences were also observed but in an opposite direction (Afonso-Pereira et al., 2018). From the duodenum to the ileum segments, female rats expressed a significantly higher P-gp level when compared with their male counterparts ($p<0.05$). In the colon, however, no significant differences in P-gp expression were observed, with both male and female rats exhibiting low levels. With the same strain of rats and same techniques, the difference between these two studies may due to the state of rats, fasted-state rats were used in current work whereas the fed-state rats were used in previous study. The published literature has provided extensive data supporting the food effect on P-gp regulation such as fruit juices, spices, herbs, cruciferous vegetables and so on (Zhang et al., 2009). However, the sex-related food effect on intestinal P-gp is lacking. Table 2.7 compared the current study with the previous studies. More work is required to understand the difference between the current studies and previous studies.

Table 2.7 Comparison of intestinal P-gp expression in rats from the different studies.

	Rat strain	**Technique**	**Rat states**	**Results**
MacLean et al., 2008	Han-Wistar from CRL	Western-blotting with villin	Fed state	No sex difference
Afonso-Pereira F et al., 2017	Wistar from HAR	Western-blotting with beta-actin	Fed state	Female expressed higher P-gp than male
Current study	Wistar from HAR	Western-blotting with beta-actin	Fasted state	Male expressed higher P-gp than female

A similar condition existed in humans, previous studies regarding the sex differences in intestinal P-gp function have been inconsistent. A study conducted by Mouly et al. (Mouly et al., 2003) investigated the P-gp expression level in the duodenum, the jejunum and the ileum obtained from four humans but did not mention sex differences as a variable. Potter. et al. reported that P-gp expression was higher in males when compared with females (Potter et al., 2004). Consequently, two studies were later published and claimed that there was no sex difference exhibited in P-gp intestinal expression. In one study, Paine. et al. investigated the P-gp expression in the proximal intestine (duodenum) in men and women (Paine et al., 2005). Results revealed that there were no significant sex differences, (mean P-gp expression was 0.66 vs. 0.73 in males and females, respectively). Another study explored the P-gp expression in the stomach, the jejunum and the ileum regions of the intestine (Canaparo et al., 2007). In all three portions, sex differences in P-gp expression were not observed.

Even through, more work is required to understand the inconsistent results among these studies, the work reported here provides an insight on the significant differences between the sexes and could provide an explanation for the previously reported sex difference in drug gut absorption. In Mai et al study, ampicillin and ranitidine, two P-gp substrates, were evaluated in fasted-state Wistar rats between sexes (Mai et al., 2017). For ampicillin, after oral administration, the $AUC_{0–480}$ was 528±52 μg·min/mL in male and 640±43 μg·min/mL in female, while was 350±33 μg·min/mL in male and 421±49 μg·min/mL in female for ranitidine. Furthermore, an *ex vivo* permeation study was conducted later by Mai et al to evaluate P-gp effect on the intestinal permeation of ranitidine (Mai et al., 2018). It was reported that a significant ($p<0.05$) higher permeation was noticed in female rats along jejunum, ileum and colon, which was highly correlated to the P-gp expression results in current study. In addition, the observed sex difference in P-gp expression in rats may also

partially explained the previously reported sex difference in verapamil and ivermectin gastrointestinal disposition (Lifschitz et al., 2006). Consequently, the data presented here shows that by failing to evaluate drugs in both male and female rat models, there may be an increased risk of inaccurately extrapolating pharmacokinetic data into humans. As P-gp is a biological membrane efflux transporter, which is capable of modulating the transmembrane activities of drugs in different organs (Morris et al., 2003), this could be particularly consequential if evaluating a novel drug that is a P-gp substrate.

2.5.3 Sex differences in P-gp mRNA expression along the GI tract

The expression of P-gp coding mRNA, mdr1a and mdr1b, along the GI tract in male and female Wistar rats were shown in Figure 2.11. In male rats, the expression of mdr1a gene experienced a high correlation to the P-gp expression along the intestine while mdr1b maintained a low expression. In terms of female rats, the relative expression of both mdr1a and mdr1b were similar and kept in a consistent low level along the GI tract. As mdr1a and mdr1b highly involved in the formation of intestinal P-gp, the relatively higher expression of mdr1a and mdr1b in male rats compared to their counterparts supported the sex difference in intestinal P-gp expression determined by Western-blotting experiment.

It can be noticed that the mdr1a gene expression was higher than the mdr1b gene expression in both male and female rats along the gut. And also, the expression of mdr1a gene reflected more closely the change of P-gp protein expression along the intestine. The reason was due to the fact that mdr1a is the major coding gene for intestinal P-gp protein in rodents, which exhibited a higher expression compared to the mdr1b gene (Brady et al., 2002; MacLean et al., 2008).

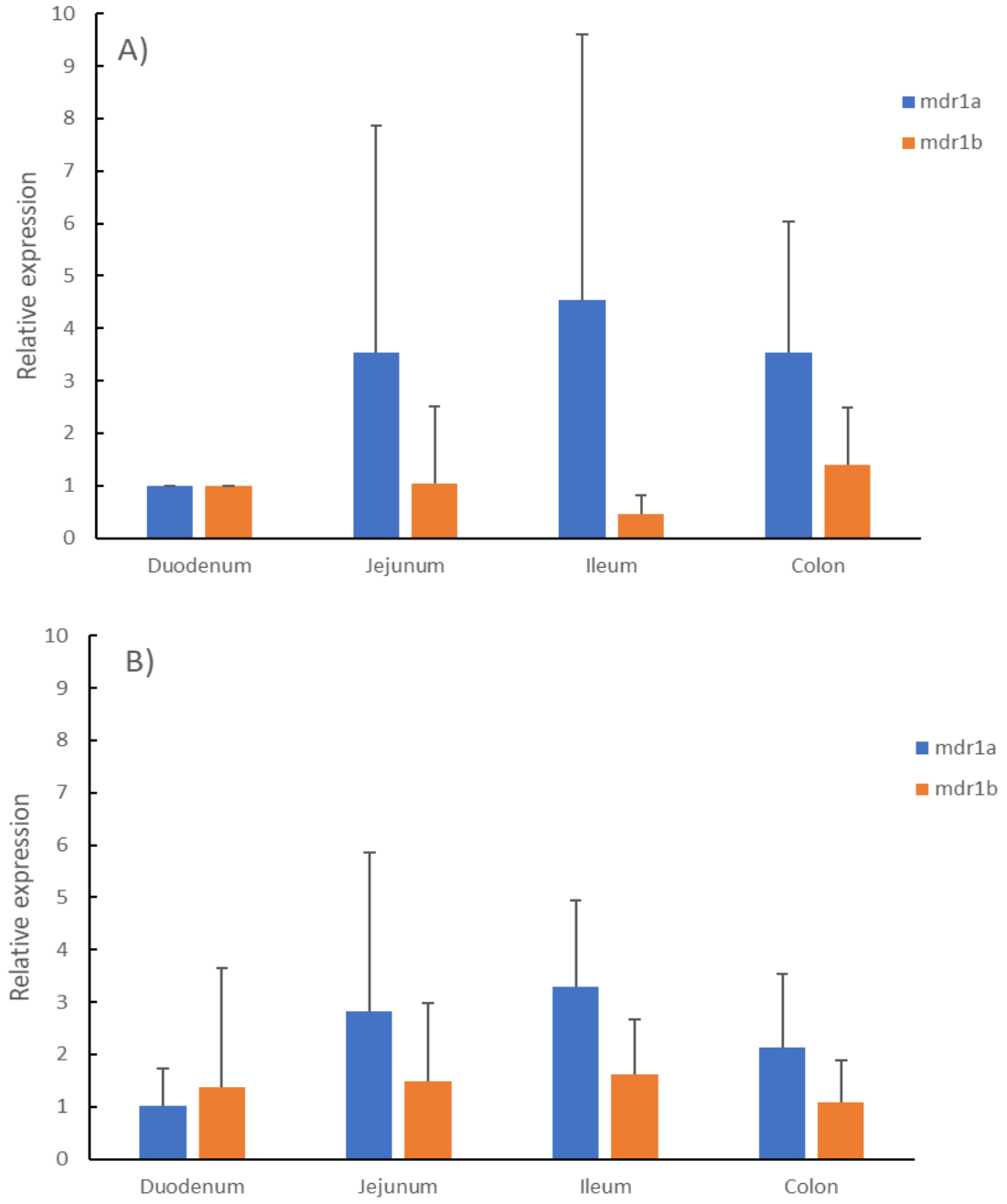


Figure 2.11 Relative expression of intestinal P-gp mRNA mdr1a and mdr1b gene in the fasted state of A) male and B) female rats (n = 6).

2.6 Conclusion

GI fluid pH, buffer capacity, surface tension and osmolality were found to be similar in the sexes. Only one significant difference was observed in duodenal buffer capacity. In terms of P-gp expression, marked sex differences were observed along the intestinal tract. Apart from duodenum, male rats exhibited a significant higher expression ($p<0.05$) of P-gp when compared with their female counterparts in jejunum, ileum and colon. In the duodenum, however, no significant differences in P-gp expression were observed, with both male and female rats exhibiting low levels. P-gp mRNA expression correlated to the P-gp protein expression, especially the mdr1a gene, along the intestinal tract. The presented study firstly reported the sex difference in gut physiology of rat, which contribute towards an increased understanding of how the GI environment and oral drug absorption is innately affected by sex. However, due to the conflict results with previous studies, more work is still needed for elucidating the participation of food as well as strain factors in the sex-related P-gp expression in rat.

CHAPTER 3:

Food, time and sex influence on rat's intestinal P-glycoprotein

3.1 Introduction

3.1.1 The effect of food and time on intestinal P-glycoprotein

3.1.1.1 External influence: Food consumption

It is widely known that food-drug interactions play a crucial role on drug performance. As such, the FDA introduced a guidance entitled "Food-Effect Bioavailability and Fed Bioequivalence Studies" for industries to understand the influence of food intake on candidate drug products (Harris et al., 2003; FDA guideline, 2002). In the intestine, food and its subsequent digestion affect drug absorption via two possible pathways; i) chemically interacting with the drug substance and/or; ii) changing the absorption environment itself (for example altering the physiological features of intestinal luminal) (Custodio et al., 2008). As an essential protective component in the intestinal luminal environment, P-gp heavily participates in the regulation of intestinal drug absorption. A number of studies have since been dedicated towards the understanding of food effects on P-gp activity.

An example of this is the investigation of fatty acids on the function of intestinal P-gp in rats. In a study by Vine *et al*., two rat group were fed with a standard diet and a diet with the addition of 18.4% (w/w) lipid respectively (Vine et al., 2002). Following a 30-day dietary period, jejunal segments were excised and mounted on Ussing chambers to evaluate the permeation of several drugs including mannitol, diazepam, glucose and digoxin. The study reported that in lipid-fed rats, the efflux transport of digoxin, a P-gp substrate, decreased by 20% when compared with standard-fed rats. Moreover, a study including ten volunteers reported the influence of chargrilled meat on intestinal (duodenum) P-gp expression in both protein and mRNA level (Fontana et al., 1999). Although it was claimed that there was no change on the expression of P-gp after twelve-day consumption of chargrilled meat, a larger variability in P-gp expression among the twelve individuals could be observed (Figure 3.1).

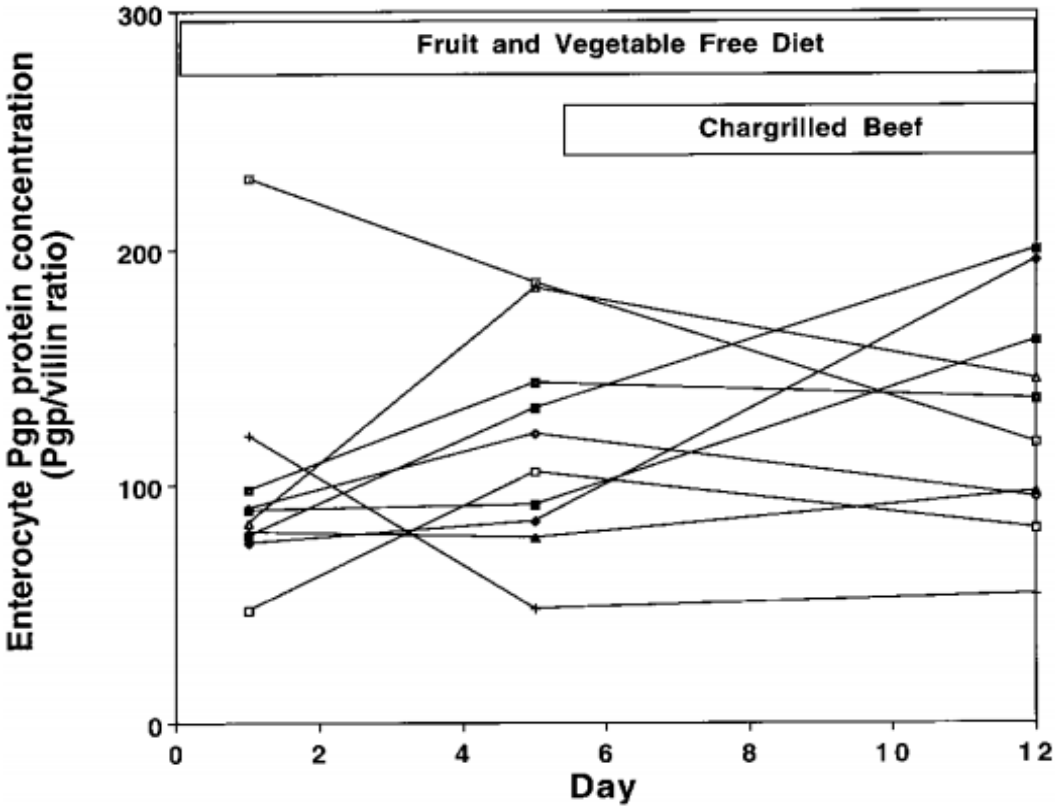


Figure 3.1 The effect of a chargrilled meat diet on enterocyte (duodenum) P-gp expression among 12 volunteers. The intestinal biopsy specimens were collected at the first, fourth and last day of the experiments, adapted from Fontana *et al*., 1999.

Apart from general food, the effect of certain food components have also been demonstrated to effect the expression of intestinal P-gp. In a study conducted by Okura *et al*., several dietary ingredients including piperine, capsaicin, daidzein, genistein, sesamin, curcumin and taurine were investigated for their modulatory effect on P-gp function via an *in vitro* LS-180 cell line (Okura et al., 2010). The accumulation of rhodamine 123, a P-gp substrate, in a LS-180 cell line significantly increased in the presence of capsaicin, piperine and sesamin but decreased with daidzein and genistein. These results suggest that capsaicin, piperine and sesamin may inhibit P-gp efflux activity whereas daidzein and genistein stimulated P-gp function to increase its efflux effect. In addition, it was reported that certain natural compounds extracted from fruits displayed a modulatory effect on the transport of P-gp substrates (Deferme et al., 2002).

In the aforementioned study, 68 standardised fruit extracts were screened based on the secretory transportation effect of cyclosporine A in a Caco-2 cell line; strawberry, mint, orange and apricot extracts showed a significant up-regulation on cyclosporine A efflux transport, although no modulatory effects were observed in the presence of grapefruit extract in the efflux of cyclosporine A across the Caco-2 monolayer. Table 3.1 highlights examples of certain food compounds or fruit extracts capable of modulating P-gp function.

Table 3.1 The examples of food effect on P-gp function, adapted from Deferme *et al*, 2003

Product	Effect on P-gp	Study model	References
Green tea	Inhibition	In-vitro (rhodamine-123, vinblastine)	Jodoin et al 2002
Rosemary extract	Inhibition	In-vitro (doxorubicin, vinblastine)	Plouzek et al 1999
St John's wort	Induction	In-vitro (rhodamine-123)	Perloff et al 2001
		In-vivo (digoxin)	Johne et al 1999; Durr et al 2000
Grapefruit juice	No effect	In-vivo (digoxin)	Becquemont et al 2001
		In-vitro (saquinavir)	Eagling et al 1999
	Stimulation	In-vitro (vinblastine, digoxin, ciclosporin A)	Soldner et al 1999
	Inhibition	In-vivo (ciclosporin A, talinolol, dextromethorphan)	Edwards et al 1999; Spahn-Langguth & Langguth, 2001; Di Marco et al 2002
		In-vitro (vinblastine, rhodamine-123, saquinavir)	Ohnishi et al 2000; Takanaga et al 1998; Tian et al 2002
Grapefruit extract (standardized)	No effect	In-vitro (ciclosporin A)	Deferme et al 2002a
Seville orange juice	No effect	In-vivo (ciclosporin A)	Malhotra et al 2001; Edwards et al 1999
	Inhibition	In-vitro (vincristine, vinblastine)	Ikegawa et al 2000; Takanaga et al 2000
		In-vivo (dextromethorphan)	Di Marco et al 2002
Orange juice	Inhibition	In-vitro (rhodamine-123, saquinavir)	Tian et al 2002
	No effect	In-vivo (ciclosporin A)	Johnston et al 1986
Orange extract (standardized)	Inhibition	In-vitro (ciclosporin A)	Deferme et al 2002a
Garlic extract (aqueous)	No effect	In-vitro (P-gp ATP-ase activity)	Foster et al 2001
Chargrilled meat	No induction	In-vivo (mRNA levels in intestine)	Fontana et al 1999
Strawberry extract (standardized)	Inhibition	In-vitro (ciclosporin A)	Deferme et al 2002a; Van Gelder et al 2002
Dietary fatty acids	Inhibition	In-vitro (digoxin)	Vine et al 2002
Milk	No effect	In-vivo (ciclosporin A)	Johnston et al 1986
Anastasia green (sweet pepper)	Inhibition	In-vitro	Motohashi et al 2001
Piperine (black pepper)	Inhibition	In-vitro (digoxin, ciclosporin A)	Bhardwaj et al 2002
Peppermint oil	Inhibition?	In-vivo (increased absorption of ciclosporin A, not due to metabolism inhibition)	Wacher et al 2001
Mint extract (standardized)	Inhibition	In-vitro (ciclosporin A)	Deferme et al 2002a
Apricot extract (standardized)	Inhibition	In-vitro, ex vivo, in situ (talinolol)	Deferme et al 2002b

3.1.1.2 Internal influence: The factor of time

The circadian timing system (CTS) is considered to be an innate 'body clock' displayed by all living organisms upon which a number of essential physiological processes and cellular functions follow. In May 2016, with the support of National Institutes of Health R13, the first symposium on "Circadian Rhythms in GI Health and Disease" was successfully held in Chicago. Following the symposium, a resounding consensus was achieved in which the individual's biological clock plays a crucial role in maintaining GI metabolic homeostasis (Bishehsari et al., 2016). As such, the factor of time may be a contributing parameter to influence intestinal P-gp variation in animals and humans (Okyar et al., 2012).

A study by Kervezee *et al*. demonstrated that the concentration of quinidine (a P-gp substrate) in the brain highly correlated to the time of day in male rats (Kervezee et al., 2014). Moreover, in an *in situ* perfusion study by Okyar *et al*., the intestinal permeability of talinolol and losartan (both P-gp substrates) were higher in the daytime than that of the night (Okyar et al., 2012). The permeability of talinolol in the ileum was significantly ($p < 0.05$) lower in the night time compared to that of in the day ($14.2 \pm 2.36 \times 10^{-5}$ and $20.8 \pm 1.64 \times 10^{-5}$ cm/s respectively). Jejunal perfusions performed in the night also displayed significantly lower permeability values ($p < 0.05$) of $14.6 \pm .79 \times 10^{-5}$ cm/s versus $19.9 \pm .93 \times 10^{-5}$ in the day respectively. The intestinal permeability of losartan was also found to be time-dependent. In experiments conducted in the day, permeability values were $10.7 \pm .50 \times 10^{-5}$ and $13 \pm 1.04 \times 10^{-5}$ cm/s in ileum and jejunum. These values, however, reduced to $8.18 \pm .75 \times 10^{-5}$ and $3.60 \pm 1.35 \times 10^{-5}$ cm/s during night time perfusions. Time-related changes of the pharmacokinetics of P-gp substrates may due to the time-related expression of P-gp. It was also demonstrated that the intestinal expression of the mdr1a gene in mice was influenced by the innate circadian organisation of the molecular clockwork (Murakami et al., 2008). In their study, the

concentration of mdr1a gene began to increase from 6:00 am and peaked in its expression at approximately 10:00 am. A decreasing trend was found in mdr1a expression towards the night time. In addition to the rat, monkey models also display P-gp variation dependent on the CTS. Iwasaki *et al*. demonstrated that the Abcb1 gene, the main coding gene for P-gp in the monkey, exhibited the highest expression level at 9:00 am when compared with the rest of the day (Iwasaki et al., 2015). In humans, a recent study reported that half of the mammalian protein-coding genome was influenced by the circadian clock albeit tissue-specific (Ruben et al., 2018).

Following the recent knowledge that 1) intestinal physiology is different between the two sexes and 2) external and internal influences such as foodstuff and the circadian rhythm can modulate P-gp expression in Chapter 2, it is therefore important to raise the research question on whether food and time can effect intestinal P-gp expression differently in males and females. Among the aforementioned studies, however, the investigation of food and time as experimental factors were mostly based on *in vitro* cell line model and/or male-bias *in vivo* experiments. The information provided was therefore insufficient to link the observed results to sex differences. As such, there is a lack of understanding on the influence of food and the innate circadian rhythm on the intestinal expression of P-gp in male and female rats, which is the essential information for drug pre-clinical study as well as linking the pre-clinical and clinical processes during the drug development.

3.1.2 P-gp quantification techniques

3.1.2.1 Limitations of Western-blotting

In biological-related research, the Western blotting technique is an analytical method ubiquitously used for the quantification of protein and RNA. However, certain limitations arise in the application of Western blotting. Firstly, the protein quantification obtained is only

relative to the reference protein. During the protein detection process, both the target protein and reference protein is detected. As such, the targeted protein can only be calculated by comparing its single value to the reference protein signal which is set as 1. The reference protein, also known as the housekeeping protein, is considered to be ubiquitously and constitutively expressed in every tissue, being essential for the maintenance of normal cellular function (Zhang and Li, 2004). Commonly used reference proteins include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin and villin (Ferguson et al., 2005). For example, villin was used as the reference protein for the determination of intestinal P-gp in studies conducted by MacLean *et al*. and Vaessen *et al*. (Vaessen et al., 2017; MacLean et al., 2008), whilst beta-actin was employed in the study by Johnson *et al*. (Johnson et al., 2006). However, a study used villin as a reference protein to assess P-gp expression in 14 human intestinal tissues despite the expression of villin mRNA being significantly low in the colon when compared to that in the small intestine ($p < 0.05$) (Englund et al., 2006). Kovalenko *et al*. confirmed the validity of the aforementioned study and further investigated villin protein expression in a rat model. Villin was consistently expressed along the intestine except colon where it was significantly lower than small intestinal segments ($p < 0.05$) (Kovalenko et al., 2013). The expression of beta-actin mRNA, however, is increased in some pathological conditions such as the presence of tumours in the kidneys, spinal injury and alcoholic hepatitis but decreases with age (Li and Shen, 2013). In addition, the complex procedure of Western blot analysis can introduce variability in the acquired results; following a Western blot experiment to quantify OATPs in HEK293 cells, Oswald *et al*. demonstrated a large variation in OATP relative expression within a day of conducting the experiment with coefficient of variations ranging from 9.6% to 94.6% (Oswald et al., 2013). One possible reason is the uncertain specificity of antibody binding process such as the cross-reactivity of primary antibody with other proteins or potentially a lack of protein functionality (Alegria-Schaffer et al., 2009; MacPhee, 2010).

Consequently, due to the various reference proteins available and multi-step analyses for the Western blot technique, relative quantification results are not precise. As such, intestinal protein expression levels obtained from different conditions such as the fasted or and fed-state, as well as potential effect of time, cannot be comparable.

3.1.2.2 Novel LC-MS/MS technique for P-gp absolute quantification

Mass spectrometry (MS) is a widely used technique in the field of life sciences. MS is an analytical tool that measures the mass-to-charge ratio of ions and a mass spectrum is a plot of the ion signal as a function of the mass-to-charge ratio. The spectra then can be used to determine the elemental or isotopic signature of a sample, the masses of particles and molecules, or to elucidate the chemical identity or structure of molecules and other chemical compounds. MS based protein research, however, has grown tremendously over the past 15 years and strongly advanced the development of biochemistry and cell biology studies (Gstaiger and Aebersold, 2009). Among these developments include the liquid chromatography-tandem MS based targeted protein quantification technique (LC-MS/MS) which is now a fundamental tool for the determination of absolute protein expression (Liebler and Zimmerman, 2013). Albeit a long and complicated experimental procedure in comparison to traditional immunochemical methods, LC-MS/MS provides sensitive and precise results (Figure 3.2).

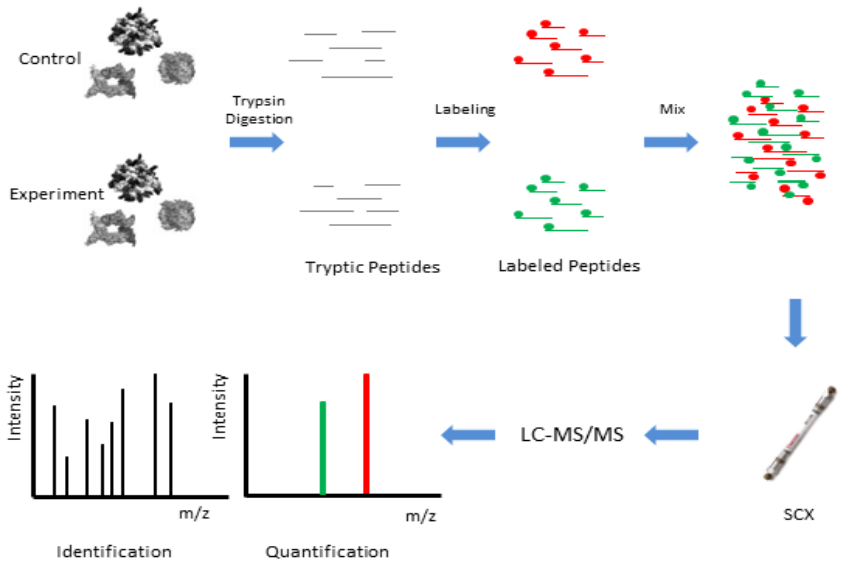


Figure 3.2 The principle of LC-MS/MS based protein quantification technique

The LC-MS/MS experiment consists of five procedures including; protein digestion, peptide separation, peptide monitoring and protein quantification. The protein matrix, which includes the target protein, is firstly digested to form a peptide matrix. One peptide with a specific amino acid sequence among all the generated peptides is selected as the target peptide based on its exclusivity. This means that only targeted protein is generated following the protein digestion procedure. For example, the peptide with the sequence AGAVAEEVLAAIR was applied in the study for human intestinal P-gp quantification (Groer et al., 2013). Liquid chromatography (LC) is then applied for the basic separation of the peptides matrix. The eluted target-peptide is then detected with tandem MS. The sensitive and precise data acquisition is mainly guaranteed by the MS detection principle shown in Figure 3.3 (Uchida et al., 2013); the eluted peptides is ionised under a very high-energy ion source. The target peptide and other proteins with similar mass will obtain a certain mass-to-charge ratio (*m/z*). As such, a filter to screen other ionised particles can be applied. The ionised target peptide and similar proteins can then be disrupted into multiple ionised sequences in the collision chamber. Sequences with a certain

m/z ratio generated from the ionised target peptide will be filtered out for detection through the third *m/z* ratio filter. By measuring the targeted peptide from the samples, the targeted protein then can be quantified correspondingly. In LC-MS/MS, an isotope-labelled target peptide is used as the internal standard to minimise procedure variability. For example, in the study by Gröer *et al*., the internal standard used was AGAVAEEVLAAIR* where Arg (R) was labelled with C-13 and N-15 (Groer et al., 2013).

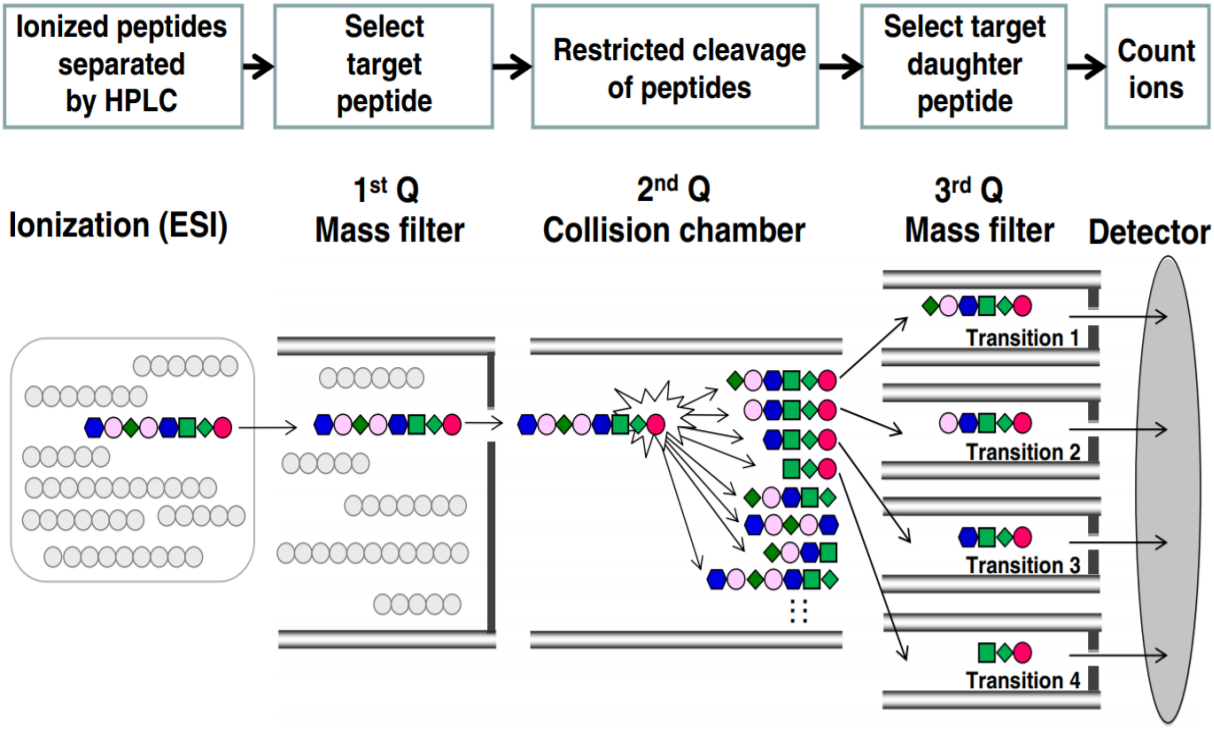


Figure 3.3 Principle of peptide selection by LC-MS/MS, adapted from Uchida et al., 2013.

MS-based targeted protein assays provide a number of compelling advantages over the Western blotting technique for the quantification of intestinal P-gp. The first advantage is being able to determine the absolute expression of any protein. Due to the specificity of mass spectrometry, the calibration curve of a target protein can be established based on its concentration alone and thus, without the need of a reference protein. This can consequently avoid any variation in protein acquisition. In addition, without the involvement of antibody, LC-MS/MS is highly

sensitive and has the ability to configure a specific assay for any protein. Moreover, it allows large-scale quantitative proteomic analysis projects based on its detection mechanism which make it more efficient than Western blotting.

Table 3.2 Comparison of Western blotting and LC-MS/MS for protein quantification

	Western blotting	**LC-MS/MS**
Principle	Ligand-binding	Mass spectrometry
Sample preparation	Protein denaturation	Protein denaturation and digestion
Separation	Gel electrophoresis	HPLC
Detection	Antibody binding	Mass spectrometry
Quantification	Relative quantification using reference protein	Absolute quantification using calibration curve
Variability	High	Low
Sensitivity	Low	High
Efficiency	Low	High

3.2 Aims

Phase I

- To develop and validate a LC-MS/MS method for the absolute quantification of intestinal P-gp in rats

- To investigate the food and sex effect on intestinal P-gp expression by quantifying the absolute expression of P-gp in male and female rats under the fasted or fed-state via the validated LC-MS/MS method

Phase II

- To understand the food and sex effect on intestinal permeation of P-gp substrates by evaluating the *ex vivo* permeability of ranitidine, ganciclovir and metformin in rats via the Ussing chamber method

- To explore the influence of time by investigating intestinal P-gp expression at different time points in both male and female rats via using the validated LC-MS/MS method

3.3 Material

Ranitidine (a P-gp and organic cation transporter (OCT) substrate) and ganciclovir (a P-gp substrate) were purchased from Sigma Aldrich (Dorset, UK). Metformin hydrochloride (an OCT protein substrate) was obtained from USV Ltd. (Mumbai, India). The standard peptide for P-gp quantification and its stable isotope-labelled internal standard were of analytical grade (purity > 95%) were synthesised and quantified via the amino acid analysis by Sigma AQUA peptides service (Poole, Dorset, UK). Iodoacetamide (CAS: 144-48-9), dithiothreitol (CAS: 3483-12-3), MS-Grade trypsin (CAS: 9002-07-7) and formic acid (CAS: 64-18-6) were bought from Thermo Fisher (UK). Ammonium bicarbonate BioUltra, chloroform, as well as the LC-MS grade acetonitrile and water were obtained from Sigma (Dorset, UK).

Phase I

3.4 Method

3.4.1 Experimental animal

12 male and 12 female Wistar rats (10 weeks old weighing approximately 250 g and 200 g respectively) were purchased from Harlan UK Ltd. (Oxfordshire, UK) and housed at room temperature of 25°C in a light-dark cycle for 12 h. Fed state rats (6 male and 6 female) were provided with free access to food (EURodent Diet 22%) and water. Fasted state rats (6 male and 6 female) were subject to an overnight fasting of 12 h prior to the experiment.

3.4.2 Intestinal tissue preparation

On the day of experiment, the rats were sacrificed by a CO_2 euthanasia chamber at approximately 8:30 am. The whole GI tract from duodenum to colon were then rapidly removed and kept in an ice-bath filled with Krebs-Bicarbonate Ringer's solution (KBR) at pH 7.4. Then the GI tract was cut into different segments: duodenum (1 cm from the ligament of Treitz); jejunum (10 cm from the ligament of Treitz); ileum (1 cm from the cecum); and descending colon. 1 cm tissue segments from the mid-part of the duodenum, the proximal part of the jejunum, the distal to mid-part of ileum and the descending colon were separated. The separated tissues were then opened along the mesenteric border and the mucosal layer was obtained by gently squeezing the serosal-side of tissue with a cover slip on ice-cold glass plate. The prepared tissue with the mucosal layer was then freshly used for the following studies.

3.4.3 LC-MS/MS study

3.4.3.1 Total protein extraction and quantification

The prepared tissue from Section 3.3.2 was placed into a glass vial containing 3 ml of freshly prepared lysis buffer (50 mM Tris, 250 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF,

1% Nonidet P40 and a protease inhibitor cocktail), and homogenised for 20 s at 10,000 rpm with a T18 digital ULTRA-TURRAX® (IKA). The homogenised tissue solution was then incubated in a 4ºC fridge for 2 h for protein extraction. Two hours later, the solution was transferred to a 1.5 ml Eppendorf tube and centrifuged with 10,000 rpm at 4ºC for 10 min. The supernatant was transferred to micro-tubes and stored at –20ºC which is stable up to 6 months. As described in Chapter 2 method, the total protein was determined following the instruction adapted from the Pierce BCA Protein Assay Kit (ThermoFisher, UK).

3.4.3.2 Protein sample digestion

50 µg total protein from the extraction sample was adjusted to 200 µl protein solution with the 50 mM ammonium bicarbonate buffer and then mixed with 4 µl dithiothreitol (20 mM). The mixed solution was then incubated for 20 min at 56ºC for the protein denaturation. After a cooling down period, alkylation was carried by adding 8 µl iodoacetamide (375 mM) and incubated for 20 min at 37 ºC in the dark. The precipitation procedure was then conducted. 600 µl cold methanol and 150 µl cold chloroform was added to the sample solution. After several tube inversions, 450 µl cold water was added followed by immediately centrifuging the sample at 15,000 rpm for 5 min at 4 ºC. After centrifugation, the upper layer (the floating protein pellet) was removed. An additional 450 µl cold methanol was added and prior to tube inversion to wash the protein pellet. The sample was then centrifuge again at 15,000 rpm for 5 min at 4ºC. Immediately after centrifugation, the supernatant was removed and then centrifuge the sample at 15,000 rpm for 1 min at 4ºC. The supernatant was then removed completely. 47 µl ammonium bicarbonate buffer (50 mM) was added to the precipitated protein pellet and resuspended by applying intermittent sonication. 5 µl trypsin solution (0.5 µg/µl) was added to the resuspend protein solution and incubated for 4 hours at 37ºC. The digestion process was halted by adding 3 µl 50% formic acid in water and 5 µl stable isotope-labelled internal standard

(200 fmol/µl) was added. The final processed sample solution (60 µl) was then centrifuged at 15,000 rpm for 5 min at 4ºC and 30 µl supernatant was then directly injected on LC-MS/MS. All sample digestion procedure steps were processed using Protein Lobind tubes (Eppendorf, Hamburg, Germany).

3.4.3.3 LC-MS/MS analysis

The P-gp proteotypic peptide and its three multiple reaction monitoring (MRM) transitions have been selected based on a published study shown in Table 3.3 (Groer et al., 2013). Calibration curves were established for all three transitions and the P-gp absolute quantification obtained from the mean values of the three calibration curves data. An Agilent 6460 triple quadrupole LC and mass spectrometer system coupled with Agilent Jet Stream technology was applied for the analysis (Agilent Technologies, Santa Clara, CA, USA). A gradient elution was applied on a Kinetex C18 column (100 X 3.0 mm, 2.6 µm, Phenomenex, Torrance, CA). The mobile phases were 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) with a flow rate of 0.5 ml/min. The gradient elution procedure started with 98% solvent A for 5 min and a linear gradient of 98% solvent A to 75% solvent A over 10 min, held at 75% solvent A for 1 min and then changed to 55% solvent A for extra 2 min. 98% of solvent A was then left running for 7 min until the end of analysation. The mass spectrometer was equipped with the electrospray ionisation and operated in the positive ion mode to monitor the three m/z transitions with 300ºC source temperature, nebuliser 45 psi, 11 L/min sheath gas flow, 500 V nozzle voltage, 20 collision energy as well as 7 cell accelerator voltage. All the chromatograms were assessed with the MassHunter Workstation software (Qualitative Analysis version B.06.00) and all samples were analysed in duplicate.

Table 3.3 Tryptic proteospecific peptide and its respective ions and mass transitions used for the P-gp absolute quantification (*isotope-labelled amino acid; the labelling of Arg (R) was done by introducing C-13 and N-15)

Molecule name	Peptide sequence	Mass	St/IS	Transition number	Q1 m/z	Q3 m/z	Dwell time (msec)	Collison Energy
ABCB1 (P-gp)	AGAVAEEVLAAIR	1268.7	Standard	1	635.3	771.3	20	20
				2	635.3	900.5	20	20
				3	635.3	971.6	20	20
	AGAVAEEVLAAIR*	1278.6	Internal Standard	1	640.3	781.4	20	20
				2	640.3	910.5	20	20
				3	640.3	981.5	20	20

3.4.3.4 LC-MS/MS method validation

The developed LC-MS/MS method for intestinal P-gp absolute quantification in the current study was validated in the following; Specificity and linearity were checked via calibration curves. The calibration curves were established by spiking the blank human serum albumin (HAS) with the standard synthetised peptide and its isotope-labelled internal standard. Initially, 1 mg/ml blank human serum albumin (HAS) was prepared and then processed for protein digestion following the description in Section 3.4.3.2. Standard peptide solutions (STDs) and internal standard peptide solution (IS) was prepared as shown in Table 3.4. The stock solution of both standard peptide and internal standard peptide was 5000 fmol/µl providing from Sigma AQUA peptides service. Different from the 3.4.3.2, 42 µl ammonium bicarbonate buffer (50 mM) was added to re-suspend the protein. 5 µl IS and STDs were added in the final analysis solution to achieve the injected amounts on column.

Table 3.4 Preparation of STDs and IS for calibration curve establishment

	0.1 % formic acid water	**Stock/STDs**	**Solution concentration**	**Volume added to HSA solution**	**Injected amounts**
STD1	120 µl	5 ul standard stock	200 fmol/ µl	5 µl	500 fmol
STD2	60 µl	60 µl STD1	100 fmol/ µl	5 µl	250 fmol
STD3	60 µl	60 µl STD2	50 fmol/ µl	5 µl	125 fmol
STD4	60 µl	60 µl STD3	25 fmol/ µl	5 µl	62.5 fmol
STD5	60 µl	60 µl STD4	12.5 fmol/ µl	5 µl	31.25 fmol
STD6	60 µl	60 µl STD5	6.25 fmol/ µl	5 µl	15.625 fmol
IS	120 µl	5 µl internal stock	200 fmol/ µl	5 µl	500 fmol

Intra-day and inter-day variation were assessed with the quality control samples (QCs). Low QC, medium QC and high QC were prepared by spiking the HAS with the standard peptide at

31.25, 125 and 250 fmol as injected amounts respectively. 500 fmol internal standard was also added in all QC samples. The intra-day variation was then assessed by analysing the QCs in the morning and afternoon on the same day, while the intra-day variation was evaluated by measuring the QC samples in three consecutive days.

Short-term stability was assessed by analysing the QC samples after 2 hours preparation at room temperature. The post-preparative stability was investigated with the samples 24 hours store at 4^{o}C after preparation. The process stability during the incubation was also evaluated by measuring the QC samples after 4 hours incubation at 37^{o}C.

Digestion efficiency was evaluated with two jejunum samples, one from male rat and one from female rat. The sample contained 100 μg protein was processed following the description in Section 3.4.3.2. In the incubation procedure, different duration times including 4, 8, 16 and 24 hours were investigated to assess if P-gp concentration differed in terms of digestion efficiency between the different incubation times.

3.4.4 Statistical analysis

The results generated from LC-MS/MS method were expressed as mean ± SD (n = 6) and analysed by one-way ANOVA and followed by a Tukey post-hoc analysis with a 95% confidence interval using IBM SPSS Statistics 16 (SPSS Inc., Illinois, USA).

3.5 Results and discussion

3.5.1 LC-MS/MS validation

3.5.1.1 Specificity and linearity

As shown in Figure 3.4, Figure 3.5 and Figure 3.6, the current method demonstrated a good specificity for determining both standard peptide and internal peptide in HSA matrix. In all three monitoring transitions, the peptide peaks were well separated, with no interference peaks detected in the vicinity of the retention times.

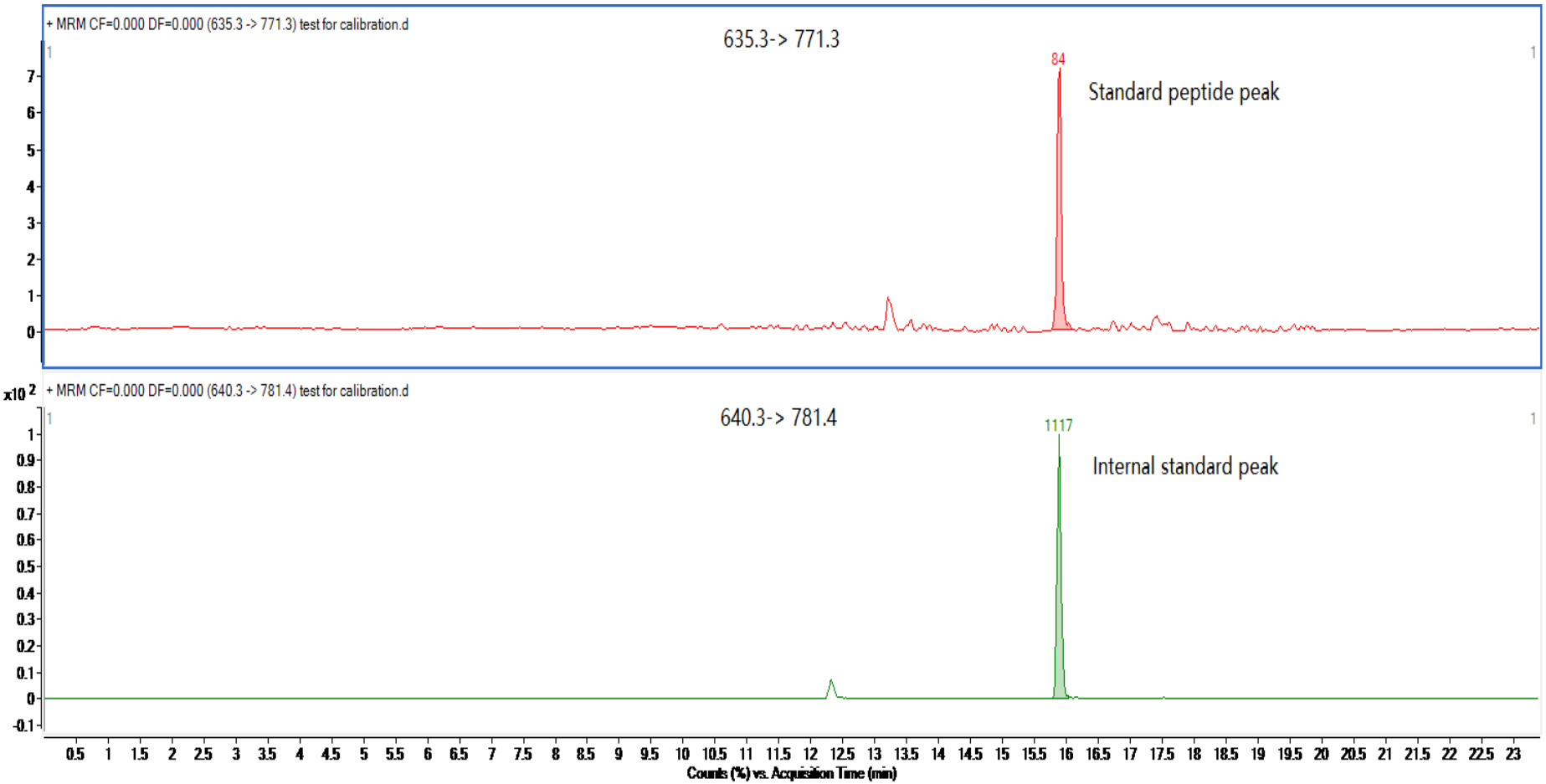


Figure 3.4 MRM chromatograms for the developed LC-MS/MS method applied to measure the first transition of proteotypic peptides (top) and their stable isotope labelled internal standard peptides (bottom) from the spiked HSA matrix.

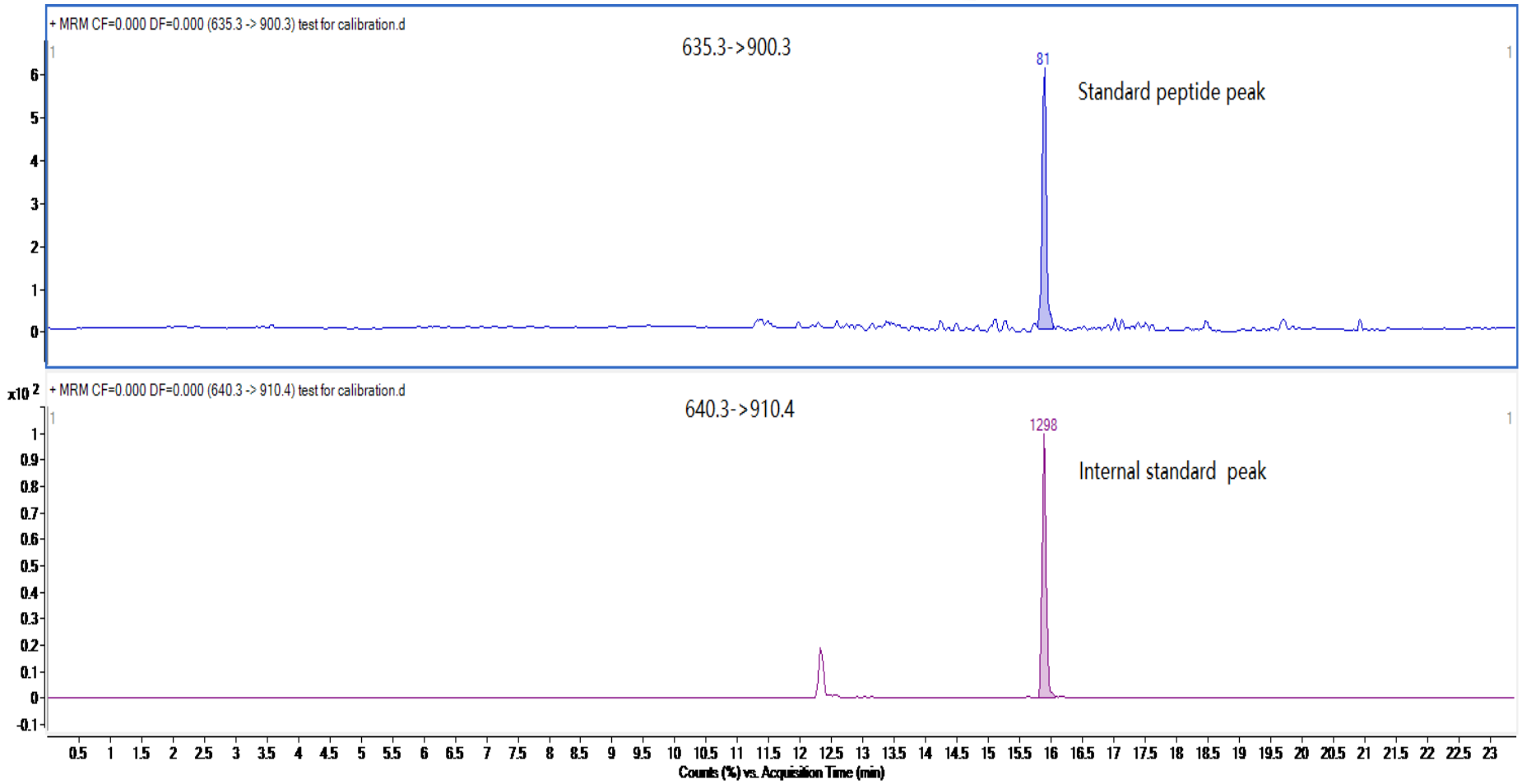


Figure 3.5 MRM chromatograms for the developed LC-MS/MS method applied to measure the second transition of proteotypic peptides (top) and their stable isotope labelled internal standard peptides (bottom) from the spiked HSA matrix.

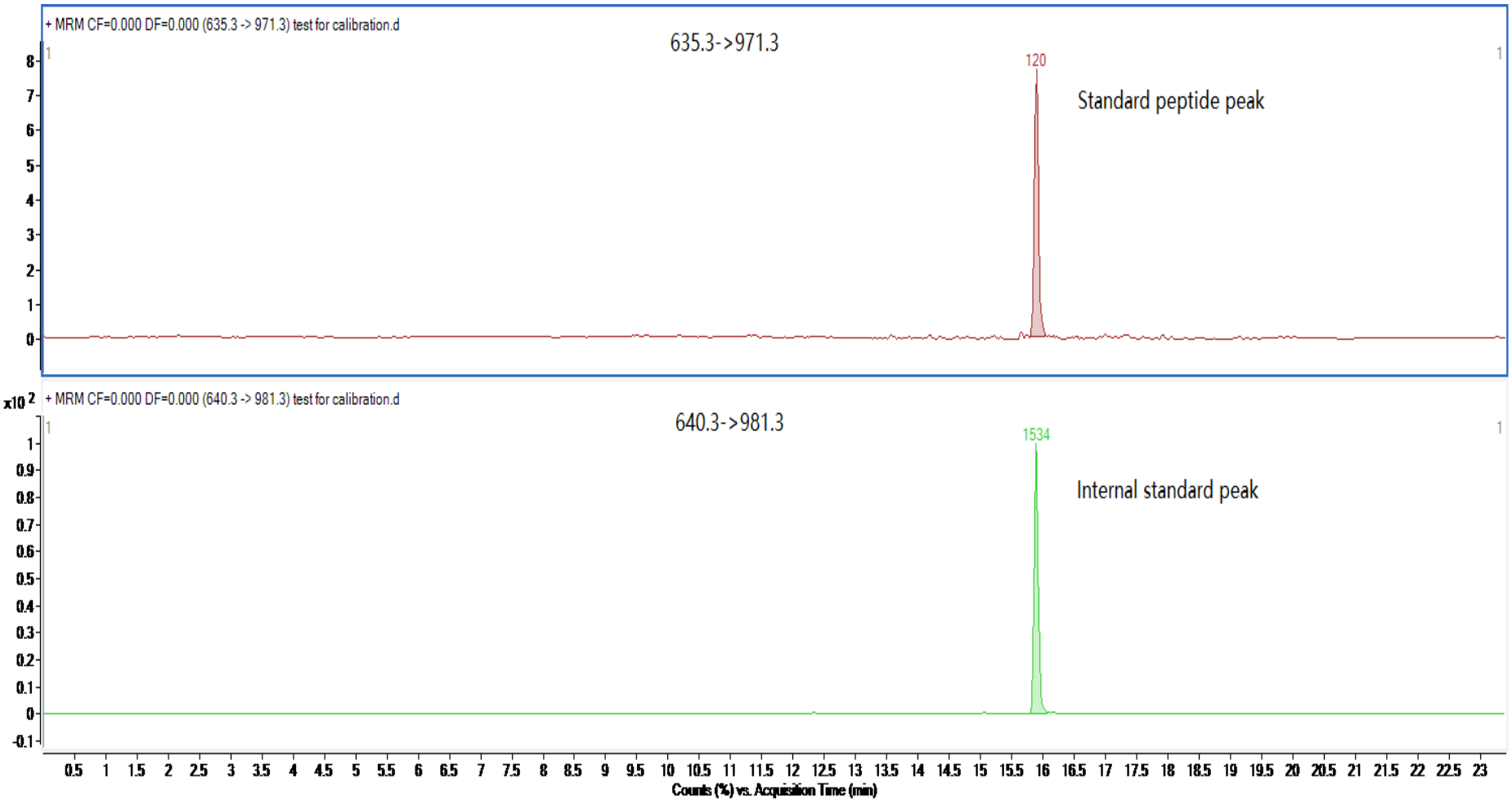


Figure 3.6 MRM chromatograms for the developed LC-MS/MS method applied to measure the third transition of proteotypic peptides (top) and their stable isotope labelled internal standard peptides (bottom) from the spiked HSA matrix.

The linearity within the range listed in Table 3.4 was investigated and shown in Figure 3.7, Figure 3.8 and Figure 3.9. For all three transitions, the regression value (R2) of calibration curves were above 0.995 and were 0.9996, 0.9966 and 0.998 for transitions number 1 to 3 respectively. Therefore, the current method demonstrated a good linearity for the P-gp specific peptide quantification within the range from 15.625 to 500 fmol.

Standard transition	Internal transition	Slope	Intercept	Regression
635.3/771.3	640.3/781.4	0.0017	0.0012	0.9996

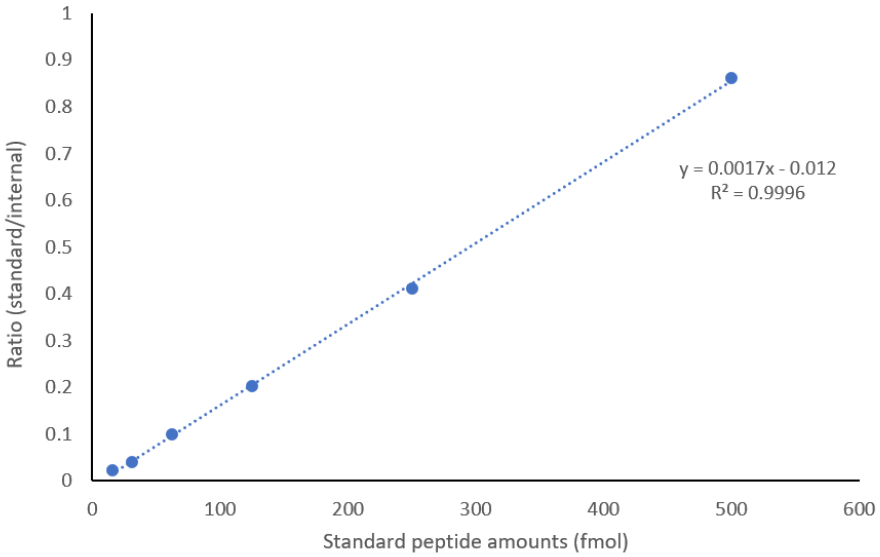


Figure 3.7 Calibration curve for monitoring the first transition

Standard transition	Internal transition	Slope	Intercept	Regression
635.3/900.5	640.3/910.5	0.0018	0.01	0.9966

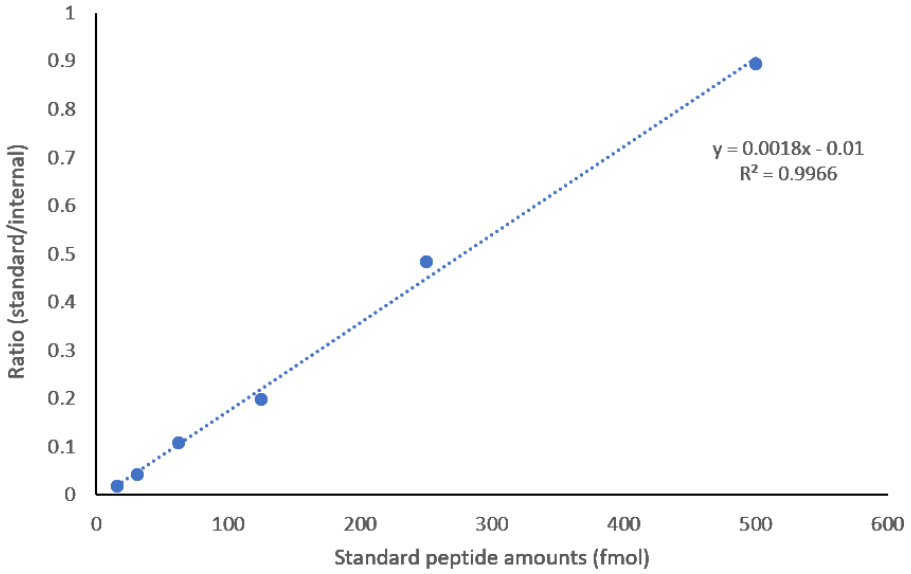


Figure 3.8 Calibration curve for monitoring the second transition

Standard transition	Internal transition	Slope	Intercept	Regression
635.3/971.6	640.3/981.5	0.0017	0.0134	0.998

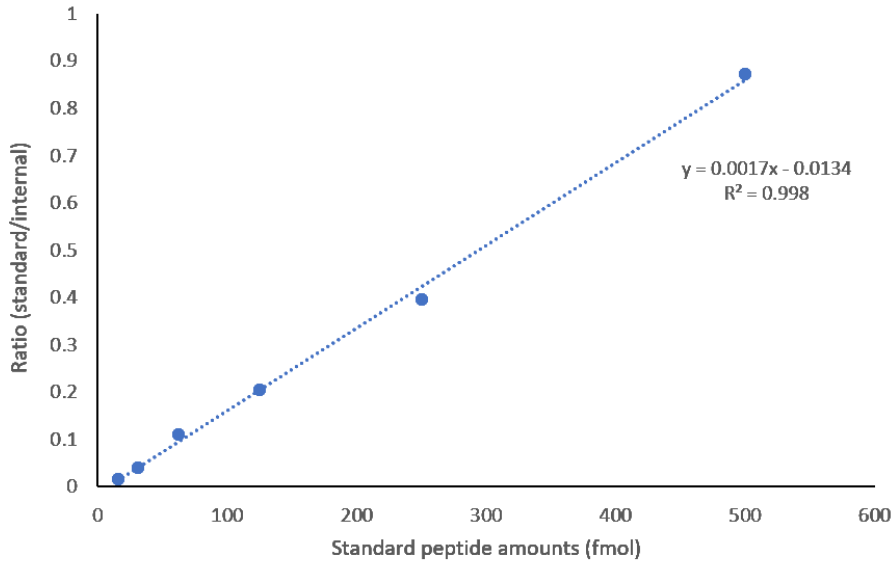


Figure 3.9 Calibration curve monitoring the third transition

3.5.1.2 Variation

The developed method displayed an acceptable variation value for both intra-day and inter-day analysation as the relative standard deviation (RSD) was not over 15% (Table 3.5). The inter-day variation ranged from 11.3% to 14.6%, which was slightly higher than intra-day variation from 6.1% to 11.1%. The greater difference in inter-day experiment was also reported in the previous study as the intra-day data displayed an RSD from 1.1% to 8.1% but the between-day exhibited an RSD from 3.7% to 13.3% (Groer et al., 2013).

Table 3.5 The intra-day and inter-day variation of QC samples with the current method, calculated as the relative standard deviation (RSD).

Variation	QC low	QC medium	QC high
Intra-day	11.1%	6.1%	9.8%
Inter-day	11.3%	14.6%	13.8%

3.5.1.3 Stability

The selected peptide for P-gp quantification exhibited acceptable stability (± 15% of the initial concentrations at low, medium and high QC samples) during storage for 2 h at i) room temperature, ii) 24 h in the cooled autosampler rack and iii) 4 h at 37^{o}C incubation which mimicked the digestion procedure in the current developed method. (Table 3.6)

Table 3.6 The stability of QC samples in the different conditions.

Stability	QC low	QC medium	QC high
2 hours at RT	96.1%	93.4%	99.6%
24 hours at 4^{o}C	91.1%	92.8%	98.7%
4 hours at 37^{o}C	94.1%	91.2%	100.9%

3.5.1.4 Digestion efficiency

As shown in Figure 3.10, the digestion process displayed no significant effect between the different incubation times from 4 hours to 24 hours ($p > 0.05$). In addition, the protein digestion efficiency in the current method exhibited same effect in male and female rats. Considering the reduced variation in the intra-day experiment from Section 3.5.1.2, 4 hours incubation of time was finally selected for the sample analysation.

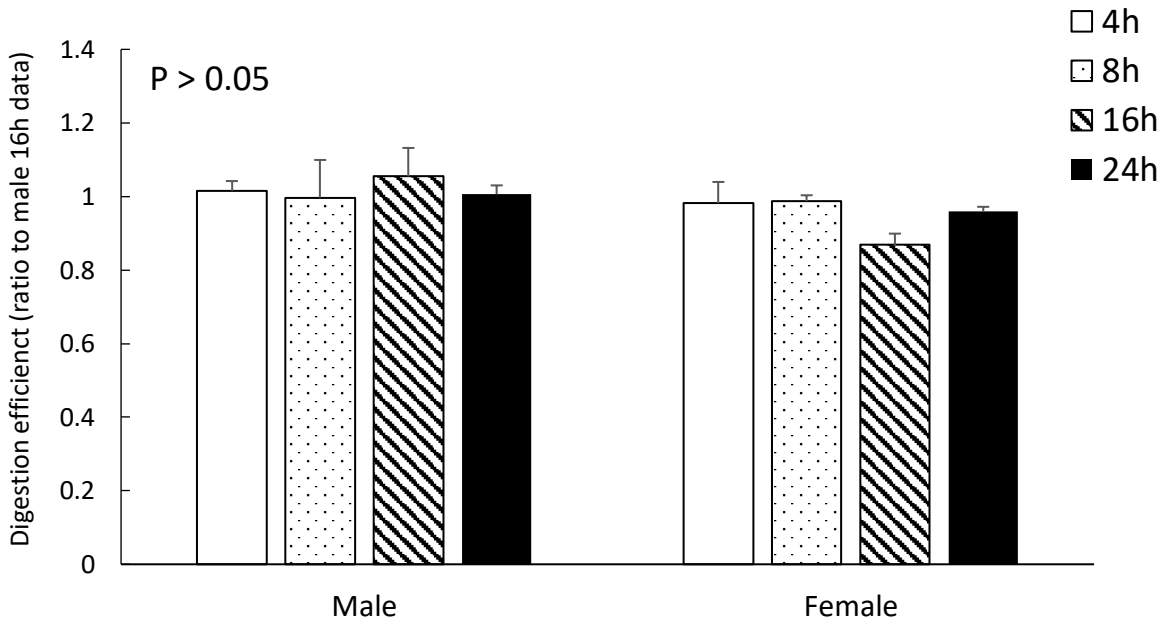


Figure 3.10 Digestion efficiency of intestinal P-gp in jejunum of male and female rat after tryptic digestion at 37°C at 4, 8, 16 and 24 h. All experiments were performed in triplicate (mean ± SD are given).

3.5.2 Sex and food effect on P-gp absolute expression

It can be observed in Figure 3.11 that the absolute expression pattern of intestinal P-gp demonstrated a stark difference between male and female in fasted-state rats. Along the intestinal tract, there was no sex difference in both duodenum and colon, while a significant sex difference ($p < 0.05$) was noticed in small intestine. Specifically, the P-gp level of jejunum segment was 2.45 ± 0.70 fmol/μg in male rats, much higher than that of in female rats (1.69 ±

0.38 fmol/μg). Similarly, ileum P-gp expression in male rats was approximately 30% higher than that of in their counterparts which was 2.75 ± 0.98 and 1.84 ± 0.24 respectively. On the contrary, as shown in Figure 3.12, a significant sex difference ($p < 0.05$) in P-gp expression was observed in rats following food intake but in an opposite direction. In both jejunum and ileum, female rats expressed much higher P-gp level than male rats, which was 3.77 ± 0.86 vs. 1.94 ± 0.22 fmol/μg and 2.54 ± 0.57 vs. 1.87 ± 0.39 fmol/μg respectively.

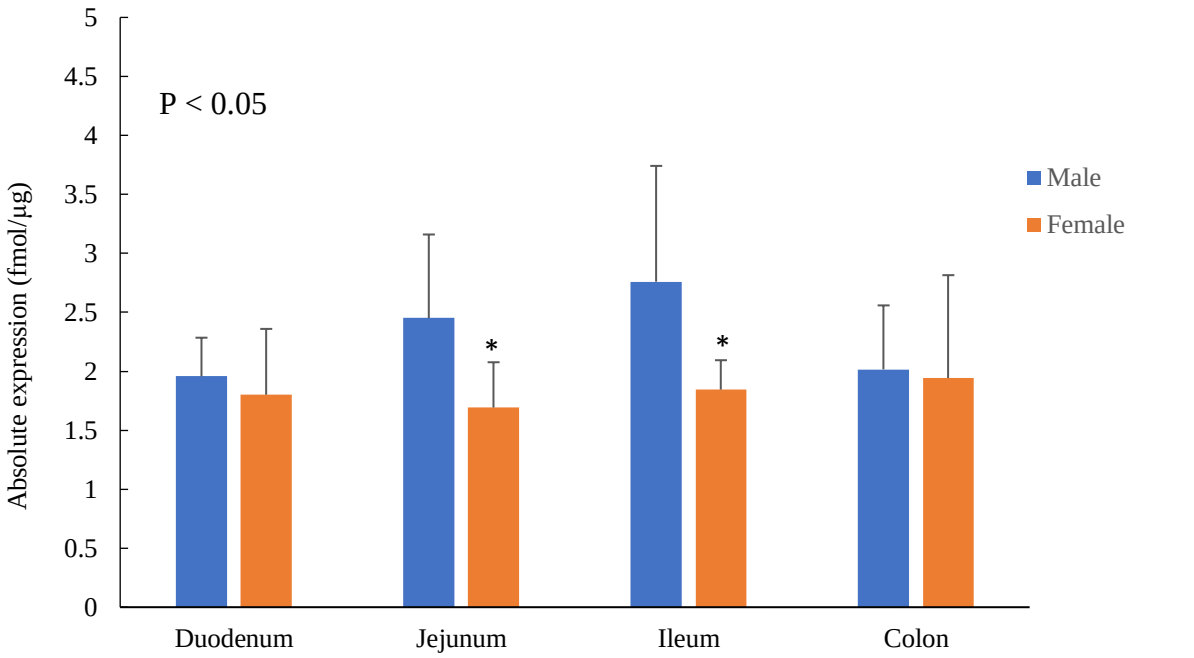


Figure 3.11 Absolute intestinal P-gp expression in male and female rats under fasted-state (n = 6). *represents a significant difference (p < 0.0.5) in P-gp expression between sexes.

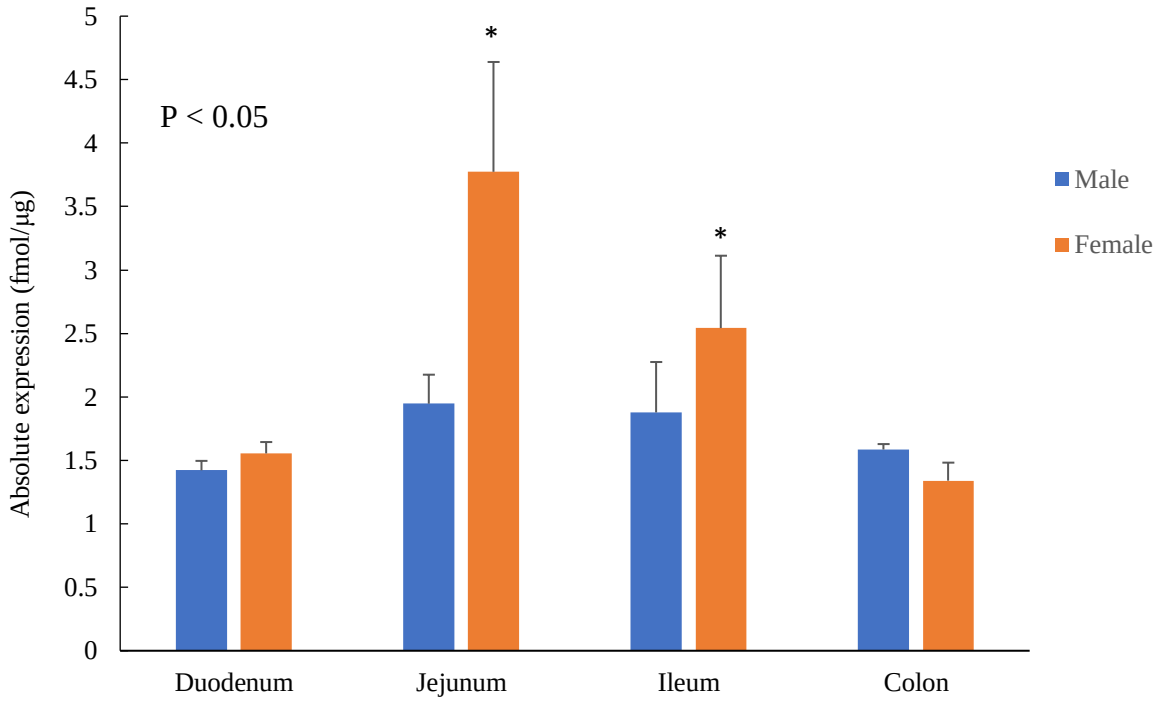


Figure 3.12 Absolute intestinal P-gp expression in male and female rats under fed-state (n = 6). *represents a significant difference (p < 0.0.5) in P-gp expression between sexes.

The P-gp expression pattern observed in Figure 3.11 correlated to the results in Chapter 2, except in the colonic segment. According to both Chapter 2 and the previously reported Western blot results, male rats demonstrated a significantly ($p < 0.05$) higher P-gp expression in the colon when compared to females (MacLean et al., 2008). The possible reason for the discrepancy may lie in the principles of these two techniques; As aforementioned in Table 3.2, the Western blot method can only garner relative target protein expression with respect to the reference protein. It has been demonstrated that the reference protein, villin, is expressed at a lower level in colon than in the small intestine among male rats (Kovalenko and Basson, 2013). Therefore, the lower expression of the reference protein such as villin and beta-actin in colon may lead to a higher calculation for relative P-gp expression following Western blot analyses. Following the validated LC-MS/MS method, however, the expression of P-gp generated is the absolute protein expression of the intestinal tissue without any interference from method limitations. In addition, the functional differences along the intestinal tract reported from previous study can support the observed data from LC-MS/MS experiments. A technique named rat precision-cut intestinal slices (PCIS) was developed in Groothuis' group and applied to investigate the intestinal P-gp activity. In their study, the intestinal permeation of P-gp substrate Rhodamine 123 (R123) was investigated along the intestinal tract. A R123 accumulation level of above 100 pmol/mg protein was observed in both duodenum and colon segments, while jejunum and ileum showed lower accumulation levels although the lowest accumulation of R123 was observed in the ileum (Li et al., 2015). A similar phenomenon was also demonstrated on human intestine. A study using Western blot demonstrated that human intestinal P-gp expression gradually increased from 0.24 in duodenum to 2.13 in colon (Bruyere et al., 2010). On the contrary, a study conducted by Drozdzik et al. in 2014 reported that the absolute expression of human intestinal P-gp increased from duodenum to ileum but dropped in the colonic segments to similar levels in the duodenum (Drozdzik et al., 2014). This study

was supported by an *ex vivo* permeation investigation that higher permeation of P-gp substrate, R123, was observed in both duodenum and colon of human tissue (Li et al., 2017).

In addition to fasted-state rats, an interesting phenomenon was observed in fed-state rats. Figure 3.13 described the change of P-gp expression from fasted-state rats to fed state rats. In male rats, the absolute expression level of P-gp dramatically decreased along the whole intestinal tract in male rats following food intake. The largest reduction in P-gp intestinal expression occurred in the ileum where levels decreased by approximately 32% from 2.75 ± 0.98 fmol/μg in fasted-state to 1.87 ± 0.39 fmol/μg in fed-state. Interestingly, in female rats, a contrasting result was observed in jejunum and ileum segments. P-gp expression increased by approximately 120% and 40% after food intake in the jejunum and ileum.

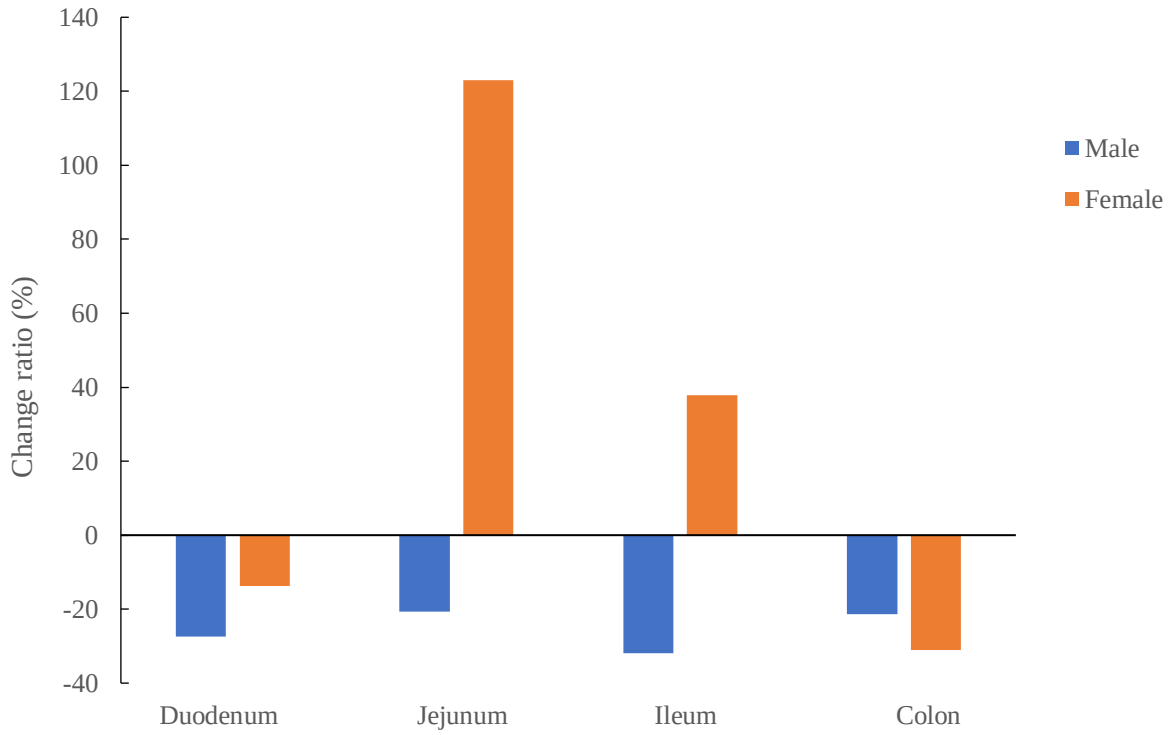


Figure 3.13 The percentage change of intestinal P-gp absolute expression level along the intestinal tract following the food consumption in rats.

Attempts have previously been made using Western blot analysis to investigate the distribution of P-gp in the rat intestinal tract. However, the conflicts and differences between the studies as summarised in Table 2.6 made it difficult to understand the sex and food effect on rat intestinal P-gp. Considering the fact that no definitive study that investigates the effect of food on P-gp expression between the sexes exists, this is the first study to understand that general food consumption itself can affect intestinal P-gp expression to different extents in males and females.

The potential reasons for the sex-dependent food effects are multifactorial. Firstly, food ingredients themselves may contribute to this observed phenomenon as aforementioned in 3.1.3. According to the manufacturer's document, the food supplied for the rats in the current study (EURodent Diet 22%) contained 32 different ingredients (LabDiet, US). Secondly, physiological changes in the intestinal luminal environment during food consumption may further contribute to the sex difference. Luminal fluid composition is normally altered from food consumption by the modification in production of bile salts, cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1). In addition, food-stimulated sex hormones may also influence the sex-dependent food effect on intestinal P-gp expression. A study proved that a diet containing menhaden oil n-3 PUFA increased serum oestradiol concentration levels from 90 pg/ml to approximately 130 pg/ml in pregnant rats (Davis et al., 2013). With the distribution of receptors in rodent intestine, sex hormones have been demonstrated to regulate P-gp expression; testosterone, the primary sex hormone in males, has been shown to induce an inhibitory effect on P-gp (Wessler et al., 2013). Conversely, another study reported that P-gp expression significantly increased after the incubation with progesterone and β-estradiol at the concentration of or greater than 10 nM and 100 nM respectively (Coles et al., 2009).

The most interesting phenomenon identified in the current study was the notable increase of P-gp in the small intestine of female rats in the fed state. It can be suggested that this may occur due to the innate protection required for successful reproduction. In the fasted-state, female rats exhibited low levels of P-gp expression. In the fed-state, however, as food contains multiple components of which some may be harmful, the body can protect itself by increasing the expression of the efflux transporter as a barrier function to hinder the absorption of potentially toxic food compounds. This mechanism could be a complex interplay of the modulation of P-gp expression, enzyme reaction and the defence ability of epithelial cells which may be further influenced by sex hormones. It was reported that oestrone and oestradiol both increase intestinal enzyme activity in female rats. By administrating b.i.d. 1 mg/kg oestrone and oestradiol for two days in female rats, the intestinal CYP-450 enzyme concentration increased from 0.03 ± 0.01 nmol/mg in the control group to 0.16 ± 0.01 and 0.09 ± 0.01 nmol/mg in the oestrone and oestradiol treated groups respectively (Brady et al., 2002). In addition, a study investigated ileum tissues obtained from both male and female rats that were exposed to harsh conditions (such hypoxia for 40 min and acidosis at pH 6.8) and normal conditions (normoxia at a normal pH of pH 7.3) via an Ussing chamber experiment (Homma et al., 2005). Cytokine and nitric oxide concentration levels in the Ussing chamber were subsequently measured to evaluate the immune-inflammatory response. Fluorescein Isothiocyanate-dextran (FITC-dextran, molecular weight of 4,300 Da) was checked to assess the barrier function of the intestinal lumen. As a result, the female intestinal tissue showed a higher anti-inflammatory response and an enhanced intestinal barrier function when compared with males. More interestingly, the addition of oestradiol in male rats relieved the intestinal injury and enhanced their anti-inflammatory ability.

Current results firstly elucidated that food and sex displayed a joint influence on intestinal P-gp expression in rats. Following a better understanding of the differences in intestinal physiology in the sexes, further considerations were applied. For example, the project aimed to identify the extent of the differences in P-gp expression on potential alterations on P-gp function, such as effects on intestinal permeation of drug. In addition, the expression pattern of P-gp in male and female rats from the fasted-state to fed-state should also be elucidated and whether this change is a quick food-stress response or a gradual change over time under different conditions. Therefore, Phase II studies were carried out in the following section, in order to better understand the food and sex effect on P-gp.

Phase II

3.6 Method

3.6.1 Experimental animal

12 fasted-state rats and 12 fed-state rats as described in Section 3.4.1 were used for the Ussing chamber experiment. In addition, for the time-related P-gp change experiment, five different groups of rats (A, B, C, D, E) were established by fasting the rats for different times as shown in Figure 3.. In each group, there were 6 male and 6 female rats. 12 fed-state rats (6 male and 6 female) described in 3.4.1 were also involved as control group.

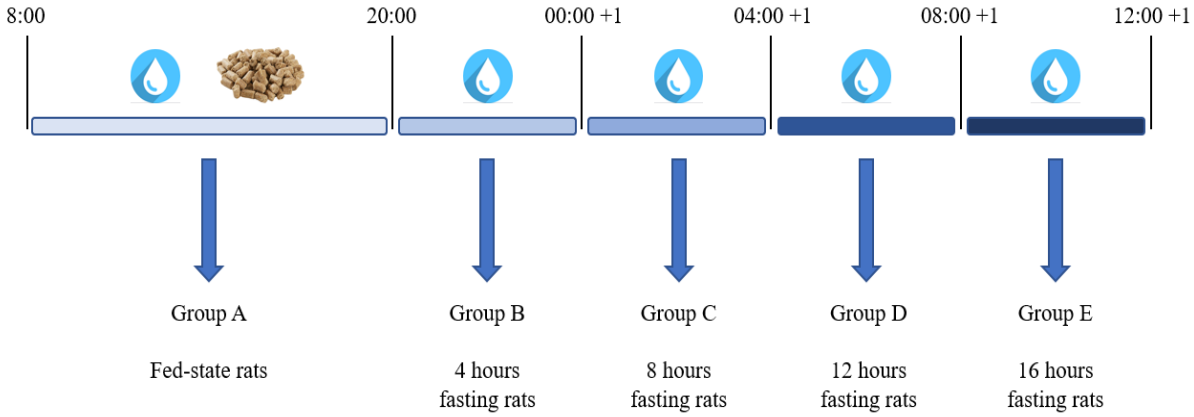


Figure 3.14 Establishment of five rat groups for time-related P-gp change experiment

3.6.2 *Ex-vivo* permeation study

3.6.2.1 Evaluation of drug permeation via Ussing chamber

Drug solutions (3 mM ranitidine, 3 mM metformin and 1.96 mM ganciclovir) were freshly prepared in a KBR solution and stored in a 37°C incubator for the experiment. Intestinal tissues from the jejunum and ileum were obtained following Section 3.4.2. The well-prepared mucosal tissues were then mounted in the vertical Ussing Chamber (Harvard Apparatus Inc., Holliston, MA, U.S.A) as flat sheets on a 0.28 cm^2 segment holder with needles for stability purposes.

The chambers were tightly screwed with high spring-tension retaining rings and the entire assembly was maintained at 37°C with a circulating water bath for a 30 min equilibrium period. Tissue integrity was evaluated every 30 min during the experiment by measuring tissue transepithelial electrical resistance (TEER) with an EVOMX meter (World Precision Instruments Inc., WPI, Hertfordshire, United Kingdom). The jejunal and ileal segments that presented a value of TEER lower than 40 Ω•cm^2 and 50 Ω•cm^2 respectively at the beginning of experiment was regarded as poorly viable and excluded immediately. The tissue was not considered viable whenever TEER values decreased more than 15% from the value measured at the end of equilibration period.

The study began with a 20 – 30 min equilibrium period. 5 mL KBR solution was added to both the apical (mucosal surface) and basolateral (endothelial surface) chambers, gassed with an O_2/CO_2 (95%/5%) gas mixture. Following the emptying of the chamber, 5 ml of fresh KBR solution was added in the basolateral chamber whilst 5 ml of the drug solution was added in the apical chamber. During the experiment, 100 μL solution from basolateral chamber was withdrawn every 30 min. The experiment lasted for 2 h and the intestinal permeation was evaluated by analysing the drug amount in the withdrawn samples (mucosa to serosa, M – S). An equal volume of fresh KBR solution was replaced immediately.

3.6.2.2 Chromatographic analysis

Chromatographic analysis was performed with a high-performance liquid chromatography (HPLC) system (Agilent Technologies, 1260 Infinity) equipped with a pump (model G1311C), an auto-sampler (model G1329B) and a diode array UV detector (model G1314B). The methods were summarised in Table 3.7. In particular, the evaluation of ranitidine was achieved by using a 5 μm Luna SCX (Phenomenex, UK) column and a mobile phase mixture of 20:80

(acetonitrile):(0.1 M sodium acetate pH = 5.0) with a flow rate of 2 mL/min. In the case of metformin, a Luna C18 (250 mm × 4.6 mm I.D./5 μm) column (Phenomenex, UK) was applied with a flow rate of 1 mL/min. The ganciclovir samples were quantified by HPLC using a Luna C18 (250 mm × 4.6 mm I.D./5 μm) column (Phenomenex, UK) with a flow rate of 1 mL/min. The mobile phase consisted of 0.5% formic acid water and acetonitrile (95:5, v/v). The UV detector was set at 275 nm.

3.6.2.3 Data analysis

The apparent permeability coefficient (Papp) was calculated for the evaluation of ranitidine, ganciclovir and metformin permeation study by using the following equation:

$$Papp(\mathrm{cm/s}) = \frac{Q}{C \cdot A \cdot t}$$

where Q (μmol) is the total amount of drug that permeated to the basolateral chamber throughout the incubation time, C (μmol/mL) is the initial drug concentration in the apical chamber, A (cm^2) is the diffusion area of the Ussing Chamber and t (s) is the time of experiment.

3.6.3 LC-MS/MS study

Rat in each group was sacrificed by a CO_2 euthanasia chamber at the end of time point immediately. After that, the procedures for tissue preparation, protein extraction, protein digestion as well as analysation were following the description in Section 3.4.

3.6.4 Statistical analysis

All results are expressed as mean ± SD (n = 6) and were analysed by one-way ANOVA and followed by a Tukey post-hoc analysis with a 95% confidence interval using IBM SPSS Statistics 16 (SPSS Inc., Illinois, USA).

Table 3.7 Summary of the HPLC methods for ranitidine, ganciclovir and metformin quantification

Drug	Column	Temperature (°C)	Mobile phase	Flow rate (ml/min)	UV detection wavelength (nm)	Injection (µl)	Reference
Ranitidine	SCX (250 mm × 4.6 mm I.D./5 µm)	50	0.1 M Sodium Acetate Buffer (pH 5.0, 80%); Acetonitrile (20%)	2	320	40	Ashiru et al., 2007
Ganciclovir	C18 (250 mm × 4.6 mm I.D./5 µm)	40	0.5% Formic acid water (95%); Acetonitrile (5%)	1	275	20	Dou et al, 2018
Metformin	C18 (250 mm × 4.6 mm I.D./5 µm)	25	10 mM Sodium Dihydrogen Phosphate Buffer with 10 mM Sodium Dodecyl Sulfonate (pH 7.0, 60%); Acetonitrile (40%)	1	234	50	Mai et al., 2017

3.7 Results and discussion

3.7.1 Intestinal permeation of ranitidine, metformin and ganciclovir

According to Table 3.8 and Figure 3.15, the permeability of ranitidine and ganciclovir exhibited a sex difference in the fasted state of the rat intestine. In both the jejunum and ileum regions, ranitidine P_{app} in female rats was higher than that of male rats which were 8.24 ± 1.29*10^{-6} cm/s compared with 6.78 ± 1.24*10^{-6} cm/s in the jejunum respectively, and 9.97 ± 0.52*10^{-6} cm/s versus 6.67 ± 0.10*10^{-6} cm/s in ileum. With regards to ganciclovir, the permeability in the jejunum and ileum were 7.64 ± 1.48*10^{-6} cm/s and 5.17 ± 0.63*10^{-6} cm/s respectively, which were lower than that of female rats (10.11 ± 1.51*10^{-6} cm/s and 7.87 ± 0.53*10^{-6} cm/s). However, no significant sex difference was identified when evaluating the intestinal permeability of metformin as it is not a P-gp substrate.

Interestingly, from the fasted-state to fed-state, the permeability of ranitidine in males achieved a 22.7% and a 27.3% increase in the jejunum and ileum respectively, whilst that of females achieved a 25.8% and a 41.3% decrease. Similarly, ganciclovir exhibited a decrease in the female rat jejunum (36.2%) and ileum (37.8%) from the fasted-state to fed-state. In male rats, however, 83.6% and 97.3% increases were observed in the jejunum and ileum respectively. The permeability of metformin remained consistent in the different sexes in both fasted or fed states. Consequently, the permeability of both ranitidine and ganciclovir exhibited an increasing trend in males but decreased along the intestine in female rats after food intake. No sex differences or food effects were observed on metformin small intestine permeability.

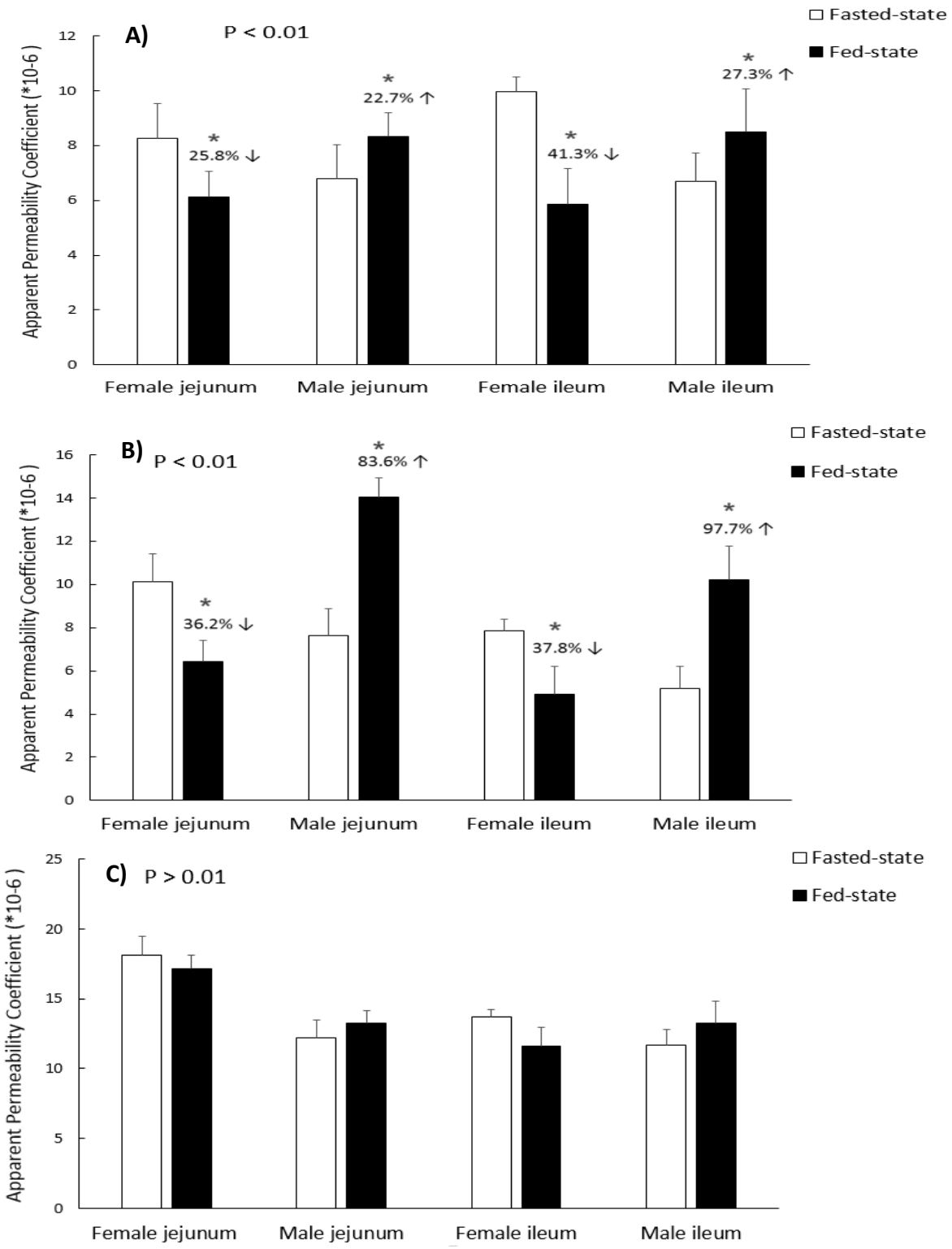


Figure 3.15 Permeation of A) ranitidine, B) ganciclovir and C) metformin in the jejunal and ileal regions of fasted vs. fed state male and female rats (mean ± SD, n = 6).

Table 3.8 Apparent drug permeability coefficients (cm/s $x10^{-6}$) in the fed and fasted state of male and female rats (mean ± SD, n = 6)

		Male jejunum	Female jejunum	Male ileum	Female ileum
Ranitidine*	Fasted-state	6.78 ± 1.24	8.24 ± 1.29	6.67 ± 0.10	9.97 ± 0.52
	Fed-state	8.32 ± 0.89	6.12 ± 0.95	8.49 ± 1.5	5.84 ± 1.32
Ganciclovir*	Fasted-state	7.64 ± 1.48	10.11 ± 1.51	5.17 ± 0.63	7.87 ± 0.53
	Fed-state	14.02 ± 0.67	6.47 ± 0.87	10.22 ± 1.81	4.89 ± 1.35
Metformin	Fasted-state	12.22 ± 0.26	18.16 ± 1.74	11.72 ± 1.69	13.74 ± 0.55
	Fed-state	13.28 ± 0.82	17.18 ± 0.86	13.25 ± 1.89	11.63 ± 1.64

*represents a significant difference ($p < 0.0.5$) in the permeability coefficients between the fasted and fed state rats

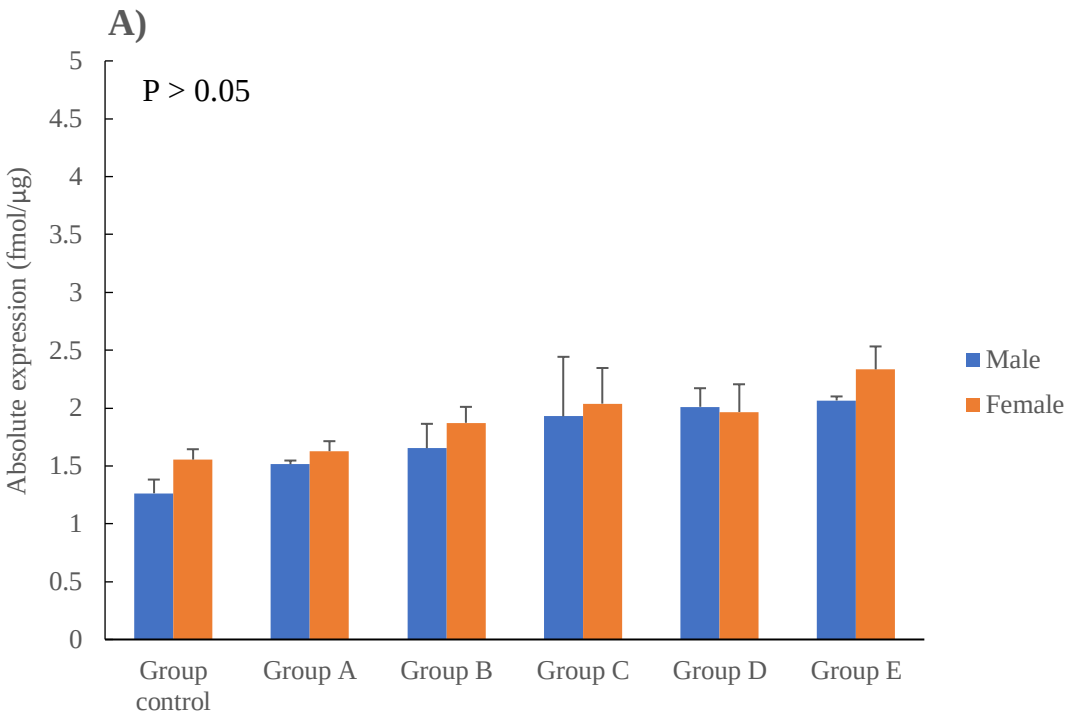
With the Ussing chamber experiment, it has been demonstrated that the observed P-gp expression change with the sex and food factors can be represented in the P-gp substrates intestinal absorption. Ranitidine and metformin share the same OCT protein absorption mechanism; however, ranitidine is also a P-gp substrate (Konig et al., 2013; Leibach and Ganapathy, 1996; Muller et al., 2005; Bourdet and Thakker, 2006; Collett et al., 1999; Liang et al., 1995). As a result, the change of permeability of ranitidine highly correlated with the change of intestinal P-gp protein expression level factoring in food and sex whilst metformin remained constant in all conditions. In addition, ganciclovir, another P-gp substrate, experienced a greater modification in intestinal permeability following food intake compared to ranitidine. The reason might due to the fact that ganciclovir is non-substrate of any uptake transporters while ranitidine is also an OCT protein substrate, which may partially offset the P-gp influence and contribute towards the lower permeation (Muller et al., 2005; Bourdet and Thakker, 2006; Collett et al., 1999; Li et al., 2011; Shah et al., 2007). As a result, the influence of sex and food on P-gp expression reported in Phase I can heavily affect the intestinal absorption of oral-administrated drugs which are P-gp substrates. In pre-clinical study, sex and food need to be well considered when designing experiments for evaluating P-gp effect on potential drug candidates.

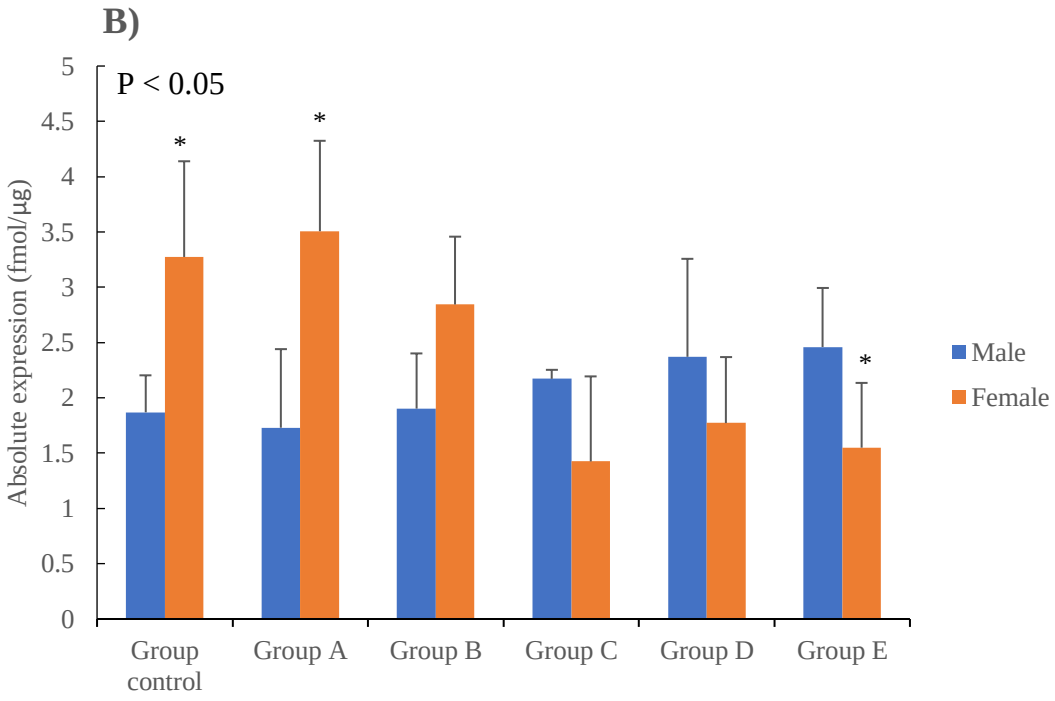
3.7.2 Time-related change of intestinal P-gp expression

Along the whole intestinal tract, no difference was observed between the control group and group A for both male and female rats (Figure 3.). No significant change ($p > 0.05$) between 8:00 am (control group) and 8:00 pm (group A) demonstrated that P-gp maintained the same expression level under fed-state condition in rats for both sexes. From group A onwards, however, P-gp expression started to change over time differently between sexes when only water was accessible. In both duodenum and colon parts, a slight increase trend was observed

in both male and female rats from group A to group E without sex difference. On the contrary, small intestinal P-gp expression displayed reversal pattern between male and female from group A to group E. In group A, fed-state female rats exhibited a significant higher ($p<0.05$) P-gp expression compared to their male subjects in both jejunum and ileum. As the fasting progress, the P-gp in female rats gradually decreased while that of in male rats achieved consistent increase. After 8 hours fasting (Group C), P-gp expressed in jejunum and ileum in male rats exceeded their counterparts. The difference was expanded in the rest of groups and a significant higher P-gp expression was noticed in male rats after 12 and 16 hours fasting (Group D and E).

Control group	Group A	Group B	Group C	Group D	Group E
Fed-state at 8:00	Fed-state at 20:00	4 hours fasting	8 hours fasting	12 hours fasting	16 hours fasting





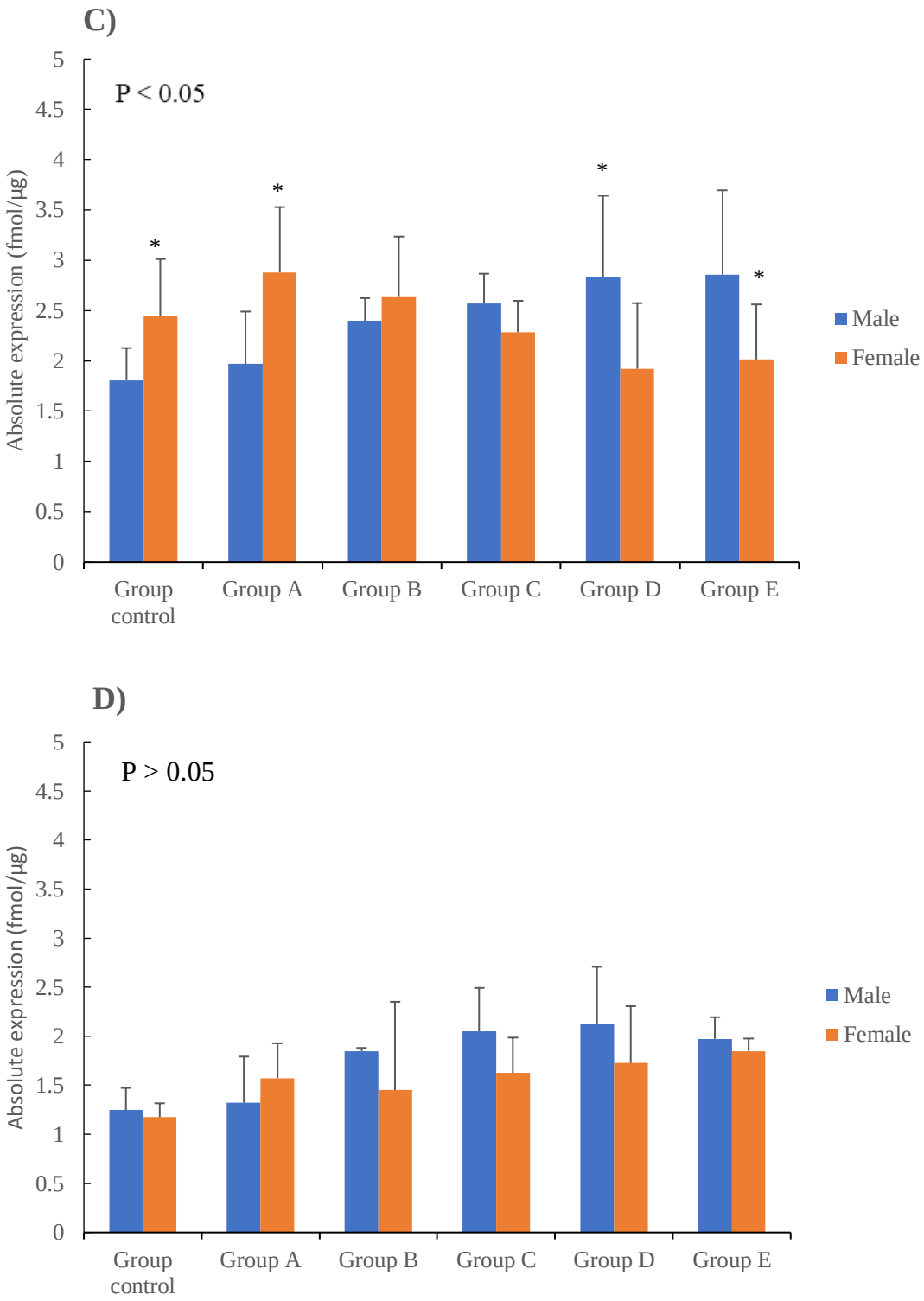


Figure 3.16 Time-related P-gp expression along the intestinal tract (A: duodenum, B: jejunum, C: ileum, D: colon) in both male and female rats (mean ± SD, n = 6). * represents a significant difference (p < 0.0.5) in P-gp expression between sexes.

As aforementioned in Section 3.5.2, both the food-intestine interaction and the change of intestine lumen environment during food consumption may contribute to the food induced P-gp expression change. In one previous study, it was demonstrated that the average time for both stomach and small intestine emptying solid meal in male rats was 6.41 hours (Quini et al., 2012). Moreover, another study conducted by Tuleu *et al.* proved that the emptying time for three types of pellet ranged from 4.72 to 6.82 hours in female rats (Tuleu et al., 1999). The time for intestine emptying food was highly correlated to the time when P-gp expression reversely changed between sexes in Figure 3., which happened between 4 hours fasting (Group B) and 8 hours fasting (Group C). Furthermore, a study conducted by Ando *et al.* investigated the change of P-gp mRNA, mdr1a, over 24 hours in male mice, which can partially support the current results. It was known that mdr1a is the main coding gene for intestinal P-gp in rodent (Brady et al., 2002). The study reported that the expression of mdr1a performed a considerable increase during the 4h to 8h of zeitgeber time (Ando et al., 2005), as zeitgeber time 0 defined as the starting point when rodent start its rest state and stop eating food (Refinetti, 2004). Apart from the correlation with food consumption activity, the P-gp change observed in the current study might also related to the circadian rhythm effect. A 24 hours circadian rhythm effect on intestinal P-gp gene expression, mdr1a, was observed in mice (Murakami et al., 2008). It was demonstrated that the mdr1a gene exhibited low expression level during 2:00 to 6:00 and 20:00 to 22:00 but achieved a sharp increase from 8:00 to 14:00, which is similar to the P-gp expression reported herein, low expression level at 8:00 and 20:00 but peaked at 12:00 in the following day. Moreover, a study conducted by Savolainen and her colleagues reported that the activity of P-gp in blood-brain barrier of male rats was reduced from 15:00 to 21:00 but highly active at 3:00 and 9:00 time points (Savolainen et al., 2016).

In a nutshell, P-gp expression in duodenum and colon maintained the similar level between sexes in both fasted-state and fed-state. Small intestinal P-gp from fed-to-fasted state achieved a large increase in male but decrease in female and the changing point was between 4 hours and 8 hours fasting, which was closely related to the food emptying time. Therefore, the results also supported the previous discussion that food substance and its induced environment change contribute to the observed P-gp expression difference in male and female rats

3.8 Conclusion

In this chapter, a novel protein analysation method, LC-MS/MS, was developed. Following validation, this method was utilised to be the first to quantify and report that the absolute intestinal P-gp expression is drastically affected by food, and to different extents in male and female rats. In fasted-state rats, males exhibited a statistically significant higher P-gp expression in jejunum and ileum compared to their female counterparts ($p < 0.05$). With food intake, however, the absolute expression of intestinal P-gp decreased in male rats but increased in female rats. As a result, P-gp expression level in female was much higher than male in fed-state rats. Subsequently, the observed sex and food effect on P-gp expression was represented on the P-gp functional activity. The intestinal permeability of P-gp substrates, ranitidine and ganciclovir, was affected by the sex and food which displayed a significant sex difference in both fasted and fed rats ($p < 0.05$). Conversely, no sex difference was noticed in the intestinal permeability of metformin which is a non-P-gp substrate. Furthermore, the conversion of P-gp expression pattern from fed-sate to fasted-state was investigated via five different time points. It has been noticed that small intestinal P-gp from fed-to-fasted state achieved a large increase in male but decrease in female and the changing point was between 4 hours and 8 hours fasting, which was closely related to the food emptying time. Given the results obtained in this chapter, the influence of food and sex should be acknowledged and implemented when using animal models for the early stage development of oral pharmaceutical products that are known or identified to be P-gp substrates.

CHAPTER 4:

The sex-related GI physiological difference in Sprague Dawley rat and human

4.1 Introduction

4.1.1 History of laboratory rats

Rats have been used in the medical sciences for over 200 years and have been extensively applied in laboratory work all over the world (Shanks et al., 2009). A single type of wild brown rat was accidentally introduced as stowaways in England from northern Europe in the 18th century. The rapid increased in rodent population resulted to a variety of social and health issues from the lack of sanitation at the time. Due to the public's resentment with the German royal house that ruled Hanover, Great Britain and Ireland from the 17th to 20th century, the English population collectively associated their social problems and the increase in rodent populations with the monarchy. As such, this particular strain of rat was coined as the Hanover rat (Baker et al., 1979). The binomial nomenclature of the wild brown rat, *Rattus norvegicus*, was devised by the English naturalist, John Berkenhout, who was responsible in popularising the binomial system (Berkenhout, 1772). Berkenhout believed that rats migrated to England from Norwegian ships in 1728 and wrote his assumption in *Outlines of the Natural History of Great Britain* which was published in 1796. Through the rampant increase of the wild brown rat, the rat-catching and rat-baiting industry quickly surged in the late 18th and early 19th century; rat-catchers previously trapped and bred rats to enter into rat-baiting contests which was a popular sport where a pit was filled with rats and timed for how long it took for a terrier to kill them all. Over time, breeding the rats for these contests may have produced variations, one of which included the albino mutant *Rattus norvegicus*.

In 1906, the Wistar Institute developed a strain of the outbred albino mutant *Rattus norvegicus* and coined it as the Wistar rat, aimed to be specifically used for biological and medical research. It was notably the first rat offered to serve as a model organism for research at that time as majority of laboratories were using common house rodents. Nowadays, more than a hundred strains of rats have been developed with more than half of them originating from the

Wistar rat (Clause, 1998). For example, the Long–Evans rat is an outbred rat developed by Drs. Long and Evans in 1915 by crossing several Wistar females with a wild grey male rat. In addition, the Sprague Dawley (SD) rat is an outbred multi-purpose breed of albino rat used extensively in medical and nutritional research due to its docile nature and ease of handling. The SD rat was firstly produced from Wistar females and a hybrid male with unknown origins from the Sprague-Dawley farms, later known as the Sprague-Dawley Animal Company, in Madison, Wisconsin, in 1925. The history of the laboratory rat has been illustrated in Figure 4.1.

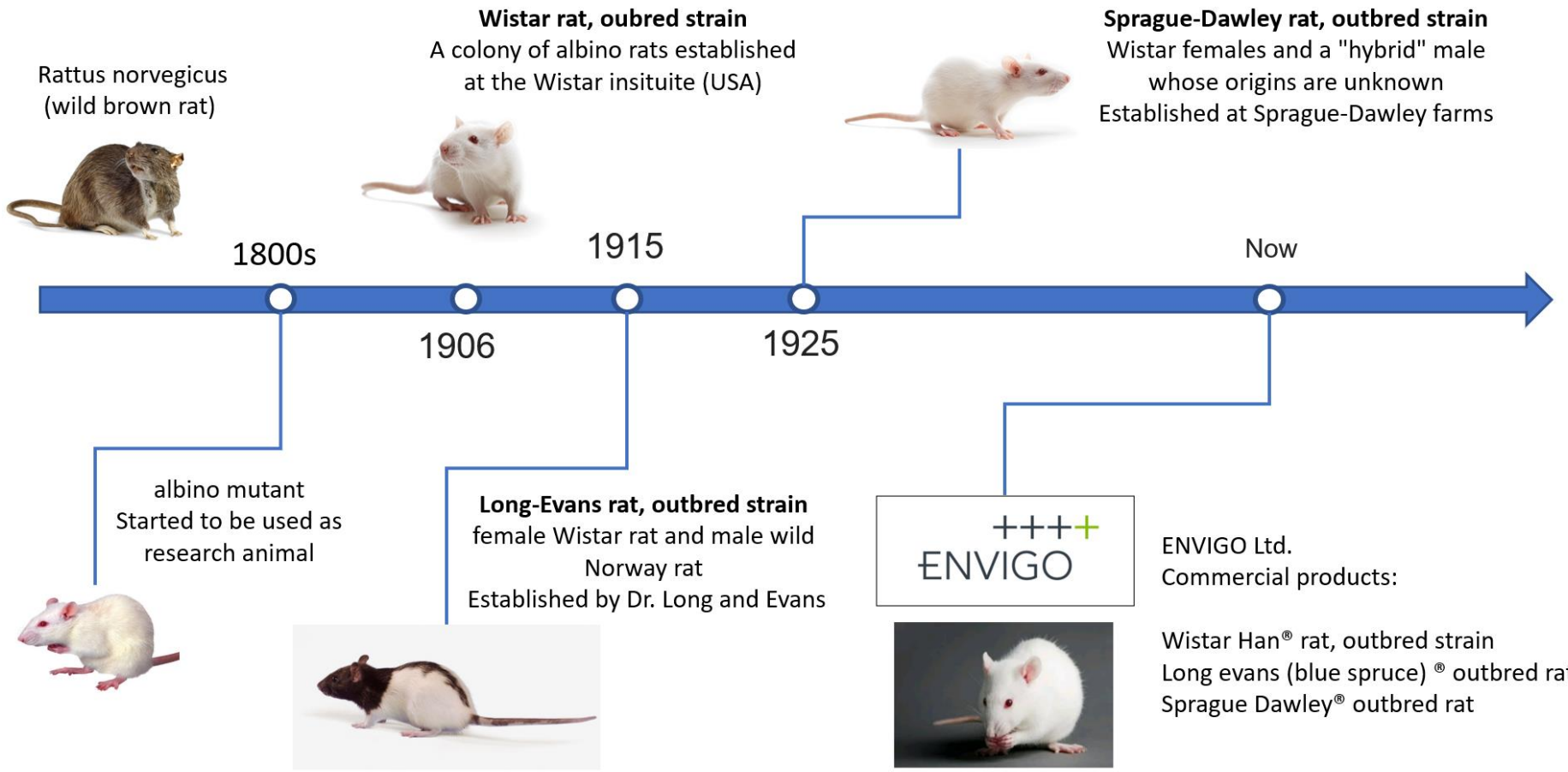


Figure 4.1 The history of laboratory rat.

4.1.2 Commercial strains of rat

Among all the strains of laboratory rats, the Wistar and SD rats are the most common rodent strains used in pre-clinical studies in the pharmaceutical arena. A literature review including over 1000 studies from 1969 to 2012 reported that the most frequent strains employed for oral bioavailability studies in rat were SD and Wistar, accounting for 49% and 35% respectively (Musther et al., 2014). As shown in Table 4.1, all four strains of rat have multi-functional applications in research including general studies, ageing research, toxicology, oncology, diet-induced obesity, surgical models, nutrition, safety and efficacy testing. However, even with the same wide range of application and outbred properties, the main difference between these two strains falls on their price. According to the quoted price from Envigo Ltd., an established animal procurement company, the Long-Evans adult rat and Wistar Unilever rat are more expensive than the Wistar Han rats and SD strain. In addition, it has been reported that spontaneous tumours may develop from 24 months of age in Wistar Unilever rats. As a result, due to its affordable price and multi-function use, Wistar rats and SD rats have been commonly used in both academic and medical research.

However, the potential effect of rat strain has often been overlooked in research with the Wistar and SD rat being used interchangeable in pre-clinical studies. A literature review published in 2015 compared the gastrointestinal physiology between human and pre-clinical animals and linked the similarity of gastrointestinal physiology with the similarity of drug intestinal performance between human and animals (Hatton et al., 2015). In the study, a database of different rat strains was generated, however, the gastrointestinal physiology properties of the rats reported were mainly derived from information on the Wistar rat whereas data on drug performance originated from the SD rat.

Table 4.1 Comparison of commercial strains of rat served in biomedical research, adapted from Envigo.com, 2019.

	Long-Evans	Wistar Unilever® rat	Wistar Han® rat	Sprague-Dawley rat
Breed	outbred	outbred	outbred	outbred
Application	General studies, behavioural research, diet-induced obesity	General studies, toxicology, safety and efficacy testing, aging	General studies, safety and efficacy testing, ageing, oncology, surgical model	General studies, safety and efficacy testing, ageing, nutrition, diet-induced obesity, oncology, surgical model
Adult body weight (10 – 12 weeks)	F: 220 – 260 g M: 330 – 380 g	F: 190 – 240 g M: 290 – 370 g	F: 190 – 210 g M: 260 – 330 g	F: 180 – 250 g M: 280 – 380 g
Price per animal	F: £75 M: £54	£47	F: £18 M: £25	F: £21 M: £26
Life span		few spontaneous tumours through 24 months of age		

F - female rat; M - male rat.

4.1.3 Correlation of rats and humans on drug performance

Despite being the most commonly used strains and interchangeably implemented in pre-clinical studies, data interpretations of Wistar and SD rats may obscure potential strain difference in drug performance. A statistical study investigated the correlation of oral drug bioavailability between humans and pre-clinical animals (Musther et al., 2014). Following the analysis of 184 compounds, Musther et al. demonstrated a low correlation between humans and mice, rats or dogs with respect to oral drug performance whereby R^2 was 0.253, 0.287 and 0.374 respectively (Figure 4.2). Among the aforementioned animals, non-human primates exhibited the highest correlation at 0.694. However, upon analysis, the rat database generated in the study pooled all pre-clinical results from different rat strains which consisted of 49% SD rat, 35% Wistar rat and 16% other strains rat including CD, Albino, Long-Evans, Fisher 344, Lewis and unknowns. As such, the correlation of oral drug performance in the SD and Wistar rat with humans may therefore be different to that seen in the unknown rat species, for example. Consequently, a re-analysation of the pooled results reported in the study is required for an accurate report of the oral drug bioavailability correlation between humans and rats.

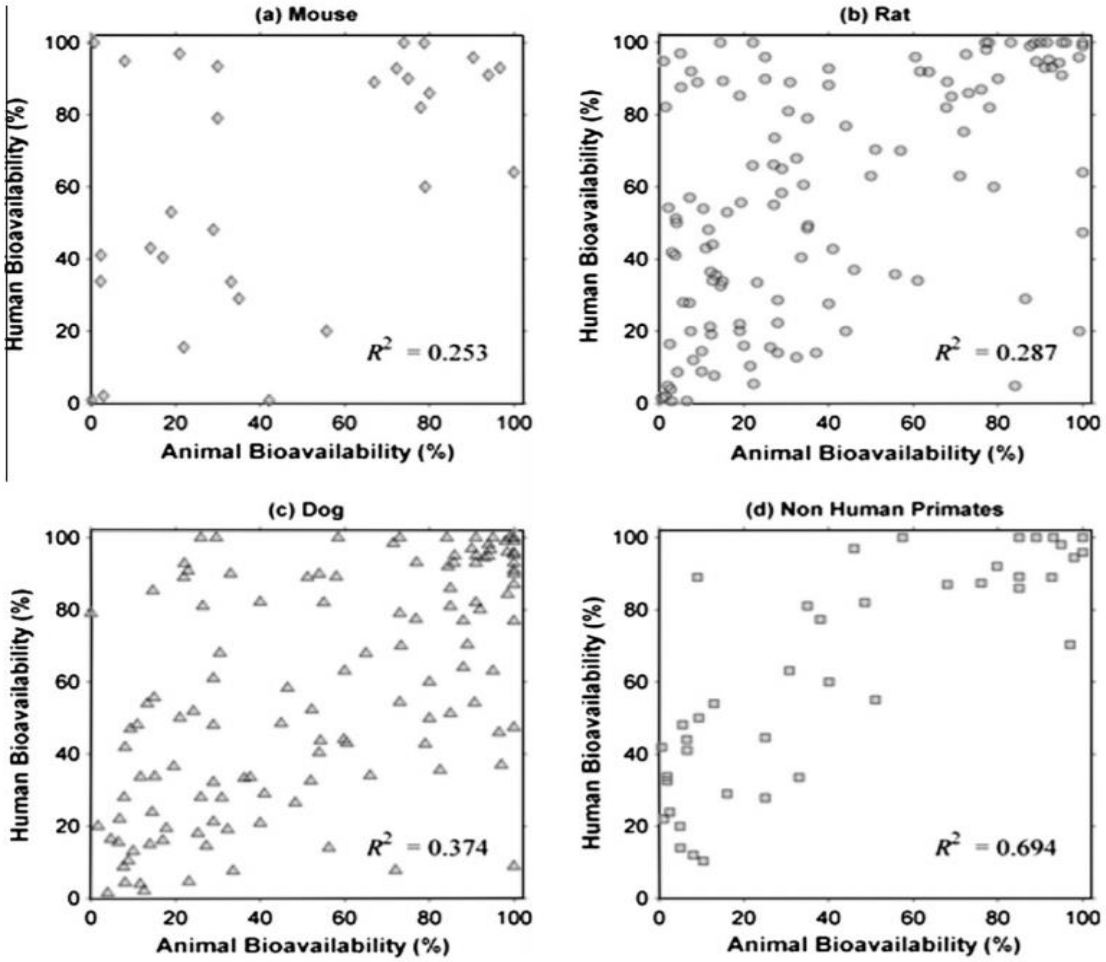


Figure 4.2 Plots for the linear regression analysis by separated by species (in percentages) with the coefficient of determination (R^2) for the linear regression shown in each plot. (a) Mouse F vs. human F; (b) Rat F vs. human F; (c) Dog F vs. human F and (d) Non-human primates (NHP) F vs. human F, adapted from Musther et al., 2014.

The sex-related strain differences in drug intestinal permeation has been observed in recent studies. A study reported the intestinal permeation of metoprolol and verapamil in four strains of rats and in both genders using a *in situ* closed loop perfusion model (Oltra-Noguera et al., 2015). The study reported a significant sex difference ($p < 0.05$) on the intestinal permeation of verapamil in Wistar, Long-Evans and CD/IGS rat bar SD rat. In addition, the mean permeability value (P_{app}) of verapamil in SD rat was the highest compared to that in other

strains. As verapamil is a P-gp substrate, its intestinal permeation can therefore be affected by intestinal P-gp activity. The sex difference in the intestinal perfusion of verapamil in Wistar rats highly correlated to the sex difference in intestinal P-gp expression reported in the studies in Chapters 2 and 3. As such, the variation on GI physiology of the rat strains may contribute to differences in oral drug performance.

Given the reported strain difference, a concern arises from the results of a well-cited research article. In the study conducted by Cao *et al*., 48 drugs were selected for oral bioavailability and 14 drugs were assessed the correlation determination of intestinal permeability between humans and rats (Cao et al., 2006). The report claimed that between humans and rats, no correlation could be drawn from oral drug bioavailability (R^2 = 0.29), although a high correlation of R^2 = 0.8 was seen in drug intestinal permeability. However, a high correlation between drug intestinal permeability was reported in humans although this was only limited to SD rats; the correlation between other strains, most importantly with the Wistar rat, however, remain unknown. A summary of strain difference in drug performance has been listed in Table 4.2.

Table 4.2 The summary of strain difference in drug performance from previous studies.

	SD rat		Wistar rat	
	Male	Female	Male	Female
Metoprolol intestinal permeability [1] (Papp ± SD (cm/s))	$4.43*10^{-5} \pm 3.78*10^{-6}$	$3.82*10^{-5} \pm 2.97*10^{-6}$	$7.13*10^{-5} \pm 5.92*10^{-6}$	$6.28*10^{-5} \pm 7.19*10^{-6}$
Verapamil intestinal permeability [1] (Papp ± SD (cm/s))	$7.81*10^{-5} \pm 2.89*10^{-6}$	$7.55*10^{-5} \pm 8.71*10^{-6}$	$6.07*10^{-5} \pm 5.85*10^{-6}$	$6.85*10^{-5} \pm 5.44*10^{-6}$
Ganciclovir intestinal permeability [2] (Papp ± SD (cm/s))	*UN*	*UN*	$(7.64 \pm 1.48)*10^{-6}$	$(10.11 \pm 1.51)*10^{-6}$
Ranitidine intestinal permeability [2] (Papp ± SD (cm/s))	*UN*	*UN*	$(6.78 \pm 1.24)*10^{-6}$	$(8.24 \pm 1.29)*10^{-6}$
The correlation of drug intestinal permeation between human and rat (R2) [3]	0.8		*UN*	
The correlation of drug oral bioavailability between human and rat (R2) [4]	0.2927		0.4103	

UN: unavailable data

[1] Oltra-Noguera et al. (2015); [2] Dou et al. (2018); [3] Cao et al. (2006); [4] Musther et al. (2014)

4.1.4 Current knowledge of strain variation in GI physiology

In order to understand the potential effect of strain differences in oral drug pre-clinical studies, it is firstly important to elucidate any strain variations in GI physiology. A minor pH difference along the GI tract was reported in SD and Wistar rats (Table 4.3). A study conducted by Christfort et al. demonstrated that pH was the lowest in stomach and maintained a high level along intestinal segments of GI tract in male SD rats (Christfort et al., 2019). A similar trend was reported in a study discussed in Chapter 1 although the stomach pH in the male Wistar rat was higher than that of the male SD rat.

Table 4.3 The pH along the GI tract in male SD and Wistar rats.

	Forestomach	**Glandular stomach**	**Proximal small intestine**	**Distal small intestine**	**Caecum**	**Colon**	
pH in SD rat [1]	2.0 ± 0.5	2.9 ± 0.7	7.5 ± 0.3	7.8 ± 0.3	7.6 ± 0.2	7.6 ± 0.2	
	Fundus	**Antrum**	**Duodenum**	**Jejunum**	**Ileum**	**Caecum**	**Colon**
pH in Wistar rat [2]	5.1 ± 0.7	4.9 ± 0.3	6.5 ± 0.2	6.7 ± 0.1	7.3 ± 0.1	6.8 ± 0.1	6.7 ± 0.1

[1] Christfort et al. (2019); [2] Data from Chapter Two

In addition, strain differences in intestinal enzyme activity have been reported. UDP-glucuronosyltransferases (UGTs) are one of the intestinal enzymes that are responsible for the glucuronidation of a wide range of structurally diverse endogenous and exogenous substances (Tukey and Strassburg, 2000; Mackenzie et al., 2005). By conjugating glucuronic acid to substrates, UGTs can increase the water solubility of the toxic substrates and enhance their excretion through bile and urine (Dutton, 1980). According to Takaya *et al.*, the mRNA of UGTs isoforms (Ugt1a1, Ugt1a6, Ugt1a7, Ugt1a3) exhibited a higher expression level in small

intestine of Wistar rats than in SD rats (Kutsukake et al., 2019). A sex-related strain difference was further reported in the study as well. As shown in Figure 4.3, female rats displayed a significantly higher ($p < 0.001$) expression level for both Ugt1a7 and Ugt1a6 when compared with their counterparts in SD rats. Conversely, significant sex differences ($p < 0.001$) were also identified in the expression of Ugt1a and Ugt1a7 in Wistar rats but with a higher expression in male (Kutsukake et al., 2019).

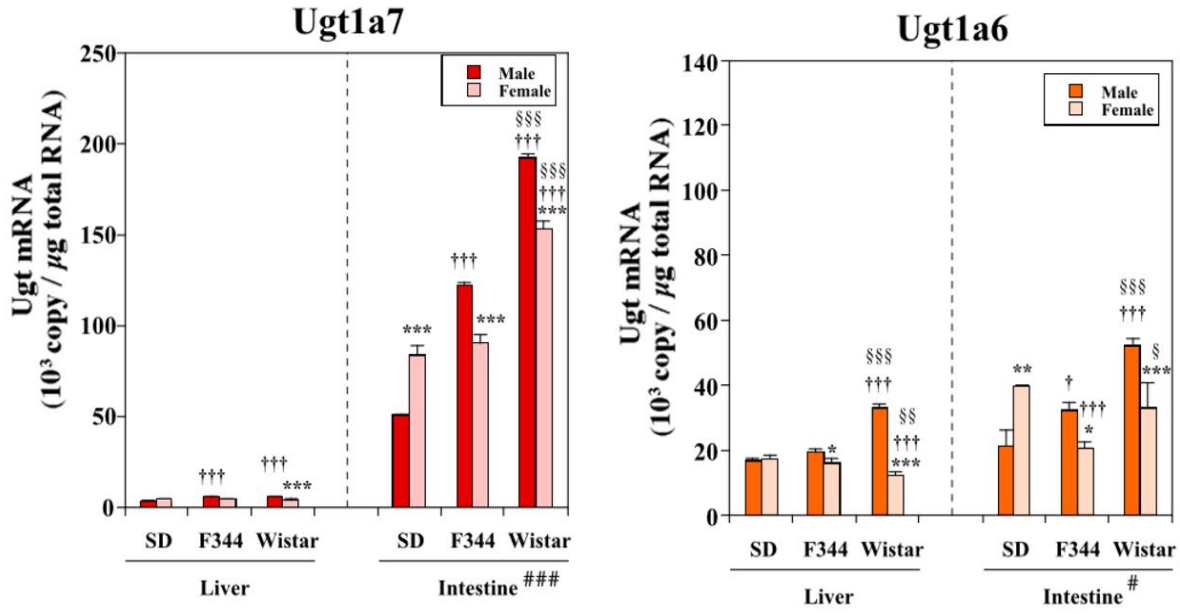


Figure 4.3 Absolute quantification of Ugt mRNAs in the liver and small intestine from male and female SD, F344, and Wistar rats. *P, 0.05; **P, 0.01; ***P , 0.001 (compared with males); †P , 0.05; ††P , 0.01; †††P , 0.001 (compared with SD rats); #P , 0.05; ##P , 0.01; ###P , 0.001 (compared with liver), adapted from Kutsukake et al., 2019.

Another study investigated the difference in enzyme activity between Wistar and SD rat strains. The study demonstrated that the activity of alkaline phosphatase and maltase in duodenum segment of Wistar rats were approximately twice as high when compared with SD rats where no strain differences were observed for trehalase and ATPase activities (Hietanen et al., 1972).

Despite a number of high impact discoveries as aforementioned, there is still a lack of a comprehensive understanding between the differences of strain on GI physiology. The luminal environment and fluids have been well characterised in Wistar rats as disclosed in Chapter 2. As such, the luminal property with the consideration of sex and potential food effect is worth exploring in the Sprague Dawley rat model. In addition, the identified sex-related strain differences on the intestinal permeability of P-gp substrate, verapamil (Table 4.2) is a promising starting point for the exploration of sex-dependent strain differences in P-gp expression. More importantly, considering the correlation differences between humans and different rat strains (Section 4.1.3), the comprehensive understanding of human gastrointestinal physiology at a fundamental level is needed for the comparison with different rat strains. As such, this information can contribute to the better selection of rat strains for pre-clinical studies. Herein, therefore, the potential sex effect on GI physiology including luminal fluids properties and intestinal P-gp expression was investigated in SD rats. Moreover, human intestinal P-gp expression was also measured for the advancement of human-rat correlation to development better animal model selection in pre-clinical studies.

4.2 Aims

- To investigate potential sex differences in luminal fluids by characterising pH, surface tension, osmolality and buffer capacity in male and female SD rats

- To evaluate the intestinal permeability of ganciclovir (P-gp substrate) and quantify the absolute intestinal P-gp expression in male and female SD rats via Ussing chamber permeability studies and LC-MS/MS respectively

- To quantify the absolute human male and female intestinal P-gp expression via the validated LC-MS/MS method to identify the correlation of GI properties between humans and rats

4.3 Materials

Ganciclovir (P-gp substrate) was purchased from Sigma Aldrich (Dorset, UK). The standard peptide for P-gp quantification and its stable isotope-labelled internal standard were of analytical grade (purity > 95%) were synthesised and quantified via the amino acid analysis by Sigma AQUA peptides service (Poole, Dorset, UK). Iodoacetamide (CAS: 144-48-9), dithiothreitol (CAS: 3483-12-3), MS-Grade trypsin (CAS: 9002-07-7) and formic acid (CAS: 64-18-6) were bought from Thermo Fisher (UK). Ammonium bicarbonate BioUltra, chloroform, as well as the LC-MS grade acetonitrile and water were obtained from Sigma (Dorset, UK). HPLC-grade water, methanol, peroxide-free tetrahydrofuran and trifluoroacetic acid were purchased from Fisher Scientific (Loughborough, UK) for luminal fluids characterization. NaOH and HCl (0.1 M standards) were used for buffer capacity determinations and were procured from Sigma Aldrich (Dorset, UK). Krebs-Bicarbonate Ringer's solution (KBR), pH 7.4, composed of 10mM D-glucose, 1.2mM CaCl2, 1.2mM MgCl2, 115mM NaCl, 25mM NaHCO3, 0.4Mm KH2PO4 and 2.4mM K2HPO4 (Clarke, 2009). Lysis buffer was freshly prepared with 50mM Tris, 250mM NaCl, 5mM EDTA, 1mM Na3VO4, 1mM PMSF, 1% Nonidet P40 and protease inhibitor cocktail in phosphate-buffered saline (PBS). All other chemicals and kits are noted individually in the following methods.

4.4 Methods

4.4.1 Human subjects

A total of 30 patients (17 males and 12 females) were enrolled in the study. Human samples were collected from the patients who underwent pancreatic cancer (for jejunum and ileum collection) and colon cancer surgery (for colon collection) at the Third Affiliated Hospital of Sun Yat-sen University (Guangzhou, China) between May 2016 and January 2019. The age of the patients ranged from 30 to 79 years old with the mean value of 46.6. The weight of patients ranged from 49 kg to 86 kg. All patients have signed an informed consent. The clinical

characteristics of each patient, including age, sex, preoperative tumour size, tumour number and the familial history of cancer, were recorded. The details of the patient information can be found in Table 4.4.

Table 4.4 Patient information in this study.

Volunteer	Sex	Age (yr)	Weight (kg)	Disease Type	Sample Region
1	M	60	55	pancreatic cancer	middle to distal jejunum
2	M	56	86	pancreatic cancer	distal jejunum
3	M	44	77	pancreatic cancer	middle to distal jejunum
4	M	48	66	pancreatic cancer	middle to distal jejunum
5	M	32	75	pancreatic cancer	distal jejunum
6	M	39	65	pancreatic cancer	middle to distal jejunum
7	M	36	79	pancreatic cancer	distal jejunum
8	M	33	62	pancreatic cancer	ileum
9	M	42	60	colon cancer	ileum
10	M	30	76	pancreatic cancer	ileum
11	M	50	81	pancreatic cancer	ileum
12	M	78	75	colon cancer	ileum
13	M	62	71	pancreatic cancer	ileum
14	M	66	67	pancreatic cancer	ileum
15	M	79	76	colon cancer	ileum
16	M	32	61	pancreatic cancer	ileum
17	M	32	68	colon cancer	ileum
18	F	34	60	pancreatic cancer	proximal jejunum

19	F	43	55	pancreatic cancer	middle to distal jejunum
20	F	56	55	pancreatic cancer	distal jejunum
21	F	30	50	pancreatic cancer	middle to distal jejunum
22	F	55	61	pancreatic cancer	distal jejunum
23	F	61	65	pancreatic cancer	distal jejunum
24	F	52	57	pancreatic cancer	proximal jejunum
25	F	51	66	pancreatic cancer	middle to distal jejunum
26	F	39	55	pancreatic cancer	ileum
27	F	33	49	pancreatic cancer	ileum
28	F	52	65	colon cancer	ileum
29	F	37	57	colon cancer	ileum
30	F	42	52	colon cancer	ileum

M - male; F - female.

4.4.2 Experimental animal

Male and female Sprague Dawley® outbred rats (10 weeks old weighing approximately 250 g and 200 g respectively) were purchased from Harlan UK Ltd. (Oxfordshire, UK) and housed at room temperature of 25°C in a light-dark cycle for 12 h. Rats were provided with free access to food (EURodent Diet 22%) and water and were subject to an overnight fasting of 12 h prior to the experiment.

4.4.3 Luminal fluids characterization

6 male and 6 female SD rats were sacrificed by CO_2 asphyxiation in the morning at around 8:30 am. Then, as described in Chapter 2.4.2, the pH of gastrointestinal tract was measured *in situ* using a pH meter (HI99161) equipped with an FC202 electrode (Hannah Instruments,

Bedfordshire, UK). For each GI segment two *in situ* measurements were taken, one at the proximal opening (A) and the second at the distal one (B). After that, the whole GI tract was promptly extracted and divided into stomach, duodenum, jejunum, ileum, caecum and colon. The gastrointestinal sections were emptied into 1.5 mL Eppendorf tubes and centrifuged (Centrifuge 5415D, Eppendorf AG, Hamburg, Germany) at 13000 rpm for 20 minutes. The supernatant obtained was kept at -80°C until analysed. The evaluation of the osmolality, surface tension as well as buffer capacity of the luminal fluids were processed following the procedure described in Chapter 2.4.2.

Due to the small amount of fluids available from some of the intestinal segments, some tests were run in pooled samples, in which fluids from the same segment of different animals were mixed to increase the available volume to perform the tests.

4.4.4 *Ex vivo* permeation study

Drug solutions (1.96 mM ganciclovir) were freshly prepared in a KBR solution and stored in a 37°C incubator for the experiment. The intestinal tissues of jejunum and ileum were prepared from male and female rats following the procedure elucidated in Chapter 3.4.2. Then, the well-prepared mucosal tissues were mounted in the vertical Ussing Chamber (Harvard Apparatus Inc., Holliston, MA, U.S.A) as flat sheets on a 0.28 cm^2 segment holder with needles for stability purposes. The chambers were tightly screwed with high spring-tension retaining rings and the entire assembly was maintained at 37°C with a circulating water bath for a 30 min equilibrium period. Tissue integrity was evaluated every 30 min during the experiment by measuring tissue transepithelial electrical resistance (TEER) with an EVOMX meter (World Precision Instruments Inc., WPI, Hertfordshire, United Kingdom). Any duodenal, jejunal, ileal and colonic segments that presented a value of TEER lower than 20 $\Omega \bullet cm^2$, 40 $\Omega \bullet cm^2$, 50

$\Omega \cdot cm^2$ and 70 $\Omega \cdot cm^2$ respectively at the beginning of experiment was regarded as poorly viable and excluded immediately. The tissue was not considered viable whenever TEER values decreased more than 15% from the value measured at the end of equilibration period.

The study began with a 20 – 30 min equilibrium period. 5 mL KBR solution was added to both the apical (mucosal surface) and basolateral (endothelial surface) chambers, gassed with an O_2/CO_2 (95%/5%) gas mixture. Following the emptying of the chamber, 5 ml of fresh KBR solution was added in the basolateral chamber whilst 5 ml of the drug solution was added in the apical chamber. During the experiment, 100 μL solution from basolateral chamber was withdrawn every 30 min. The experiment lasted for 2 h and the intestinal permeation was evaluated by analysing the drug amount in the withdrawn samples (mucosa to serosa, M – S). An equal volume of fresh KBR solution was replaced immediately.

4.4.5 Absolute quantification of intestinal P-gp

The total protein solution from human subjects and rats were prepared followed the description in Chapter 3.4.3. As shown in Table 4.5, human (*Homo sapeins*) and rats (*Rrattus norvegicus*) shared the same specific peptide from the tryptic P-gp protein. Therefore, the same P-gp proteotypic peptide and its selected three multiple reaction monitoring (MRM) transitions were applied in the study for human and rat intestinal P-gp determination, shown in Table 4.5.

Table 4.5 Protein specificity of tryptic peptides used for protein quantification in human, mouse, rat, dog and horse, adapted from Groer et al., 2013.

Protein	Peptide	Human (Homo sapeins)	Mouse (Mus musculus)	Rat (Rattus norvegicus)	Dog (Canis lupus)	Horse (Equus ferus caballus)
ABCB1	AGAVAEEVLAAIR	√	√	√	√	√
ABCC2	LTIIPQDPILFSGSLR	√	X	√	√	X
ABCC3	IDGLNVADIGLHDLR	√	X	X	√	√
ABCG2	SSLLDVLAAR	√	√	√	√	√
OATP1A2	EGLETNADIIK	√	X	X	X	X
OATP2B1	SSPAVEQQLLVSGPGK	√	X	X	X	X
OCT1	ENTIYLK	√	X	X	X	X
OCT3	GIALPETVDDVEK	√	X	X	√	√

√ represents the cross-species specificity; X represents no cross-species specificity

Initially, 50 µg total protein fluid from the collected samples of human or rat was processed following the steps elucidated in Chapter 3.4.3.2 for the denaturation, alkylation and digestion. All the procedures were carried out using Protein Lobind tubes (Eppendorf, Hamburg, Germany). After that, 30 µl tryptic protein sample containing the specific peptide was then injected onto LC-MS/MS. An Agilent 6460 triple quadrupole LC and mass spectrometer system coupled with Agilent Jet Stream technology was applied for the analysis (Agilent Technologies, Santa Clara, CA, USA). A gradient elution was applied on a Kinetex C18 column (100 X 3.0 mm, 2.6 µm, Phenomenex, Torrance, CA). The mobile phases were 0.1% Formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) with a flow rate of 0.5 ml/min. The gradient elution procedure started with 98% solvent A for five minutes and then a linear gradient of 98% solvent A to 75% solvent A over 10 min, held at 75% solvent A for 1 min and then changed to 55% solvent A for extra 2 min. Then solvent A changed back to the original statue (98%) and held for 7 min until the end of analyzation. The mass spectrometer was equipped with the electrospray ionization and operated in the positive ion mode to monitor the three m/z transitions with 300oC source temperature, nebulizer 45 psi, 11 L/min sheath gas flow, 500 V nozzle voltage, 20 collision energy as well as 7 cell accelerator voltage. All the chromatograms were assessed with the MassHunter Workstation software (Qualitative Analysis version B.06.00) and all samples were analysed in duplicate.

4.4.6 Statistical Analysis

The experiments were performed at least six times and data were expressed as mean ± standard deviation (S.D.). Significant differences among groups were analysed by one-way ANOVA followed by a Tukey post-hoc analysis with a 95% confidence interval using IBM SPSS Statistics 16 (SPSS Inc., Illinois, USA).

4.5 Results and discussion

4.5.1 Sex difference in luminal fluids in SD rats

4.5.1.1 pH

As shown in Figure 4.4, the gastrointestinal fluid pH in SD rats was lowest in stomach section with pH of around 3.8. Sharp rise in pH was observed from fundus to proximal duodenum (duodenum A) then remained relatively stable along the rest of the gastrointestinal tract. There was no significant sex difference ($p < 0.05$) in fluids pH along the entire GI tract.

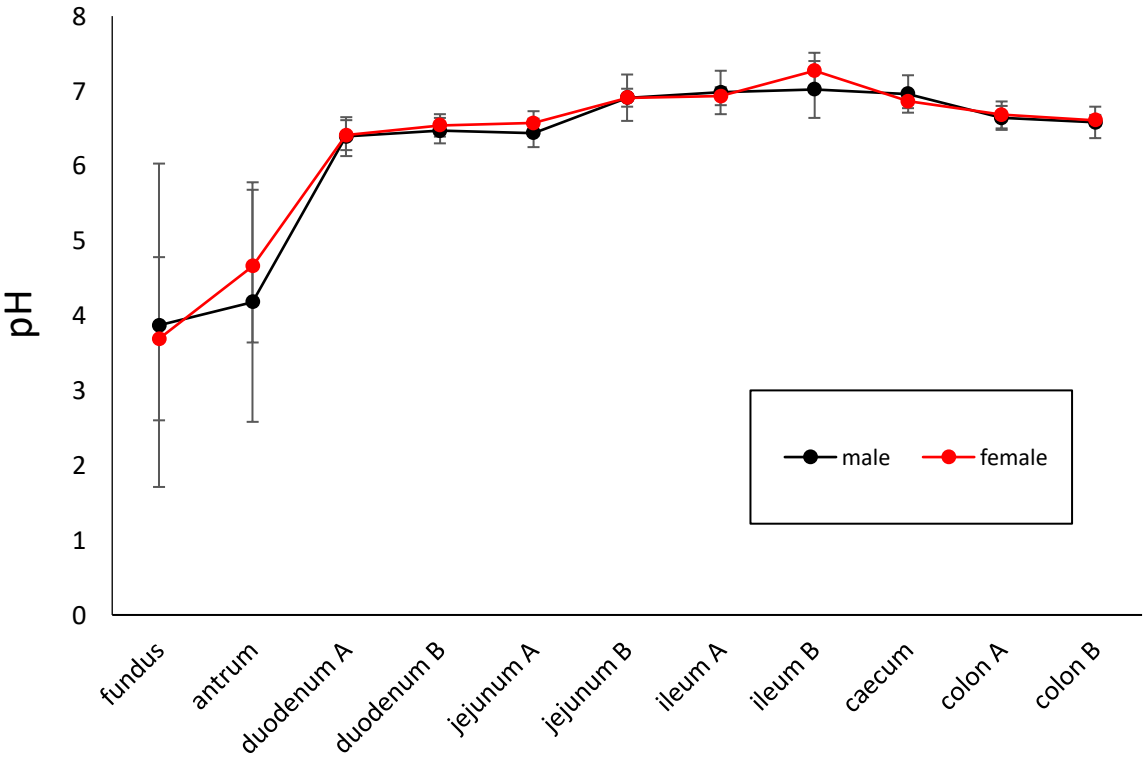


Figure 4.4 pH (mean± standard deviation (SD)) of luminal environment of the gastrointestinal tract section in male and female SD rats under fasted state (n=6). A- denotes proximal portion of segment; B- denotes distal portion of segment.

The overall trend of the pH along the GI tract was similar between the male and female SD rats and corresponding to the trend in published literature (Christfort et al., 2019; Afonso-Pereira et al., 2018; Merchant et al., 2015). In rats, fundus (forestomach) is a holding chamber for food and does not have glands, hence the pH is higher in fundus compared to antrum (Uehara et al., 2018). Antrum contains G cells (gastrin-secreting enteroendocrine cell) that secrete the hormone gastrin. Gastrin stimulates the secretion of gastric acid by the parietal cells present in gastric glands and causes highest acidity in stomach. As secretin is produced in duodenum, it inhibits gastric acid secretion from stomach and stimulates pancreas to release bicarbonate which increases the pH of chyme and causes a huge rise in pH in the duodenum (Utiger, 2017). Fermentation, a process where digested food from small intestines is broken down by bacteria anaerobically occurs mainly in proximal colon which explains for the drop of pH in caecum and colon. This also reflects the ability of rats as herbivores to break down cellulose (Nakatsuji et al., 2018; Farmer et al., 2014).

In addition, comparing the Figure 2.4 and Figure 4.5, it can be noticed that there was no sex difference in pH along the gut for both SD and Wistar rats. More interestingly, it demonstrated that the pH pattern along the GI tract in SD rats was identical to that of in Wistar rats except the antrum part of stomach. In stomach, SD rats displayed a lower pH value and larger variation compared to Wistar rats. The potential reason for the lower pH value in SD stomach might due to the higher secretion of gastric acid. Another possible reason is the limitation in the experiment. The flowability of the stomach fluids in the fasted-state rat may result in the variation of pH measurement, e.g. the pH probe might be in contact with the mucous layer lining the surface of gastrointestinal tract and causes fluctuation in reading. In human, the pH trend was similar to both SD and Wistar rat except stomach where human displayed a lower pH (1.6-2.7) (Kalantzi et al., 2006).

4.5.1.2 Buffer capacity

As shown in Figure 4.5, the highest buffer capacity was observed in stomach for male with the value of around 20. The buffer capacity decreased until jejunum segment and stabilised before a drastic decline was observed at colon segment. Female rats were spotted to have highest buffer capacity in jejunum segment with the value of around 18 and then declined gradually until colon segment. There was no significant difference ($p < 0.05$) between male and female rats under fasted state except in stomach segment.

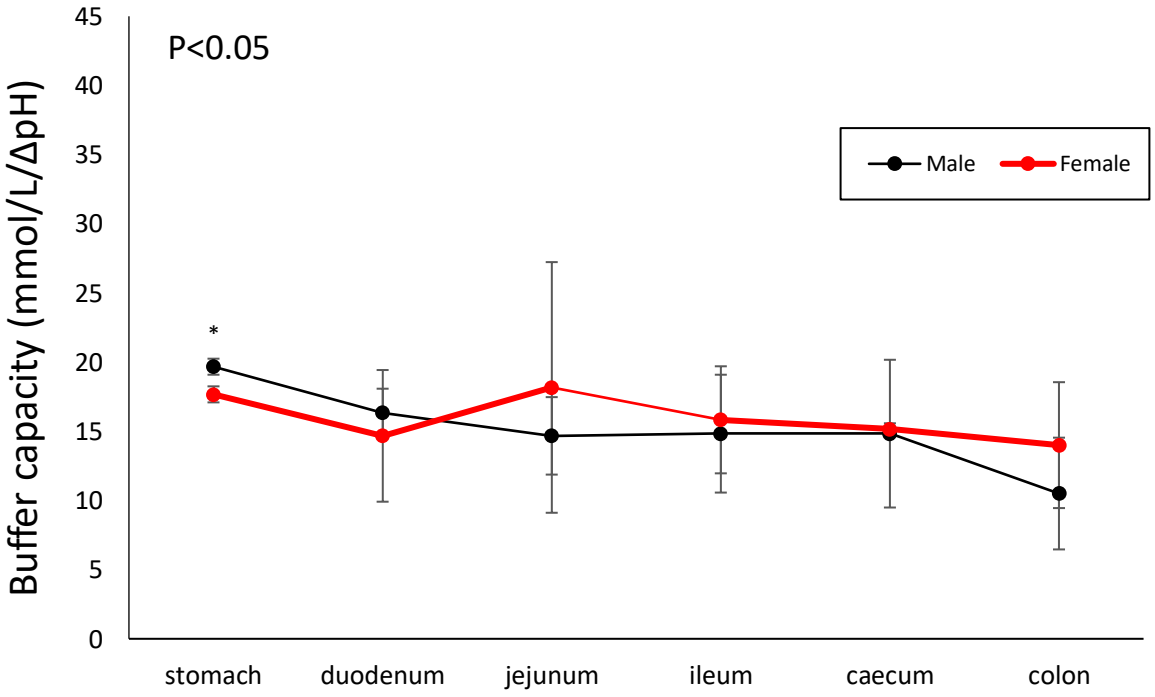


Figure 4.5 Buffer capacity (mean± standard deviation (SD)) of gastrointestinal fluid of the gastrointestinal tract sections in male and female SD rats (n=6). * denotes statistically significant difference ($p < 0.05$) between the two groups compared in each graph above.

Overall, the trend for buffer capacity of luminal fluid along the GI tract was similar in both male and female rats, gradually decreased from proximal intestine to distal intestine. The

observed sex difference in stomach buffer capacity may due to the coprophagy. Several studies have demonstrated that the adult male laboratory rats consumed their maternal faeces (Novakova and Babicky, 1989). In addition, the observed variation of buffer capacity in both male and female rats represented the individual variability as the samples were pooled in two groups of three animals respectively to obtain an average from six reading.

Compared to the buffer capacity obtained in Wistar rats (Figure 2.5), the pattern of luminal fluid buffer capacity along the intestine was similar between the two commonly used strains of laboratory rats, which were both in the range from 10 to 25 mmol/L/ΔpH. In human, a similar range of buffer capacity was reported, 6.9~27.6 mmol/L/ΔpH, according to the previous studies (Kalantzi et al., 2006; Litou et al., 2016; Reppas et al., 2015; Diakidou et al., 2009). However, instead of gradually decreasing from stomach to colon which happened in both Wistar and SD rat, human buffer capacity achieved higher value in both stomach (18~27.6 mmol/L/ΔpH) and in distal intestine (19.2~21.4 mmol/L/ΔpH).

It has been demonstrated that the buffer capacity of luminal fluids was heavily affect by the ingested food (Afonso-Pereira et al., 2018). Highest buffer capacity in stomach of male rats was associated with the large amount of chyme that is slowly released into the duodenum. As the chyme is released to duodenum, the buffer capacity decreases by around 13mmol.L^{-1}ΔpH^{-1}. In addition, it was reported that the short-chain fatty acids (SCFAs) contributed to the overall buffer capacity of gastrointestinal fluid (Liu et al., 2014). Therefore, with no ingested substance under fasted-state rats, the buffer capacity along the GI tract maintained a baseline value and there was no sex and strain difference. The observed buffer capacity pattern of rat gastrointestinal fluid with the consideration of sex and strain could potentially have an impact

on both the solubility and permeability of drug since the pH of gastrointestinal fluid highly dependent on the buffer capacity.

4.5.1.3 Osmolality

The osmolality of gastrointestinal fluid in both male and female SD rats showed a sharp increase from stomach to duodenum, then declined distally (Figure 4.6). Female rats were found to have a higher osmolality than male rats across the gastrointestinal tract except stomach segment. Statistically significant differences ($p<0.05$) were found in three segments: stomach, jejunum and colon.

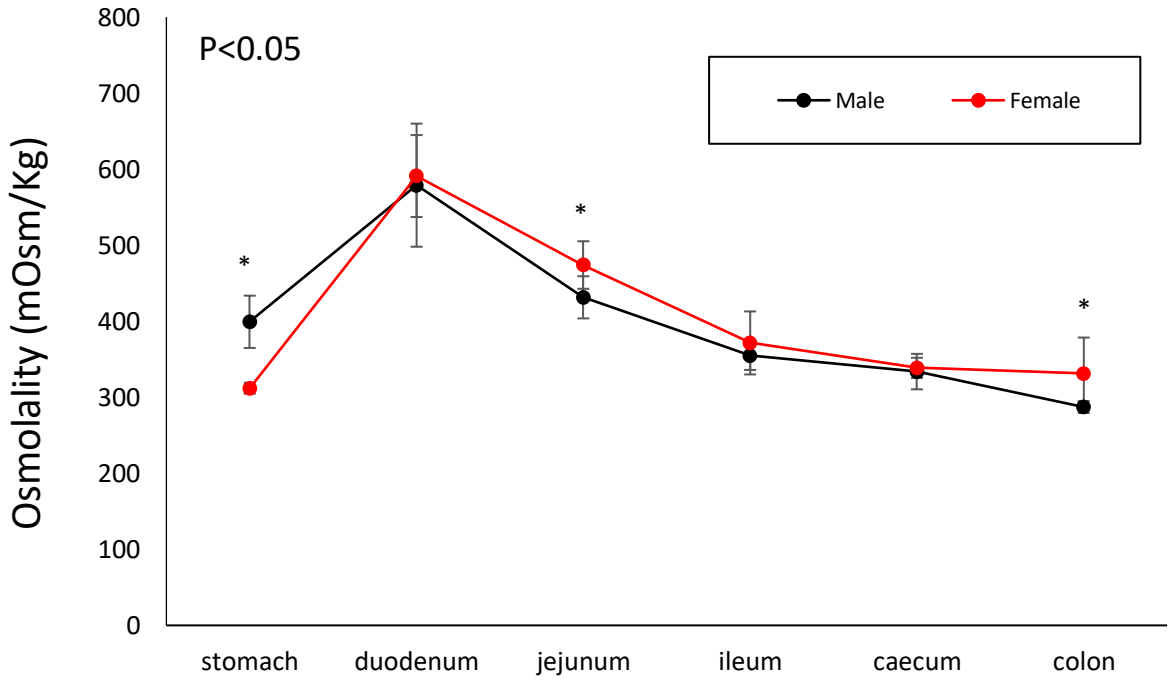


Figure 4.6 Osmolality (mean± standard deviation (SD)) of gastrointestinal fluid of the gastrointestinal tract sections in male and female SD rats (n=6). * denotes statistically significant difference ($p< 0.05$) between the two groups.

The osmolality of SD rat luminal fluids exhibited the similar pattern to that of in Wistar rat and the potential reason might due to the generation mechanism of the luminal fluid. In current

study, after overnight fasting, the main components contained in luminal fluids will be own organ secretions such as bile salts. As rodents do not have gall baller, duodenum is the place initially collect the secretions and therefore achieve a high concentration (Oldham-Ott et al., 1997). With the spread of the secretion from the duodenum to distal intestine, the concentration of the bile salts tends to decrease (Vonk et al., 1978). As a result, the osmolality peaked in duodenum and gradually decreased along the GI tract. In stomach, the fluid of male rat displayed a higher osmolality compared to their counterparts and the potential reason is may be due to the coprophagy phenomenon reported in the previous study (Novakova and Babicky, 1989). In addition, the significant higher osmolality value observed in female jejunum and colon may related to the higher gastrointestinal fluid volume in male (Freire et al., 2011). Compared to the rat, human displayed a relatively lower osmolality along the GI tract, which was 117~206 mOsm/kg in upper intestine and 60~144 in distal intestine (Litou et al., 2016; Reppas et al., 2015; Diakidou et al., 2009).

It is important to note that the amount of water ingested by rats before sacrificed was not possible to be controlled and this could affect the osmolality of gastrointestinal fluid. Osmolality is the measurement of concentration of solute in the solvent, hence if more water is ingested, the osmolality is reduced. As aforementioned in Chapter 2, the change in ionic contents in gastrointestinal fluid may influence the solubility of ionisable drug and reduce absorption of drug (Horter and Dressman, 2001). Therefore, this finding is important due to the alteration of ionic content may further influence drug ionisation, and hence, limit drug absorption. The higher GI osmolality in rodents than human need to be concerned during the early drug development.

4.5.1.4 Surface tension

The following figure represents how surface tension of gastrointestinal fluid changes across the gastrointestinal tract of male and female SD rats. As shown in Figure 4.7, the surface tensions of gastrointestinal fluid of male rat were significantly different ($p < 0.05$) from female rat in stomach section of the gastrointestinal tract. The surface tension of male rat was highest in stomach segment with value of around 65 mN/m and steeply fell in duodenum segment then increased gradually until caecum before slight drop was observed. For female rats, the lowest surface tension of around 48 mN/m was found in stomach section. The value then slightly rose in jejunum and remained the same until colon section.

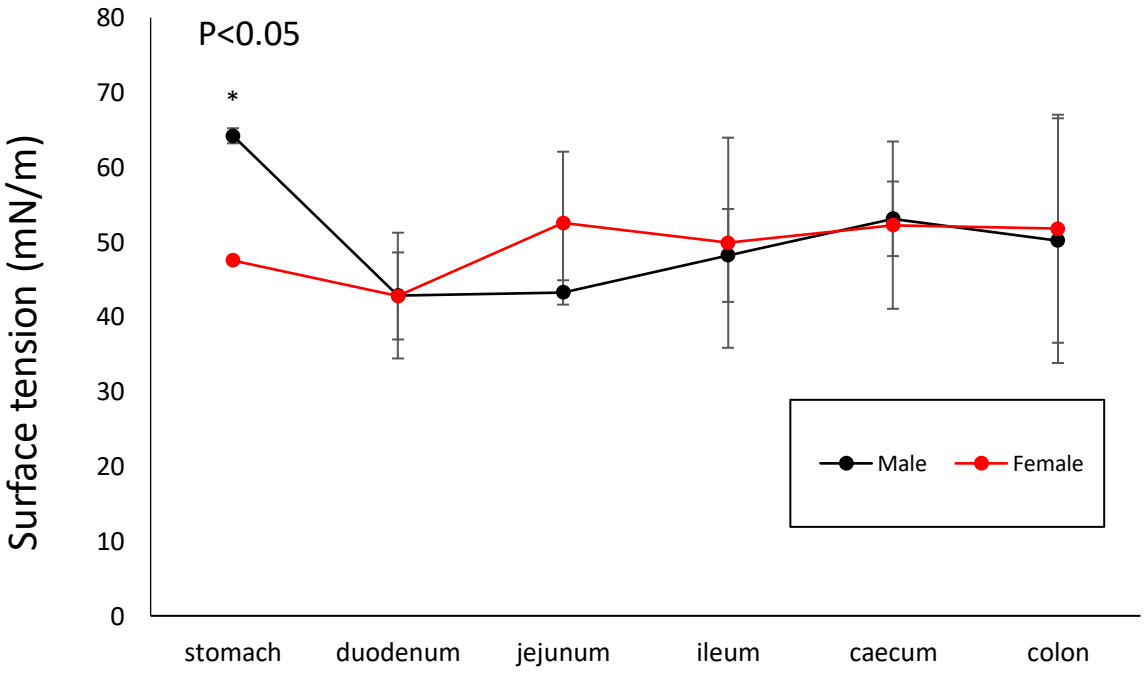


Figure 4.7 Surface tension (mean± standard deviation (SD)) of gastrointestinal fluid of gastrointestinal tract sections in male and female SD rats (n=6). * denotes statistically significant difference ($p < 0.05$) between the two groups.

Surface tension of gastrointestinal fluid has a crucial influence on the drug absorption by affecting wetting behaviour and solvation of poorly soluble drug (Fuchs and Dressman, 2014). Most of the time, surfactants are added in drug formulation to lower the surface tension to increase drug particle solvation and wettability, which improve drug dissolution.

The overall trend of fluid surface tension value along the gut was similar between SD rats and Wistar rats as shown in Figure 2.7 and Figure 4.8. In SD rats, a higher surface tension ($p< 0.05$) in stomach of male rats compared to female rats was observed. To our knowledge, there is no published literature comparing surface tension of male and female rats under fasted state. We hypothesise that the result observed might be related to inherently larger pool of bile acid in female rats compared to male rats which reduces the surface tension of gastrointestinal fluid (Turley et al., 1998). In addition, the faeces consumption reported in adult male rat may also contribute to the higher surface tension of stomach fluids observed in current study (Novakova and Babicky, 1989). Compared to the rats, human luminal fluids possessed a slightly lower surface tension which ranged from 32.7 to 43 mN/m but a similar profile along the GI tract (Diakidou et al., 2009; Clarysse et al., 2009).

4.5.2 Sex difference in intestinal P-gp expression and activity of SD rats

4.5.2.1 Sex difference in intestinal P-gp expression

As shown in Figure 4.8 , P-gp expression is significantly increased from the proximal to distal segments of the small intestine. Absolute P-gp levels significantly increased by 289% from the duodenum (0.56 ± 0.06 fmol/μg) to the ileum (2.18 ± 1.02 fmol/μg) in males ($p < 0.05$). Small intestinal P-gp expression in female Sprague Dawley rats resembled the same trend as males where P-gp levels significantly increased by 181% from the duodenum to ileum (from 0.69 ± 0.06 fmol/μg to 1.96 ± 0.84 fmol/μg respectively) ($p < 0.05$). P-gp expression in the colon, however, significantly decreased from specific regions of the small intestine in both sexes.

Colonic P-gp levels significantly decreased by approximately 41% and 65% from the jejunum and ileum (1.16 ± 0.18 fmol/µg and 2.18 ± 1.015 fmol/µg to 0.69 ± 0.14 fmol/µg) in males respectively. In females, P-gp expression in the colon decreased by nearly 38% and 59% from the jejunum and ileum respectively (1.30 ± .29 fmol/µg and 1.96 ± 0.84 fmol/µg to 0.81 ± 0.16 fmol/µg) ($p < 0.05$). Colonic P-gp levels resembled similar levels to that expressed in the duodenum. In addition, no significant differences were determined in the intestinal expression of P-gp between males and females ($p > 0.05$).

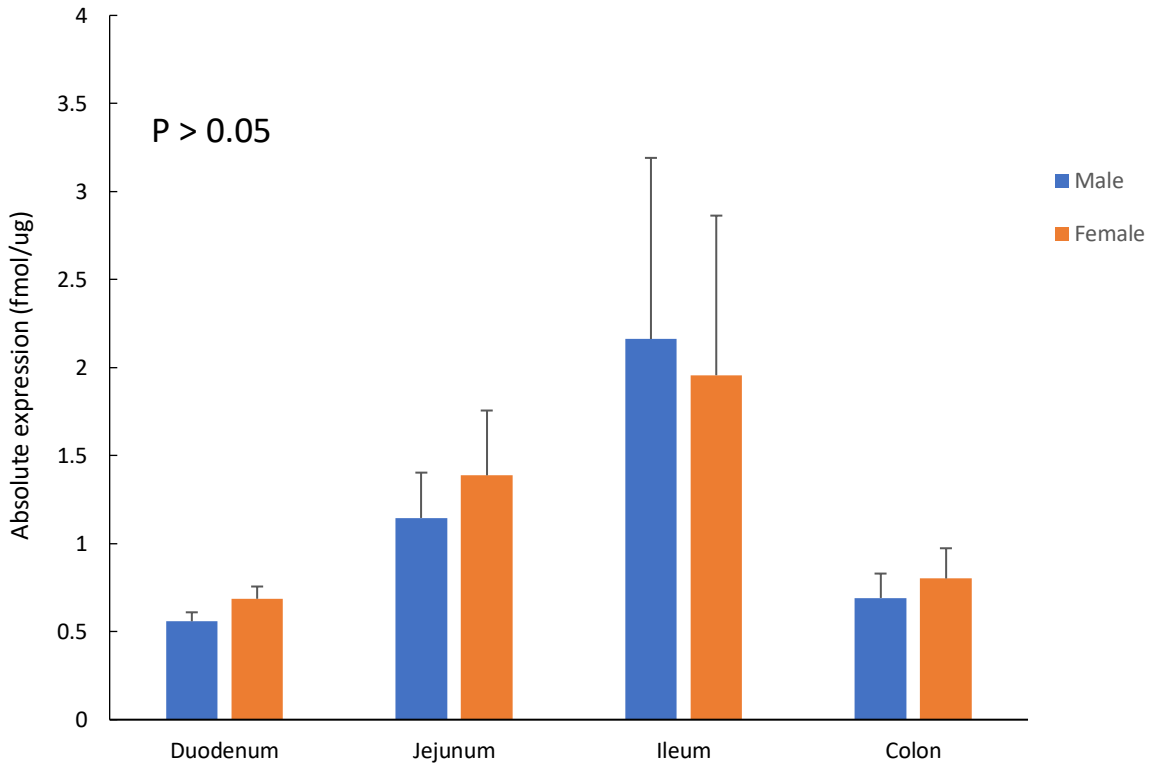


Figure 4.8 Absolute intestinal P-gp expression in fasted Sprague Dawley rats in males and females. Data are presented as mean ± SD (n = 5).

In this study, P-gp expression significantly increased from the proximal to distal regions of the small intestine in both males and females prior to a significant reduction in protein levels in the colon from the ileum. Previous work conducted by Brady et al. (Brady et al., 2002) investigated

the relative gene expression of P-gp (mdr1a mRNA) in the complete intestine of male SD rats. It was demonstrated that the tissue distribution of rat mdr1a mRNA was found to significantly increase from the proximal to distal segments of the small intestine although decreasing from ileum to colon (Figure 4.9).

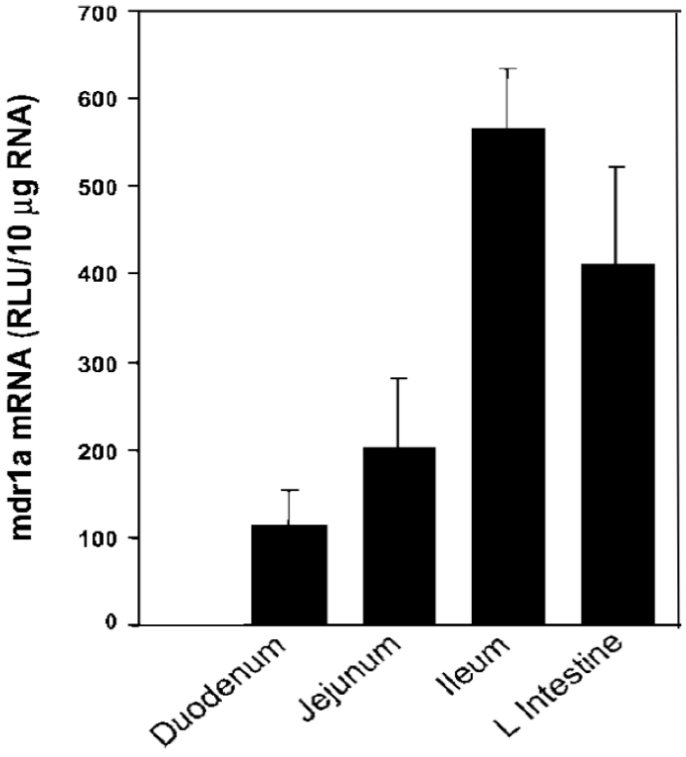


Figure 4.9 Tissue distribution of rat mdr1a mRNA in the male SD rat. Data are presented as mean ± SD (n = 5) (Brady et al., 2002).

As the strong positive correlation between mdr1a expression and P-gp protein abundance with high levels of P-gp expression associated with high levels of mdr1a has been reported in a study conducted by Mai et al. (Mai et al., 2018), the work reported by Brady et al. therefore supports the work outlined for male Sprague Dawley rats herein due to the positive correlation in mdr1a

expression and P-gp protein level attained via LC-MS/MS with the same trend observed in females.

Moreover, the P-gp expression pattern observed in current study was represented in drug intestinal permeability level from the previously published studies. Oltra-Noguera et al. investigated the influence of strain and sex on the gastrointestinal absorption of a strong P-gp drug substrate, verapamil (Oltra-Noguera et al., 2015); no differences were identified in the intestinal absorption of verapamil between male and female SD rats which may be due to similar expression levels from the duodenum to colonic regions of the intestine. In addition, due to its poor bioavailability following oral administration, a promising anti-cancer agent, SNX-2112, was assessed for its potential interactions with P-gp. Liu et al. showed that the absorption of SNX-2112 was mediated by P-gp in the intestine in male SD rats (Figure 4.10) (Liu et al., 2014).

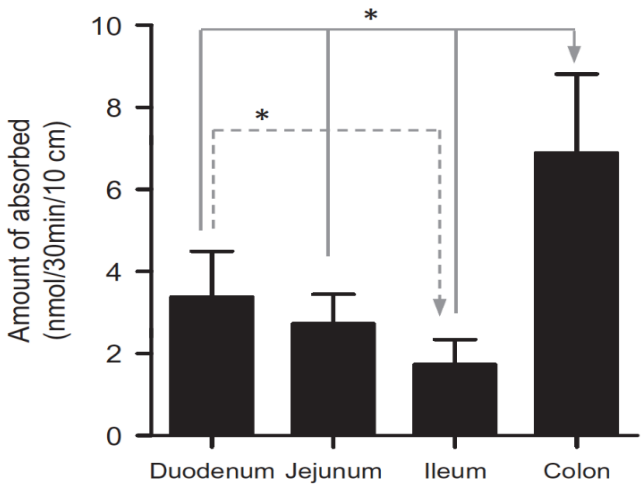


Figure 4.10 P-gp-mediated efflux limited intestinal absorption of SNX-2112 in different segments of male Sprague Dawley rat intestinal. Data are presented as mean ± SD (n = 4). * Denotes statistical significance ($p < 0.05$) (Liu et al., 2014)

Figure 4.9 and Figure 4.10 further support the results of the study investigated herein as the concentration of SNX-2112 (mmol/30 min/10 cm) absorbed was lowest in the ileum. As P-gp is highly expressed in the ileum when compared with any other segmental region of the small intestine, a higher concentration of P-gp substrate can be pumped out of the cell membrane to decrease permeation. The highest amount of SNX-2112 was absorbed in the colon which is subsequently where P-gp is least expressed. However, in contrast to Figure 4.10, there were no significant differences in duodenal and colonic P-gp expression in male and female SD rats (Figure 4.8) ($p > 0.05$). The study conducted by Liu et al. reported that exact reason as to why absorption in the colon was less limited by P-gp mediated efflux is unknown (Liu et al., 2014). However, it can be suggested SNX-2112 is potentially mediated by other ABC efflux membrane transporters in the colon such as multi-drug resistant protein 2 (MRP2) and MRP3 (Drozdzik et al., 2014). Although, SNX-2112 may also have a higher affinity for solute carrier membrane transporters (otherwise known as uptake transporters) than P-gp which can mediate its absorption from the lumen and into the cell membrane (Drozdzik et al., 2014). This could therefore be another reason as to why the absorption of SNX-2112 is significantly higher in the colon than in the small intestine. The role of membrane transporters in the colon and its influence on drug absorption is poorly understood, however, and should addressed in future studies.

In contrast to the P-gp expression in SD rats, the P-gp expression pattern was different as reported in Chapter 3 (Figure 3.13). P-gp expression increased from the proximal to distal regions of the small intestine in male Wistar rats, however, in their female counterparts, P-gp levels were significantly lower in the jejunum and ileum. Male SD intestinal P-gp expression described herein compliment the absolute intestinal P-gp expression levels for male Wistar rats whereby expression is increased from the proximal to distal regions of the small intestine.

However, as no significant differences were observed ($p > 0.05$), SD rats do not exhibit sex differences between males and females in intestinal P-gp expression as found in Wistar rat. These stark differences in normal intestinal physiology highlight the need of assessing the potential influence of strain and sex in the pre-clinical arena which may lead to differences in drug pharmacokinetics or efficacy. As P-gp is a biological efflux membrane transporter capable of modulating the transmembrane activity of many drug products in different organs, the findings reported here could be particularly consequential if evaluating a novel drug is a P-gp substrate. If the SD rat was used in the pre-clinical development of a new P-gp drug substrate, an entirely different result may occur comparing the study using Wistar rat. It is therefore important to consider the rat strain in order to optimise pharmaceutical research for the better development of medicines for humans.

4.5.2.2 Sex difference in intestinal P-gp activity on ganciclovir permeation

As shown in Table 4.6 and Figure 4.11, the permeability of ganciclovir exhibited similar value between male and female SD rat with no statistically significant ($p>0.05$). In male SD rats, the Papp of ganciclovir was sex $10.98 \pm 3.47*10^{-6}$ cm/s and $10.99 \pm 1.69*10^{-6}$ cm/s in jejunum and ileum respectively. A similar value was observed in female rats as the Papp of ganciclovir was $9.82 \pm 2.5*10^{-6}$ cm/s in jejunum and $8.64 \pm 2.61*10^{-6}$ cm/s in ileum.

Table 4.6 Apparent drug permeability coefficients (cm/s $x10^{-6}$) in male and female SD rats (mean ± SD, n = 6).

	Male SD rat	**Female SD rat**
Jejunum	10.98±3.47	9.82±2.51
Ileum	10.99±1.69	8.64±2.61

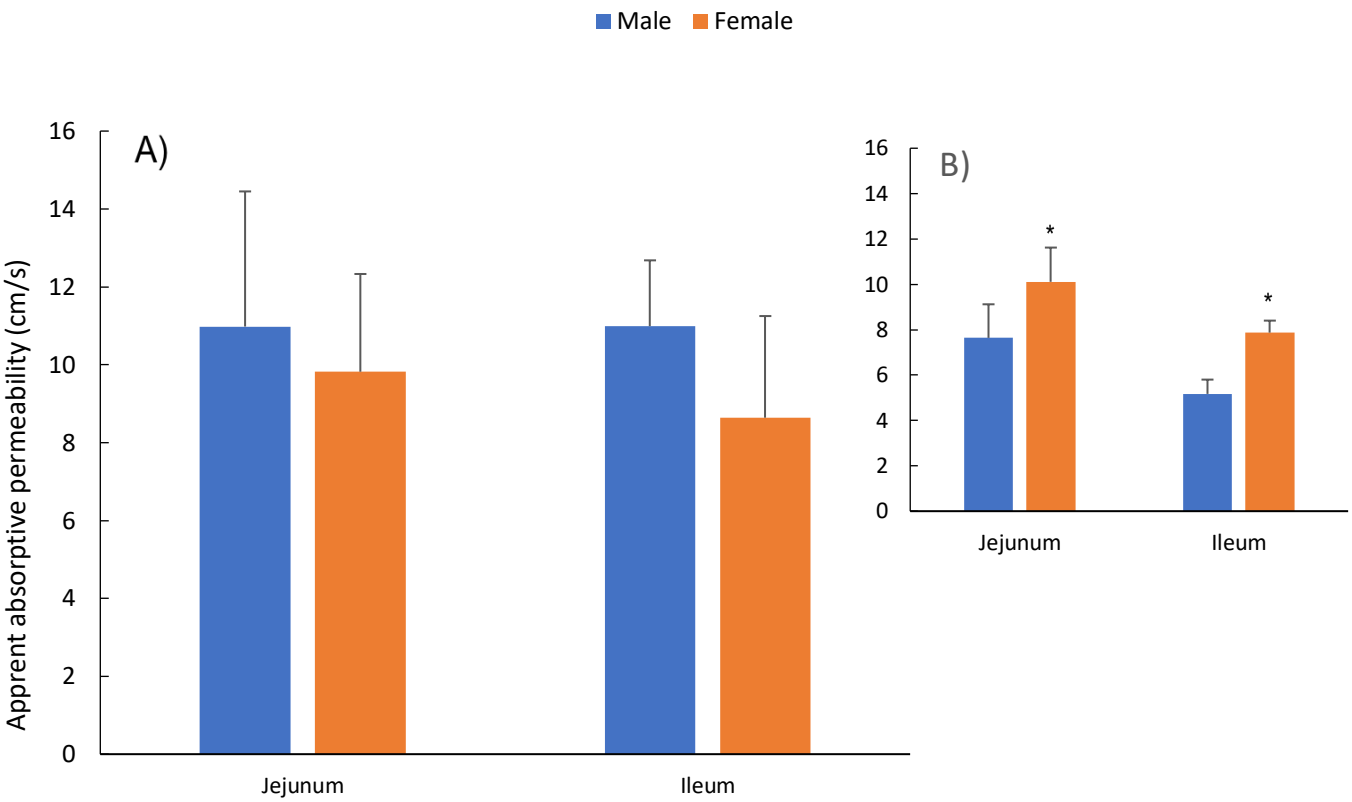


Figure 4.11 Apparent permeability coefficients of ganciclovir in A) male and female SD rats (n=6); B) male and female Wistar rats (n=6, data from Chapter 2). * denotes a statistical significance ($p<0.05$) between males and females.

In Chapter three, it has been well discussed that ganciclovir is P-gp substrate and can be modulated by intestinal P-gp expression. As shown in Figure 4.11, the higher value of P_{app} in female Wistar rat than that of in male rat supported the P-gp expression results in Wistar rats. Likewise, the no sex-related difference in ganciclovir SD rat permeation results highly supported that there is no sex difference in intestinal P-gp expression in SD rat. The data displayed here is an additionally evidence to prove that SD rat do not have sex difference in the intestinal P-gp expression and do not show any sex-related difference in intestinal permeation of P-gp substrates.

4.5.3 Sex difference in human intestinal P-gp expression

According to Table 4.7 and Figure 4.12, the absolute level of P-gp protein was quantified in human jejunum and ileum to identify P-gp expression profile along the intestinal tract. As seen from Figure 4.12, in male subjects, there is a constant increase in P-gp content along the small intestine from proximal to distal parts from 2.81 ± 0.75 fmol/µg in jejunum to 4.56 ± 0.88 fmol/µg in ileum. The findings herein were in good agreement with a study where the absolute P-gp content from six organ donors were quantified using LC-MC/MS (Drozdzik et al., 2014). Drozdzik *et al*. it demonstrated a 3-fold higher P-gp level in the distal ileum compared to the duodenum or the proximal jejunum. In terms of female subjects, a lower expression level was observed in both jejunum and ileum segments at 1.45 ± 0.57 fmol/µg and 2.79 ± 0.46 fmol/µg respectively. The notable sex difference ($p < 0.05$) observed herein was consistent with the previous published study which reported a higher enterocyte P-gp content in the male small intestine when compared to females (Schuetz et al., 1995, Potter et al., 2004).

Table 4.7 Absolute expression of intestinal P-gp for each patient (fmol/µg)

Men			Women		
Patient No.	Jejunum	Ileum	**Patient No.**	Jejunum	Ileum
1	1.09	-	**18**	0.59	-
2	3.32	-	**19**	1.85	-
3	2.41	-	**20**	1.94	-
4	2.89	-	**21**	1.14	-
5	3.05	-	**22**	1.95	-
6	2.22	-	**23**	1.86	-
7	2.97	-	**24**	0.68	-
8	-	6.43	**25**	1.60	-
9	-	4.40	**26**	-	2.71
10	-	3.92	**27**	-	3.53
11	-	3.87	**28**	-	2.88
12	-	4.28	**29**	-	2.31
13	-	5.01	**30**	-	2.52
14	-	3.50	-	-	-
15	-	4.99	-	-	-
16	-	4.11	-	-	-
17	-	4.02	-	-	-

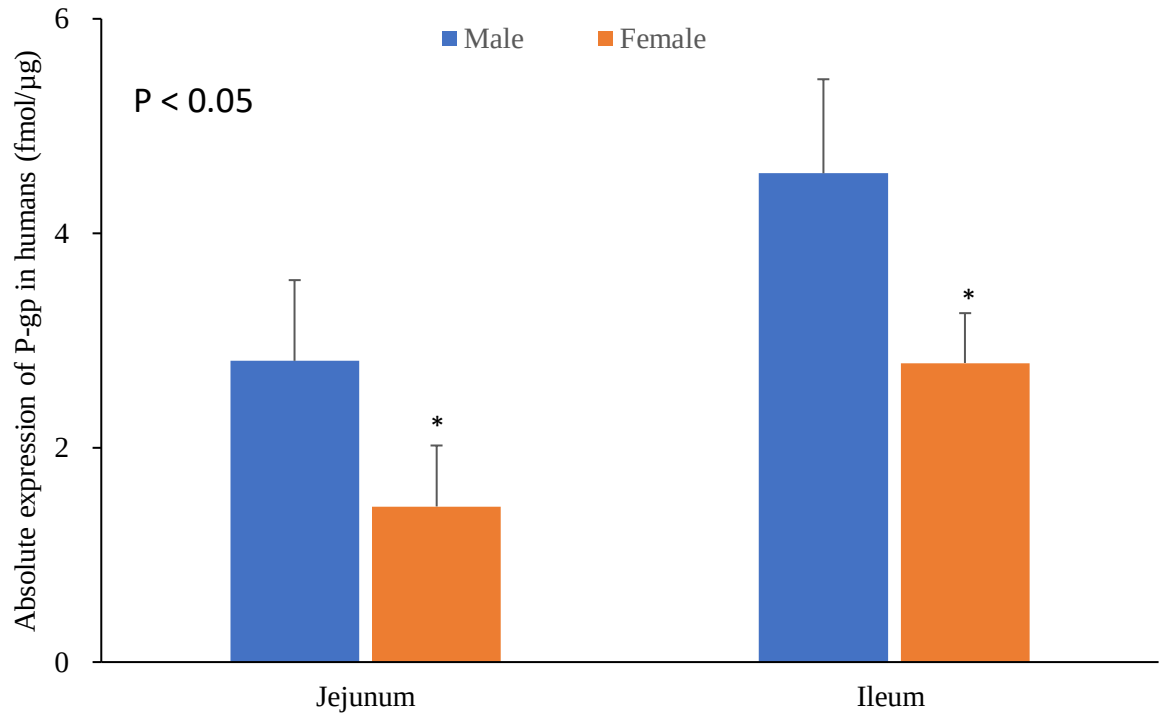


Figure 4.12 Absolute expression of P-gp along the human small intestine, n = 30.
* denotes statistically significant difference ($p < 0.05$) between males and females.

As a well-abundant protein in human intestine, the mapping of P-gp expression along the human intestine reported herein has highlighted that the lower P-gp level and related efflux in the proximal (in contrast to the distal) small intestine may provide an explanation as to why the proximal segment is the ideal absorption site for drugs that are P-gp substrates. It also demonstrated how compounds which reduce the gastrointestinal motility, such as sodium acid pyrophosphate, mannitol and sorbitol, can significantly increase the oral absorption of concomitantly administered P-gp substrate drugs (Adkin et al., 1995a, Adkin et al., 1995b, Chen et al., 2007). In addition, with the novel LC-MS/MS technique, this study was the first to investigate P-gp expression in both men and women. The interesting observation regarding the sex difference in intestinal P-gp abundance may therefore contribute to the understanding of sex difference in clinical drug performance and therapeutic effect listed in Table 1.1.

In addition, as shown in Figure 4.13, both men and male rats exhibited a similar P-gp expression profile along the GI tract in which P-gp expression constantly increased from proximal to distal parts (jejunum < ileum) of small intestine. However, an interesting difference can be noticed with the consideration of sex among these subjects. P-gp expression increased from the proximal to distal regions of the small intestine in Wistar male rat, however, in their female counterparts, P-gp levels were significantly lower in the jejunum and ileum. These results showed good correlation with absolute intestinal P-gp expression in human small intestinal tissues as female P-gp expression was also lower (significantly so in the ileum) than their male counterparts. However, as no significant differences were observed ($p > 0.05$), SD rats do not exhibit sex differences between males and females in intestinal P-gp expression as found in humans. As a result, the study herein has identified that the Wistar rat showed a better correlation with humans in terms of P-gp expression in both sexes when compared with SD rat and therefore, could be an appropriate model to predict the small intestinal sex differences in drug absorption for P-gp substrates in humans.

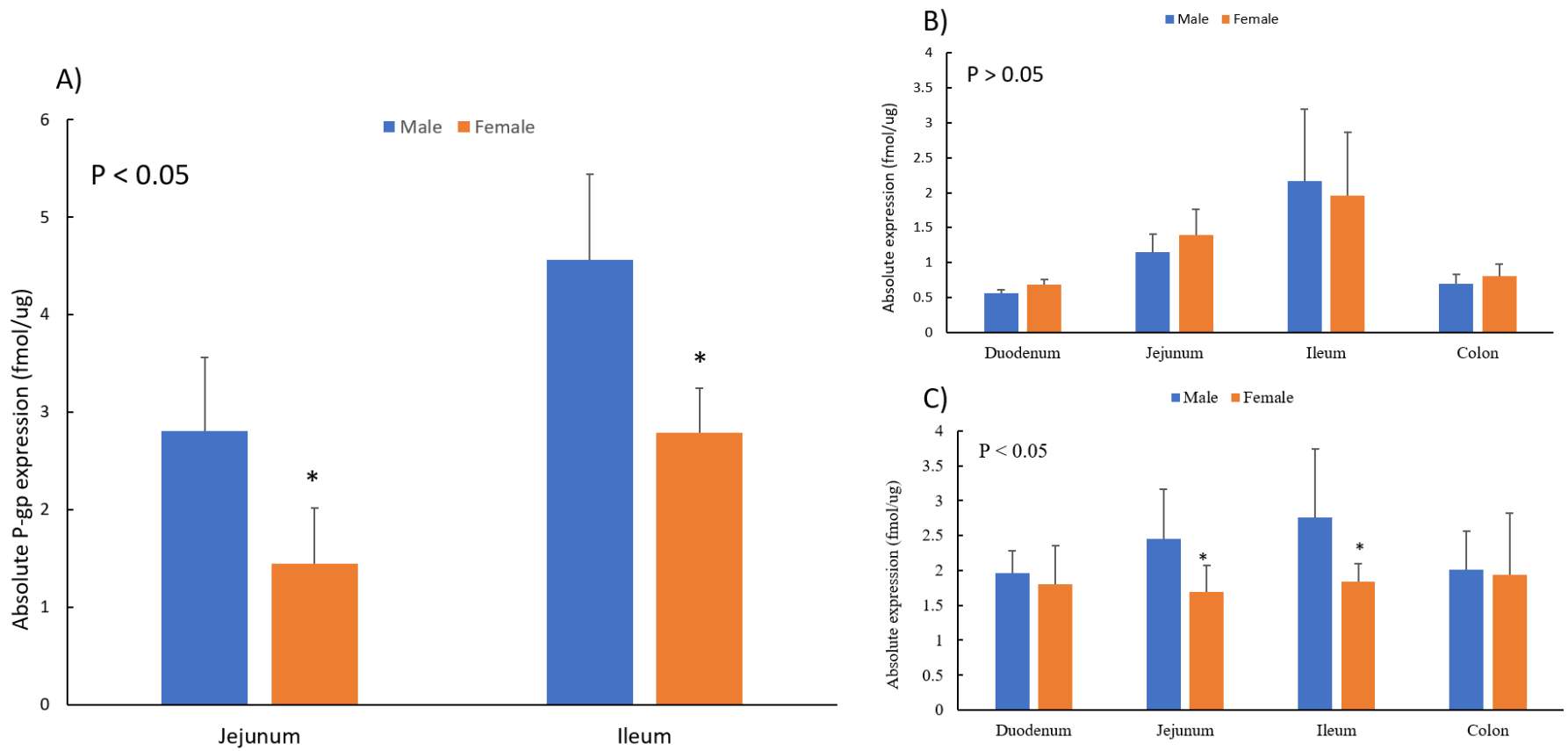


Figure 4.13 Comparison of absolute expression of intestinal P-gp in A) human, B) SD rats and C) Wistar rat (data from Chapter 3). * denotes statistically significant difference ($p < 0.05$) between the male and female groups

In addition, the *in vivo* correlation of oral drug bioavailability between human and rats further supports that the Wistar rat may be a better pre-clinical model than SD rat. As aforementioned, the study conducted by Helen et al investigated the oral bioavailability of 184 compounds and demonstrated that the correlations of oral drug performance between human and rat low (R^2 = 0.287) (Figure 4.2). However, the result was obtained by pooling the data from studies using different strains of rats. After re-analysation, a strain difference in the correlation of oral bioavailability was observed in the 186 compounds studied. As shown in Figure 4.14, the coefficient of determination between human and Wistar rats was the highest (0.4103) compared to the other strains of rat, which was even higher than human and dog correlation (0.374). SD rat achieved a similar R^2 value to the pooled rat data while the other strains of rat display a relatively lower value, which was 0.2927 and 0.1582 respectively. Even though the factor of sex was not considered, it can be claimed that the similarity of sex-related P-gp expression along the gut between humans and the Wistar rat can contribute to the good correlation of oral drug bioavailability.

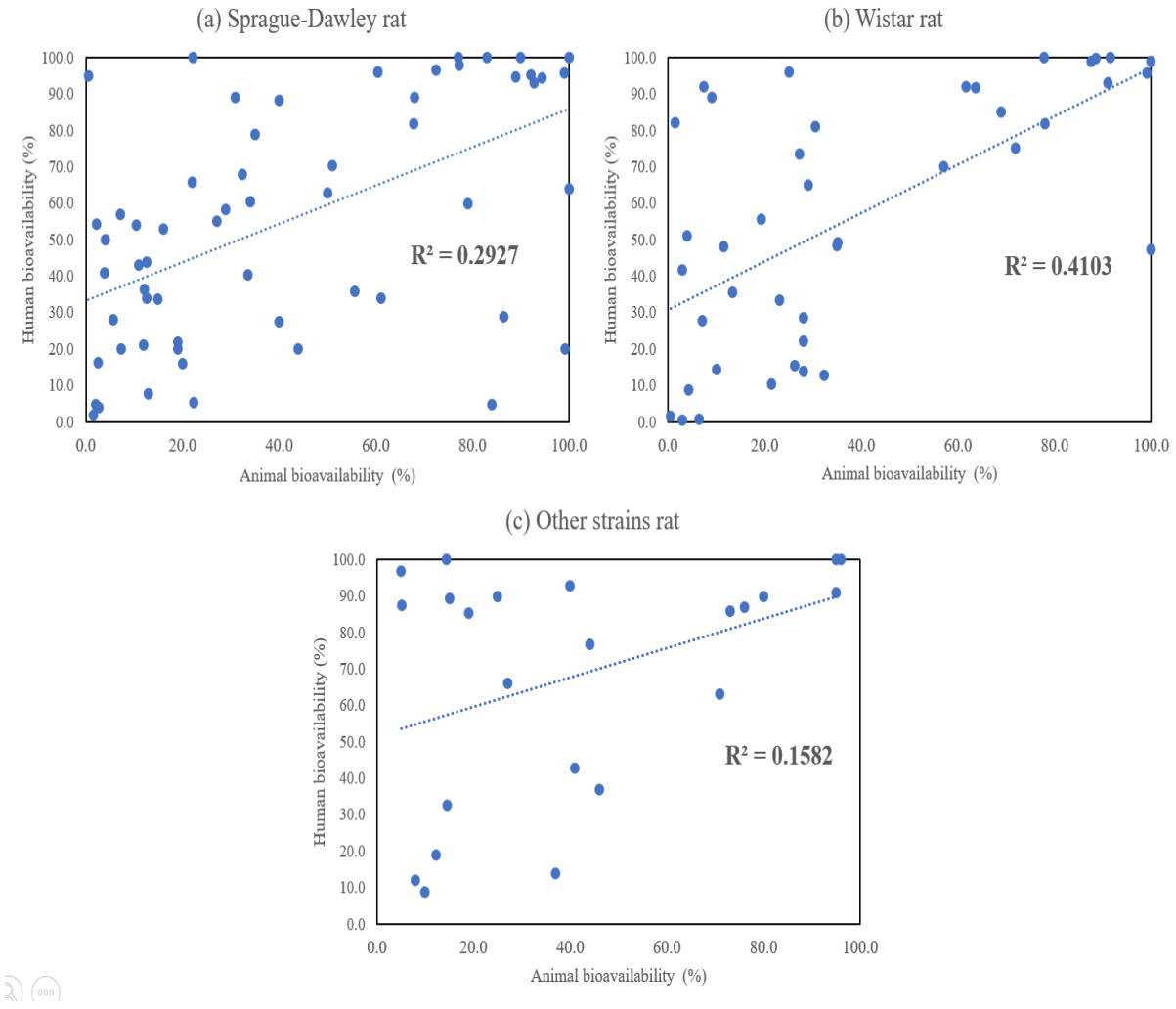


Figure 4.14 Re-analysation of Figure 4.2. (a) Sprague-Dawley rat F vs. human F （60 drugs）; (b) Wistar rat F vs. human F (41 drugs) and (c) Other strains rat F vs. human F (23 drugs).

For the first time, these findings provided a comparable picture of P-gp expression along the intestine of human and preclinical animal models, and demonstrated a good correlation in the small intestine between human and Wistar rat in both sexes. These stark differences in normal intestinal physiology highlight the need of assessing the potential influence of strain and sex in the pre-clinical arena which may lead to differences in drug pharmacokinetics or efficacy. As P-gp is a biological efflux membrane transporter capable of modulating the transmembrane activity of many drug products in different organs, the findings reported here could be

particularly consequential if evaluating a novel drug is a P-gp substrate. If the Sprague Dawley rat was used in the pre-clinical development of a new P-gp drug substrate, an entirely different result may occur during first in human clinical trials due to innate differences in human intestinal physiology. It is therefore important to consider the rat strain in order to optimise pharmaceutical research for the better development of medicines for humans.

4.6 Conclusion

Although traditionally ignored, the selection of rat strain and sex are important factors to consider during early stage drug development as data from different strains may not always be comparable. The work herein is the first to characterise the luminal fluids as well as the absolute P-gp expression along the GI tract in male and female SD rats. It was found that the stomach fluids of male SD rats exhibited a higher buffer capacity, osmolality and surface tension compared to their counterparts. The possible reason may due to the reported coprophagy phenomenon in adult male rats. There was no significant strain difference in the properties of luminal fluid between SD rats and Wistar rats. However, a sex-related strain difference was noticed in intestinal P-gp abundance. Unlike the Wistar rat having sex difference in small intestinal P-gp expression, no significant sex difference observed in P-gp abundance along the GI tract in SD rat ($P>0.05$). In both male and female rats, P-gp levels increased from the proximal to distal regions of the small intestine (duodenum < jejunum < ileum) but decreased in the colon segment which was similar to the duodenum level.

More interesting, it was the first study to quantify the intestinal P-gp in men and women and correlate the data with the data obtained from rats. In view of Figure 4.13, both Wistar rats and human displayed a significant sex difference in absolute P-gp expression in small intestine, while no significant difference was shown in the absolute P-gp protein abundance of SD rats. Our finding firstly reported that there was a sex-related strain difference in gut physiological properties between SD rat and Wistar rats, which Wistar rat exhibited a higher correlation with human in terms of small intestinal P-gp abundance as well as oral drug bioavailability. It also serves as a reminder that the factor of strain and sex need to be taken into consideration when choosing the rat model for the pre-clinical study.

CHAPTER 5:

Conclusion and future work

5.1 Conclusion

It is well-known that there is sex difference in drug performance and male and female patients tend to react differently to the same therapeutic regimen in the clinical practice. It has been reported that women are 1.5 – 1.7 times more likely to develop a drug side-effect compared to their counterparts and there are at least 40% pharmacokinetic differences identified between men and women for 6% to 7% of new active pharmaceutical ingredients after a sex-based analysis. With the awareness of the sex-specific differences in drug performance among patients, extensive studies have been conducted to further elucidate the factor of sex in body physiology and drug behaviour. In addition, both sexes are required to be represented in all phases of clinical trials to avoid sex differences in drug efficacy and side effect according the guideline released from FDA in 1993, "Guideline for the study and evaluation of gender differences in the clinical evaluation of drugs".

However, although the acknowledgement and involvement of sex as a variable in clinical trials have since been applied, such standardisations have not been made in the pre-clinical arena. Consequently, a significant sex bias currently exists in animal-based experiments as researchers continue to neglect the factor of sex. As pre-clinical studies are vital for translation into applicability in human clinical trials, the gap between pre-clinical and clinical study may obscure key sex differences such as an enhanced, reduced or other unexpected effects in specific sexes. Therefore, the current work aimed to fill the gap and investigate the potential sex difference among the pre-clinical animals.

The rat, is the most commonly used animal in pre-clinical study albeit potential sex differences in gut physiology is less known. In Chapter Two, the sex difference in gut luminal fluids and membrane transporter (P-gp) was investigated in Wistar rat, which is a widely used rat strain in early drug development. The characterisation of GI fluids for pH, buffer capacity, surface

tension as well as osmolality were found to be similar between the sexes. A significant sex difference, however, was observed in duodenal buffer capacity, by which female Wistar rats displayed a higher buffer capacity than males ($p < 0.05$). Apart from luminal fluids, an unexpected resulted was identified in the expression of the membrane transporter, P-gp. P-gp is the main efflux transporter expressed on the apical membrane of intestine and heavily affect the P-gp substrate drug absorption. With similar abundance levels in duodenum, male Wistar rats exhibited a significant higher expression of P-gp when compared with their female counterparts in jejunum, ileum and colon ($p < 0.05$). The same sex phenomenon was also observed in P-gp mRNA expression by which male mdr1a gene displayed a higher expression along the intestinal tract. However, following better initial understanding, more concerns were raised to fully elucidate the Wistar rat. The stark sex difference in P-gp observed in Chapter Two garnered conflicting results to previously published studies due to the inconsistency in experimental methods. In the current study, rats were under the fasted-state although other investigations explored P-gp relative expression in the fasted-state. In addition, other species of rats, such as the SD rat, were also explored for P-gp expression. Moreover, the inconsistencies of the reference protein used in Western-blotting experiments for P-gp quantification may also contribute to the controversial results.

In Chapter Three, a novel LC-MS/MS technique was developed and validated to quantify absolute expression of intestinal P-gp, thereby eradicating potential interferences in the use of a reference protein. In addition, with the novel technique, the sex difference in P-gp expression was evaluated in both fasted-state and fed-state Wistar rats. The developed method showed good specificity and linearity ($R^2 > 0.995$) for the P-gp specific peptide quantification within the range from 15.625 to 500 fmol. The method displayed acceptable variation as the relative standard deviation (RSD) did not exceed 15%. Moreover, the inter-day variation for Wistar rats ranged from 11.3 to 14.6 and intra-day variation ranged from 6.1 to 11.1. In terms of

stability, the selected peptide for P-gp quantification exhibited acceptable stability (± 15% of the initial concentrations at low, medium and high QC samples) during storage for i) 2 h at room temperature, ii) 24 h in the cooled autosampler rack and iii) 4 h at 37°C incubation. Finally, the digestion efficiency of the developed method was evaluated under different incubation time, 4 hour, 8 hours, 16 hours and 24 hours. There was no significant ($p > 0.05$) difference between the different incubation times in both male and female Wistar rat. Moving forward, Chapter Three outlined that the absolute intestinal P-gp expression was drastically affected by both the consumption of food and the factor of sex. In fasted-state rats, a sex difference similar to Chapter Two was observed, except colon where there was no sex difference. With the food intake, however, the absolute expression of intestinal P-gp decreased in male rats but increased in female rats, especially in jejunum and ileum segments. As a result, P-gp expression level in females was much higher than male in fed-state rats. Subsequently, the observed sex and food effect on P-gp expression was represented on the P-gp functional activity, the intestinal permeability of P-gp substrates, ranitidine and ganciclovir. With the observation of food-related sex differences in Wistar rat P-gp abundance, a further study was conducted in this Chapter to understand the transition of P-gp expression pattern from fed-sate to fasted-state. It was identified that small intestinal P-gp from fed-to-fasted state achieved a significant increase in males but decreased in females following 4 to 8 hours of fasting. Interestingly, this closely relates to average food emptying time. Chapter Three deduces that intestinal P-gp is a dynamic protein and is reactive to changes in luminal environment and alters its function based on external conditions.

With the understanding of sex difference in GI features of the Wistar rat, another strain, the SD rat, was investigated in Chapter Four to understand the strain variation as Wistar and SD strains are the most commonly used rats in pre-clinical study and are often used interchangeably. There

were no significant strain differences ($p > 0.05$) in the overall trend of pH, buffer capacity, surface tension and osmolality along the GI tract between SD and Wistar rat. The significant sex difference ($p < 0.05$) in SD rat was observed in stomach as all four parameters were higher than female SD rat, which may relate to the consumption of faeces. When compared to humans, both SD and Wistar rat exhibited a similar pH pattern along the intestine except stomach where human displayed a lower pH (1.6 – 2.7). For buffer capacity, humans displayed a similar level when compared with the two strains of rats but displayed a different profile along the gut. A lower but similar profile of surface tension and osmolality were demonstrated between human and SD as well as Wistar rats. Interestingly, a sex-related strain difference in intestinal P-gp abundance was observed in the Chapter. Unlike Wistar rat having a sex difference in small intestinal P-gp expression, no significant sex difference was observed in P-gp abundance along the GI tract in SD rat ($p > 0.05$). In both male and female rats, P-gp levels increased from the proximal to distal regions of the small intestine (duodenum < jejunum < ileum) but decreased in the colon segment which was similar to the duodenum level. In order to compare the intestinal P-gp between human and the rats, the absolute P-gp expression in men and women was firstly investigated in Chapter Four. It was demonstrated that the overall expression of intestinal P-gp was higher in human intestinal tissue compared to both strains of rats. In addition, both Wistar rats and human displayed a significant sex difference in absolute P-gp expression in small intestine, whilst no significant difference was shown in the absolute P-gp protein abundance of SD rats. Moreover, the higher correlation of oral drug bioavailability between humans and Wistar rat ($R^2 = 0.4103$) further supported the fact that the Wistar rat is a good representation of the human gut when compared with the SD rat.

The study conducted herein firstly demonstrated that there was a significant sex difference in the GI physiology of Wistar rats, a commonly used animal in pre-clinical study. Secondly, the state of the rat (fasted or following food intake) heavily affected not only the luminal fluids but

also the expression of P-gp. Consequently, following the lack of sex-differences in GI features in the SD rat ($p > 0.05$) and the Wistar rat closely representing the human small intestine, the results herein advise that the pharmaceutical industry should strongly consider the factor of rat strain and sex as during pre-clinical studies.

5.2 Future work

The findings in this study highlight the need of a standardised pre-clinical animal model to determine potential sex differences towards the better prediction of drug absorption in human males and females during drug development. This work raises several other aspects that could be investigated in the future and divided into two aspects: cross-sectional study and longitudinal study.

Firstly, in terms of the cross-sectional study, other membrane transporters and other animal species extensively used in pre-clinical study need to be investigated. Other efflux membrane transporters that belong to the same ATP binding cassette (ABC) family as P-gp, including breast cancer resistant protein (BCRP) and multidrug resistant-associated protein 2 (MRP2) also contribute to the drug intestinal absorption. There is currently no data available in the literature for their absolute expression in Wistar and SD rat for both sexes. It would therefore be interesting to explore the expression of these efflux membrane transporters using LC-MS/MS to understand the potential sex and strain difference. Future work need not be limited to efflux membrane transporters alone but could further explore the potential influence of uptake transporters including organic anion transporter proteins (OATP). In addition, mice are another widely used animal during the early drug development apart from rat. As such, the potential sex and strain difference is worth exploring in mice as well. Moreover, larger animals such as the dog, monkey and pig are heavily involved in the last stages of pre-clinical studies. Sex differences in these larger animal, especially on membrane function (transporter

abundance and activity) are still unknown. With the novel protein quantification technique, it is worth investigating the transporter expression in these animals as well.

In addition, following the interesting observations in the current work, a longitudinal study should be invested into understanding the phenomena and mechanism behind sex differences. There is no current explanation as to why P-gp expression varies in different strains of male and female rats and more importantly, in humans. Although, a suggested mechanism could be due to a complex interplay between the modulation of P-gp expression, enzyme reaction and the defence ability of epithelial cells which may be further influenced by sex hormones. Moreover, it has been demonstrated that the function of pregnane X receptor (PXR) highly correlated to the modulation of P-gp function blood-brain barrier (BBB). Therefore, the potential correlation between the intestinal PXR and P-gp regulation is interesting to investigate. Understanding the mechanism could fundamentally optimise drug development with respect to the doses required, tailored according to sex.

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