MODULATING INTESTINAL ABSORPTION
USING PHARMACEUTICAL EXCIPIENTS

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(MPharm, MRPharmS)

Thesis submitted for the degree of
Doctor of Philosophy

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PLAGIARISM STATEMENT

This thesis describes research conducted in the School of Pharmacy, University of London between 2005 and 2009 under the supervision of Dr Abdul W 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 the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature

Date
ABSTRACT

The objective of this thesis was to determine the effects of polyethylene glycol 400 (PEG 400) on the intestinal absorption of Biopharmaceutical Classification System (BCS) Class III drugs.

The effect of different doses of PEG 400 (0.5, 0.75, 1, 1.25, 1.5 g) on the bioavailability of ranitidine was investigated in both male and female human subjects. An HPLC method for the analysis of ranitidine (and other H2 antagonists) and a mass spectrometry method for PEG 400 in urine were developed. In the male volunteers, the mean cumulative amount of unchanged ranitidine excreted in urine in the presence of 0.5, 0.75, 1, 1.25 and 1.5 g PEG 400 increased by 34, 63, 49, 43 and 6% over the control, whilst in the female volunteers, there were no differences compared to the control. All doses of PEG 400 enhanced the bioavailability of ranitidine in male subjects but not females, with the most pronounced effect in males noted with the 0.75 g dose of PEG 400 (63% increase in cumulative ranitidine excretion compared to control, p<0.05). The difference in bioavailability of ranitidine between males and females in the presence of PEG 400 was not due to differences in ranitidine metabolism or PEG 400 absorption.

In separate human studies, the effect of different doses of PEG 400 (0, 0.5, 0.75, 1.0, 1.5 and 5 g) on the bioavailability of another BCS class III drug (cimetidine) was investigated. The results mirrored that noted with ranitidine, where lower doses of PEG 400 (< 1.5 g) enhanced the bioavailability of cimetidine in male subjects. In the female volunteers, there was no difference in bioavailability in the presence of PEG 400.

The influence of PEG 400 on the bioavailability of ranitidine was also investigated in male and female rats. In a similar manner to man, there was no difference in the bioavailability of ranitidine in the absence of PEG 400. In the presence of PEG 400, there were some differences, however these were not significant. The plasma profiles of ranitidine in the male rats exhibited a single peak compared to the female rats which had multiple peaks. Mechanistic studies were carried out in an attempt to explain the findings. Cimetidine, ranitidine and PEG analogues were screened for their P-glycoprotein (P-gp) activity using an ATPase assay. Ranitidine and PEG 400 stimulated P-gp activity whilst cimetidine and PEG 200 did not. Subsequently the effects of PEG 400 on the efflux transport of ranitidine and cimetidine was investigated using Caco-2 cell lines. PEG 400 did not change the Transepithelial Electrical Resistance and mannitol flux of the Caco-2 cell lines indicating that PEG 400 does not influence paracellular transport. An indicative increased absorptive effect, though not significant, was observed for the transport of ranitidine in the presence of PEG 400 possibly due to the effect of PEG 400 on efflux transporters such as P-gp and Breast Cancer Resistance Protein (BCRP).

These findings challenge the ‘inert/inactive’ status of excipients such as PEG 400. The results of this thesis have implications for the use of PEG 400 in dosage forms (for example when compounds are being considered for biowaivers) and also highlight the importance of gender studies in pharmacokinetics.
DEDICATION

To the Almighty God...to you alone be the glory and praise

To my parents, Chief Ashley and Chief (Mrs) Oladunni Ashiru, words can never explain how much you mean to me and say enough thanks for all that you have done over the last 28 years

To my husband, Dr Adetola Oredope and our child, Oluwatumininu Elizabeth.... I love you both so much

To my siblings (Debbie, David & Victoria), you are simply the best
ACKNOWLEDGEMENTS

I would firstly like to acknowledge my supervisor, Dr Abdul Basit, wow what can I say, I am more than blessed to have you as my supervisor, your support, training, advice and supervision have been second to none. You have inspired me in so many ways. I appreciate your encouragement, support, counsel and motivation all through my PhD and especially towards submission. Dr Rajesh Patel, my industrial supervisor (GlaxoSmithKline) is also acknowledged for his support especially with provision of different materials for use in this project.

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I am forever indebted to my loving parents Chief Ashley & Chief (Mrs) Oladunni Ashiru. All their support through these years is greatly acknowledged. I would like to thank them for going to great lengths to help me in every way that I have needed it. My parents are responsible for the person I am today. I will always be overwhelmingly grateful for their guidance and prayers which has been and remains to be invaluable to my life. Their devoted love for me and my siblings and now their first grandchild is one of my most cherished gifts. Mum, as promised, here is your birthday present (my submission). Thank you so much for coming to our home and looking after my daughter and I so excellently well as I completed the writing of this thesis.

My dearest siblings, Debbie, Dave and Victoria; you guys are simply the best. I am so proud of you and honoured to be your big sis.

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To my wonderful husband, Dr Adetola Oredope, what can I say, thank you for your love and care and for putting up with me during this period of thesis submission. You are a wonderful husband and I am excited about our future together. My darling, beautiful daughter Oluwatumininu Elizabeth, thank you for being such a wonderful and peaceful baby whilst in the womb and from the day you were born, you allowed mummy to complete her PhD and write her thesis....Mummy loves you so much....To our unborn kids, I can’t wait to meet you....

Finally, I would like to give all praises to the Lord God Almighty, without whom none of my accomplishments in life would be possible. All glory and honour goes to God.

Diane A.I. Ashiru
April 2009

Post Viva

To almighty God… I praise you always.

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Special thanks to my dear ‘lil sister Victoria, for baby sitting my daughter so fantastically well as I revised for the viva and completed the thesis correction; I am so grateful. Mum and Dad, Debbie and David, thank you so much for your unwavering support and love.

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August 2009
List of Publications

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<td>ABC Transporters</td>
<td>ATP-binding Cassette transporters</td>
</tr>
<tr>
<td>AP-to-BL</td>
<td>Apical to Basolateral</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BL-to-AP</td>
<td>Basolateral to apical</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast Cancer Resistance Protein</td>
</tr>
<tr>
<td>BCS</td>
<td>Biopharmaceutics Classification System</td>
</tr>
<tr>
<td>BNF</td>
<td>British National Formulary</td>
</tr>
<tr>
<td>BP</td>
<td>British Pharmacopoeia</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium without Sodium Pyruvate with 4500 mg/mL glucose</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ES-MS</td>
<td>Electro Spray-Mass Spectrometry</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>HPLC-UV</td>
<td>High Performance Liquid Chromatography- Ultraviolet</td>
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<tr>
<td>HPLC-MS</td>
<td>High Performance Liquid Chromatography- Mass Spectrometry</td>
</tr>
<tr>
<td>LCMS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>LLOQ</td>
<td>Lower limit of quantification</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of Detection</td>
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<tr>
<td>LOQ</td>
<td>Limit of Quantification</td>
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<td>Madin Darby Canine Kidney (MDCK) cells with the mdr1 gene</td>
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<td>MMC</td>
<td>Migrating myoelectric complex</td>
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<tr>
<td>MS</td>
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</tr>
<tr>
<td>Papp</td>
<td>Apparent Permeability Coefficient</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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<td>P-gp</td>
<td>P-glycoprotein</td>
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<tr>
<td>TEER/TER</td>
<td>Transepithelial Electrical Resistance</td>
</tr>
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<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
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<td>SD</td>
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</tr>
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<td>SIM</td>
<td>Selected Ion Monitoring</td>
</tr>
<tr>
<td>UCL/UCLH</td>
<td>University College London/ University College London Hospital</td>
</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopoeia</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
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<tr>
<td>Vit E-TPGS</td>
<td>d-alpha-tocopheryl polyethylene glycol 1000 succinate</td>
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CHAPTER 1: Introduction
1.1 Overview

The International Pharmaceutical Excipients Council (IPEC, www.ipec.org) defines Pharmaceutical excipients as “substances other than the pharmacologically active drug or prodrug which are included in the manufacturing process or are contained in a finished pharmaceutical product dosage form” (IPEC, 2009). Excipients are usually listed as inactive ingredients in drug monographs (CDER/FDA, 2005). The FDA defines an inactive ingredient as “any component of a drug product other than the active ingredient” and an active ingredient is defined as “any component of a drug product intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of humans or other animals” (CFR/FDA, 2004). Generally, excipients account for majority of the weight or volume of medicinal products. For many years, excipients were often assumed to be inert and void of having any pharmacological effect (such as influencing transit of drugs through the intestine or having an effect on efflux transport). However, this is no longer the case and they are now considered essential and critical functional components of modern pharmaceutical formulations (Pifferi and Restani, 2003). Excipients have many functions within the pharmaceutical dosage form, many are responsible for the transport of the active drug to the site of the body where the drug is intended to exert its action. Some keep the drug from being released too early after ingestion in places where the drug could cause tissue damage or lead to gastric irritation or stomach upset. Excipients are also used to help drug molecules to disintegrate into particles small enough to reach the blood stream quickly. Other critical functions of excipients include aiding the identification of the drug.
product, making a product taste and look better to improve patient compliance especially in children. Excipients are present in all pharmaceutical forms including parenteral; to enhance solubilization, stabilization and preservation (Akers, 2002), ophthalmic: to enhance viscosity (Bozdag et al., 2008), rectal and vaginal (to increase the contact time between the dosage form and the rectal or vaginal mucosa) topical (to improve stability) and the classic oral formulations (to enhance their organoleptic properties; i.e. smell, taste, swallowability and local tolerability in order to improve patient’s compliance) (Pifferi et al., 1999).

Although excipients are still listed as inactive ingredients in drug monographs, there have been many reports that have challenged this. There have been several reports where excipients have had pharmacological effects and led to both beneficial and detrimental effects (Adkin et al., 1995c, Bardelmeijer et al., 2002, Basit et al., 2002, Tayrouz et al., 2003, Yamagata et al., 2007a, Yamagata et al., 2007b). Besides if excipients are added to formulations to enhance solubility and hence absorption, can they really be referred to as ‘inactive or inert’?

Although drug molecules can be administered by many different routes (oral, intravenous, intradermal, optically, intranasally), the oral route remains the most common and acceptable method of administering drugs – 84% of the fifty bestselling pharmaceutical products in the US and European markets are oral medications (Lennernas and Abrahamsson, 2005). The major site of absorption for orally administered drugs is the small intestine because of the functional specialization of the cells, the large surface area and relatively long transit time. For orally administered drugs to be absorbed into the systemic circulation they must cross the intestinal
epithelium and the intestinal epithelium is the most important biological barrier in the gastrointestinal absorption of drugs (Gan and Thakker, 1997, Hayton, 1980, Kimura and Higaki, 2002). The small intestine although an integral part of the whole gastrointestinal tract is dependent on the other parts of the digestive system.

1.2 The Gastrointestinal Tract

The digestive system consists of the oral cavity (mouth), oesophagus, stomach, small intestine and large intestine (Figure 1.1). Primarily, the digestive system ensures that ingested food which contains complex molecules is broken down into simple molecules that can be absorbed into the blood or lymph. It is also involved in the absorption of drug molecules into the blood stream. A summary of the main physiological and anatomical parameters of the gastrointestinal tract is presented in Table 1.1

The oral cavity is lined by the oral mucosa which is composed of stratified squamous epithelial cells. The functions of the oral cavity include:

1. Ingestion of the material.

2. Physical breakdown of the ingested material by the actions of the teeth, tongue and the roof of the oral cavity formed by hard and soft palates.

3. Lubrication of the material with salivary gland secretions to aid swallowing.

4. Initial breakdown (digestion) of carbohydrates and lipids.
Figure 1.1: The digestive system
Table 1.1: Anatomy and physiological parameters of the gastrointestinal tract

<table>
<thead>
<tr>
<th>Parameters → Intestinal Tract section ↓</th>
<th>Length (m)</th>
<th>Diameter (cm)</th>
<th>Absorptive Area (m²)</th>
<th>pH</th>
<th>Bacterial concentration (CFU/g of contents)</th>
<th>Transit time (h)</th>
<th>Total fluid volume (mL) (post mortem)</th>
<th>‘Free’ fluid volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>0.2</td>
<td>0.25</td>
<td>~0.11</td>
<td>1.0-4.5</td>
<td>$1 \times 10^4$</td>
<td>1.5 (variable)</td>
<td>118 ± 82</td>
<td>Fasted: 45 ± 18</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>Fed: -686*</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>6</td>
<td>2.5</td>
<td>~200</td>
<td>5–7.5</td>
<td>$1 \times 10^4$</td>
<td>3–4</td>
<td>212 ± 110</td>
<td>Fasted: 105 ± 72</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>$1 \times 10^6-10^8$</td>
<td></td>
<td></td>
<td>Fed: 54 ± 41</td>
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<td>1–60</td>
<td>187</td>
<td>Fasted: 13 ± 12</td>
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<td>Fed: 11 ± 26</td>
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</tbody>
</table>

The oesophagus is a hollow muscular tube which has the primary function of transferring ingested material from the mouth through the cardia (the distal or lower oesophageal sphincter) to the stomach. It is lined by stratified squamous epithelium, however the lowest approximately 2 cm which lies in the abdominal cavity is normally lined with gastric mucosa and covered by peritoneum (Wilson, 1989). The average pH of the oesophagus is 5.6 (Ritschel, 1991).

Transport of drugs through the oesophagus is normally very short. Transit time of tablets and capsules of varying shapes and sizes in various studies was 3-9 seconds in the erect position (Wilson and Washington, 1989). Certain pathological conditions may lead to prolonged retention of the ingested material in the oesophagus.

The exact shape and size of the stomach, a sack with an expanded J shape is variable between individuals and meals. The average length and diameter of a stomach is 20 cm and 15 cm respectively (Ritschel, 1991).

The major function of the stomach is to act as a reservoir for food. Very little drug absorption occurs in the stomach. The absorbing surface area of the stomach is 0.11 m², a much smaller surface area compared with the small intestine which has a total absorptive area of 200 m² (Martini, 2004, Ritschel, 1991). The pH of the stomach ranges from 1.0 to 4.5 depending on the absence or presence of food (Lee and Yang, 2001). It is more acidic in the fasted state, and the pH of the fluid in the stomach can rise to 4.5 after the ingestion of food (Ibekwe et al., 2008).
The rate of gastric emptying from the stomach influences both the rate and extent of drug absorption from the small intestine (Lee and Yang, 2001).

The stomach empties the three components of a meal – the liquid, digestible and indigestible solids at different rates (Wilson and Washington, 1989). The motility of the stomach changes distinctively as the emptying of a meal nears completion. In the fasted state, a distinct pattern of contratile activity known as migrating myoelectric complex occurs. There are four separate stages of phases of activity (Code and Marlett, 1975, Szurszewski, 1969). In Phase I regarded as the period of quiescence, there is little or no contraction or motor activity. This period lasts up to 1 h. The period of quiescence is followed by Phase II lasting 20 to 40 min. In this phase, the contractions are irregular and bile is secreted into the intestines. The contractions in this phase increase in amplitude and frequency until phase III; the burst phase is reached. Phase III is a short period of approximately 5 - 15 min with very strong contractions which migrate distally from the stomach to the large intestine. During this phase, the stomach lumen is cleared of undigested materials and cellular debris and hence the contractions in this phase are known as the housekeeper wave. The final phase (phase IV) is an even shorter period (0 – 5 min), separating the intense activity of phase III from the quiescence of phase I, before the cycle repeats. The MMC is thought to be modulated by motlin, gastrin, prostaglandins or signals from the central nervous system (Rao and Schulze-Derieu, 1993, Weisbrodt, 1984).

The small intestine is the region between the stomach and the large intestine. It has diameter of about 2.5 cm and averages 6 m in length (Martini, 2004). The first 25 cm of
the small intestine is the duodenum, the second 2.5 m, the jejunum and the final 3.5 m, the ileum. The duodenum serves as the mixing bowl, receiving the chyme from the stomach and digestive secretions from the pancreas and liver (Martini, 2004). The jejunum is where the chemical digestion and nutrient absorption takes place. The ileum, the final segment of the small intestine is the longest and ends in the ileocecal valve, a sphincter which controls the flow of the remaining materials from the ileum to the large intestine. Although the whole length of the small intestine contains villi and lymphatic follicles (Peyer’s patches), the duodenum has larger and more numerous villi than the ileum and the ileum contains larger and more numerous Peyer’s Patches than anywhere else in the intestine (Wilson and Washington, 1989).

The absorptive power of the small intestine is due to its increased surface area – 200 m² (Martini, 2004) contributed to by the length of the small intestine, and the presence of:

- Circular folds of the epithelium called the folds of Kerckring or plica circulares. These folds increase the surface area by a factor of 3.
- Finger-like projections – villi in the mucosa
- Mirovilli on the surface of each intestinal lining cell – the enterocyte
- Crypts of Lieberkuhn

Following the breakdown of materials ingested by the stomach, on reaching the small intestine, they are exposed to the intestinal epithelium which is a monolayer of epithelia cells which line the villi and their surrounding crypts. The villi are covered mainly with
enterocytes (absorptive cells) which are responsible for the majority of the absorption of nutrients and drug molecules from the small intestine. These enterocytes are tall columnar cells which are bound to one another and other cell types by junctional complexes including tight junctions (zonula occludens), zonula adherens (occur at cell-cell junctions in epithelial tissues, usually more basal than tight junctions) and macula adherens (spot-like adhesions randomly arranged on the lateral sides of plasma membranes). Previously the tight junction was conceived to be an impermeable barrier, however over the past 40 years, improved understanding of the architecture and function of the tight junction has led to an understanding that it is more of a dynamic and complex multiprotein structure which is selectively permeable to certain hydrophilic molecules (Ward et al., 2000).

The pH values of the human intestinal fluids range from 5 to 7.5 going from the duodenum through the jejunum and finally to the ileum (Kararli, 1995).

The total fluid volume in the small intestine measured post mortem is 212 ± 110 mL (Gotch et al., 1957), this is higher than the 118 mL measured in the stomach (Gotch et al., 1957) and 187 mL in the large intestine (Cummings et al., 1990). Recent research has however shown that the volume of intestinal fluid is not constant throughout the gut lumen, rather there are separated pockets filled with liquid and there are also ‘dry segments’ with no intestinal fluid randomly scattered along the small intestine (Schiller et al., 2005). Also the ingestion of water did not necessarily guarantee the presence of sufficient fluid in the small intestine. In fasting volunteers, there was < 100 mL of fluid
scattered in up to six pockets located primarily in the distal small intestine despite ingestion of 850 mL of water within the preceding 7 h, 150 mL of this fluid volume was served 1 h prior to MRI acquisition. Also water ingestion with a meal resulted in the further volume reduction in the segment not filled with chyme. The authors suggested that ingestion of a meal, not only leads to gastric retention of concomitantly or consecutively administered non-disintegrating dosage forms but also initiates transport of solids and fluid from the small intestine into the large intestine (Schiller et al., 2005).

Whilst fluid volume increased significantly with feeding in the stomach, the small intestinal fluid volumes decreased significantly. After a meal, the small intestinal fluid decreased significantly. This decrease was observed to be followed by a significant increase in fluid pockets and decrease in the fluid volume per pocket; from a median value of 12 mL before meal to 4 mL after the meal. In the upper small intestine, there was a slurry of ingested food. Unlike the small intestine, within the large intestine, there was an increase in the fluid volume and number of fluid pockets following the ingestion of the meal. However the volume per pocket was not significantly different.

Whilst gastric retention and emptying have been studied extensively and it has been shown on numerous occasions that many factors affect gastric emptying, small intestinal transit time was thought to independent of external factors including food and to be consistent at 3-4 h (Davis et al., 1986). However recent studies have suggested that the timing of food may affect the small intestinal transit time of a dosage from. For example a study by (Digenis et al., 1990) showed that the bioavailability from enteric-coated erythromycin beads given 30 min before food was lower due to faster small
intestinal transit time of the dosage from. A more recent study by (Fadda et al., 2009) showed that administration of tablets 45 min before food resulted in an increased small intestinal transit time over fasted and fed doses. The effect was especially pronounced for those tablets which had moved into the proximal small intestine before the food was ingested at 45 min. Studies using gamma scintigraphy have reported that the small intestinal time of orally administered drug solutions is approximately 240 min (Basit et al., 2001, Haruta et al., 2002).

Transit through the gastrointestinal tract and especially the small intestine has been reported to be altered by disease, drugs and excipients. For example patients with irritable bowel disease generally have faster intestinal transit, also, compared to normal patients, patients with ulcerative colitis have faster colonic transit (Hebden et al., 2000). Research has shown that nifedipine, a calcium channel blocker can increase transit time by approximately 26 % (Chiarioni et al., 1993). Polyethylene glycol 400 a solubility enhancing excipient at pharmaceutically relevant doses has been shown to accelerate small intestinal transit and subsequently affect the absorption of drugs (Basit et al., 2001, Basit et al., 2002, Schulze et al., 2003). Mannitol and sodium acid pyrophosphate are two other excipients which have been shown to have similar effects to PEG 400 on intestinal transit and subsequently influence the absorption of drugs (Adkin et al., 1995b, Adkin et al., 1995a, Adkin et al., 1995c, Koch et al., 1993).

The large intestine extends from the ileocaecal junction (the terminal region of the small intestine, where luminal contents are accumulated until digestion is mostly completed)
to the anus at approximately 1.5 m length. It is subdivided into caecum, colon, rectum and anal canal. For many years, it was thought that very little drug absorption occurs in the large intestine due to the small surface area and increasing viscosity of the luminal contents. However, a recent review by Tannergren et al. showed that drugs with high solubility and high permeability are well absorbed in the colon whilst drugs with low permeability are less well absorbed from the colon (Tannergren et al., 2009). The main function of the colon is storage and elimination of faecal material as well as the absorption of sodium and water.

1.3 **Drug Absorption affected by many factors**

Key parameters affecting drug bioavailability include permeability (drug transport) and solubility (Amidon et al., 1995). These parameters along with drug dissolution form the basis for the characterization of drug substances into what is termed the Biopharmaceutics Classification System – BCS (Amidon et al., 1995, CDER/FDA, 2000).

1.3.1 **Biopharmaceutics Classification System**

The developed Biopharmaceutics Classification System (Amidon et al., 1995) is now used by the US Food and Drug Administration Agency (FDA) to issue a waiver for in vivo bioavailability and bioequivalence studies for immediate release solid oral dosage forms which contain certain active moieties and active ingredients (CDER/FDA, 2000). The four classes in which a drug can belong to according to its solubility and permeability (BCS) are:
Class I  High Solubility – High Permeability
Class II  Low Solubility – High Permeability
Class III High Solubility – Low Permeability
Class IV  Low Solubility – Low Permeability

(Amidon et al., 1995)

The BCS biowaiver is currently only applicable for use with class I (high solubility-high permeability) oral immediate-release dosage forms which exhibit rapid in vitro dissolution using recommended test methods (CDER/FDA, 2000, EMEA/CPMP, 2001). Excipients used in these dosage forms should not have significant effect on the rate and extent of absorption of the drug (CDER/FDA, 2000, EMEA/CPMP, 2001). The FDA provides a database of excipients in FDA approved products and includes the maximum potency of excipients that can be contained in a particular route or dosage form of a drug product containing the excipient (CDER/FDA, 2005). Drugs having a narrow therapeutic window for example theophylline, digoxin and drugs absorbed in the oral cavity are also exempt from BCS biowaivers (CDER/FDA, 2000).

1.4 Permeability of Drugs

Drugs molecules and ions permeate (cross) through the intestinal epithelium via three main pathways (Figure 1.2): (1) passive diffusion between adjacent cells via the tight junctions (paracellular pathway); (2) passive diffusion across the plasma membrane of the epithelial cells (transcellular pathway) and (3) carrier-mediated transport (carrier-mediated transcellular pathway). Compared with the extensive surface area of cells
available for transcellular transport, the surface area for paracellular uptake is low and more restrictive due to the tight junctions (Kataoka et al., 2003, Daugherty and Mrsny, 1999a, Daugherty and Mrsny, 1999b).

Figure 1.2: Routes and mechanisms for drug transport across the lining epithelium (adapted from Hillery, 2001)

1.4.1 The Paracellular Route

Small hydrophilic molecules such as hydrochlorothiazide are transported by passive diffusion of molecules across negatively charged tight junctions between epithelial cells
this is termed paracellular absorption. It has been suggested that paracellular absorption favours cationic molecules, with cations being more easily transported through the tight junctions than non-ionic species or anions (Karlsson et al., 1999, Lee and Yang, 2001). However, a study on the localisation of drug permeability along the small intestine of rats showed that mannitol – a neutral molecule, lucifer yellow – an anion and ranitidine – a cation all had similar paracellular permeabilities (Lacombe et al., 2004). It was also a common belief that the passive diffusion of hydrophilic molecules was driven by a concentration gradient and inversely proportional to molecular weight (Lee and Yang, 2001). However, the study by Artursson et al (1993) on paracellular permeability of intestinal absorption indicated that permeability was not simply inversely proportional to a molecule’s molecular weight, but that molecular structure should also be considered (Artursson et al., 1993). For example the permeability of PEG with molecular weight 194 g/mol was 6 to 28 fold higher than the permeability of mannitol with molecular weight 182 g/mol (Artursson et al., 1993). Molecular size (cross-sectional diameter) is also an important factor when determining permeation rate. Bjarnason et al. (1994) reviewed intestinal permeability and pointed out that PEG-400 had a higher permeation rate compared with other permeability probes, mannitol, lactulose and $^{51}$Cr-ethylenediamineteraacetic acid (EDTA), inspite of PEG 400 having the highest molecular weight (400g/mol); (mannitol, 182 g/mol), lactulose (342 g/mol), $^{51}$Cr –EDTA (341 g/mol), its cross-sectional area is the smallest, 5.3Å compared with mannitol (6.7Å); lactulose (9.5Å) and $^{51}$Cr –EDTA (11.5Å). Studies have shown that there is excellent correlation between molecular size of probes and their intestinal epithelial permeation rate (Ma and Krugliak, 1996).
1.4.2 The Transcellular Route

Lipophilic drug molecules with low molecular weight are usually absorbed transcellularly by active and or passive processes across the epithelial cells (Lee and Yang, 2001). The extensive surface area available for transcellular transport makes it the desired direction of drug uptake from the intestinal lumen. The concentration gradient drives absorption and the process is governed by Fick’s law (Lee and Yang, 2001). It is determined by the physiochemical properties of the drugs and the concentration gradient across the cells.

Drugs such as levodopa and methyldopa are transported by a carrier-mediated transport by specialized membrane protein molecules e.g. amino acid and glucose transporters (Bandoh et al., 1999). Transport can be against the concentration gradient (active absorption) or along the concentration gradient (facilitated diffusion).

The paracellular and transcellular route are capable for the absorption of small molecules < 500 Da. Larger molecules e.g. proteins and peptide molecules are absorbed by endocytic processes; which is where extracellular material and fluid are engulfed following the internalization of the plasma membrane.

In addition to the physical barrier of the intestinal epithelium, it also functions as a biochemical barrier. Metabolic enzymes (Benet et al., 1999, Berggren et al., 2007), apical polarized efflux pumps (e.g. P-glycoprotein (P-gp) and Multi-drug Resistance Protein (MRP) (Benet et al., 1999, Berggren et al., 2007, Daugherty and Mrsny, 1999b)
are present in the intestinal epithelium and can affect the ability of drug molecules to cross the epithelial barrier.

The importance of active efflux of drugs by transporters is now widely recognised as one of the most important factors that regulate the oral bioavailability of drugs. P-glycoprotein (P-gp) a membrane efflux transporter which actively pumps drugs out of cells is the most widely studied and perhaps the most important of the efflux transporters in the control and disposition of its substrates (Hunter and Hirst, 1997). It is present in the apical membranes of epithelial cells of the intestine and therefore drugs that are absorbed by these cells can be pumped back into the lumen of the small intestine. P-gp can reduce the bioavailability of drugs. Inhibition of P-gp can potentially enhance drug absorption by enhancing intestinal permeability of its substrates. For example administration of digoxin with talinolol led to a significant increase in digoxin’s absorption with oral co-administration but not when talinolol was administered intravenously (Collett et al., 2005, Westphal et al. 2000). This suggests that there is an interaction at the intestinal level. Other examples of P-gp substrates include verapamil and cyclosporine. Often many new chemical entities (NCE) which are substrates for P-gp are poorly absorbed. Therefore there is substantial interest in trying to increase the bioavailability of such drugs especially anticancer and anti-HIV drugs. Administration of low molecular weight compounds which compete for the P-gp sites can enhance oral bioavailability of such P-gp substrates. However some of these ‘active’ substrates such as verapamil and cyclosporine have pharmacological activity, and can act on P-gp everywhere in the body such as the liver, kidney, brain and not just
intestinally (Takano et al., 2006). Excipients which are generally void of pharmacological activity can therefore be exploited for their effect in P-gp.

1.5 Excipient Effects on Drug Absorption

The absorption of a drug is affected by many parameters including stability, intestinal wall permeability, transport mechanisms, presence of food and first pass metabolism.

1.5.1 Excipient Effects on Intestinal Permeability

Pharmaceutical excipients can also inhibit or reduce the function of P-gp and hence enhance the absorption of both oral and intravenous P-gp substrates (Bogman et al., 2005, Cornaire et al., 2004, Hugger et al., 2002a, Hugger et al., 2002b, Johnson et al., 2002, Rege et al., 2002, Shen et al., 2006). They perhaps could be more useful for exploitation to enhance anti-cancer drugs which are P-gp substrates, rather than active drug molecules which are nonselective P-gp inhibitors that are also mostly metabolised by CYP3A4 and hence result in decreased clearance and increased toxicity of the anticancer drugs as a result of inhibiting P-gp as well as CYP34A (Benet et al., 2004). Although more selective inhibitors of P-gp have been developed which interact only with the P-glycoprotein transporter, excipients may be a more suitable and cheaper option due to multiple functions for example solubility enhancement and P-gp inhibition. A case in point being vitamin E d-alpha tocopheryl polyethylene glycol succinate (Vit E-TPGS) a solubility enhancing excipient and also an inhibitor of P-gp.

The impact of excipients on the absorption of P-glycoprotein substrates – digoxin and celiprolol were investigated in vitro and in vivo by Cornaire et al 2004. For digoxin, the most effective excipients at a concentration of 0.5%w/v in vitro with an improved
The everted gut sac technique is Labrasol > Imwitor 742 > Acconon E = Softigen 767 > Cremophor EL > Miglyol > Solutol HS 15 > Sucrose monlaurate > Polysorbate 20 > TPGS > Polysorbate 80. With celiprolol, Cremophor EL and Acconon E had no effect, however transport was enhanced by Softigen 767 > Vit E-TPGS > Imwitor 742. In vivo (rats) the excipients altered the pharmacokinetic profile of the drugs’ absorption, mostly causing early peaks of absorption. However there was no increase in the overall bioavailability. The properties required by modulator compounds to increase drug transport by P-gp inhibition include water solubility, presence of ester bonds, a saturated carbon chain of about C10 and polyoxyethylene groups (Comaire et al., 2004). Connaire et al (2004) showed the usefulness of everted gut sac system to screen pharmaceutical excipients/surfactants for their ability to enhance the intestinal uptake of P-gp substrate drugs.

In other studies, surfactants (Cremophor EL, TWEEN 80 and VitE-TPGS) also inhibited P-gp activity in vitro (Rege et al., 2002, Wandel et al., 2003). In vivo, Cremophor EL has also been shown to have a pharmacological effect when used in the formulation of P-gp substrates such as digoxin. In one study, formulating digoxin with Cremophor EL led to a 22 % increase in bioavailability (Tayrouz et al., 2003). In contrast to this though, Cremophor EL has also been shown to reduce the oral bioavailability of another P-gp substrate paclitaxel, this was attributed to the Cremophor EL possibly counteracting the pharmaceutic interaction (i.e. the entrapment of paclitaxel within micelles) (Bardelmeijer et al., 2002, Malingre et al., 2001).
1.5.2 Excipient Effects on Solubility

A common method of increasing the solubility of drugs is the use of solubilizing agents. Although the majority of oral preparations available are tablets or capsules, many drugs are available in solubilized forms as oral solutions, syrups, soft gelatin capsule or tablets. Solubilization is employed to enhance the absorption of poorly water-soluble drug molecules, aid compliance especially in children and adults who are unable to swallow and also to allow easier measurement of required doses especially in children where dosing is usually based on weight. A poorly soluble drug, so classified when its dissolution takes longer than its transit time to pass its absorptive site. This leads to poor bioavailability (Horter and Dressman, 2001).

Solubilizing excipients include co-solvents, surfactants, complexing agents, pH modifiers (Strickley, 2004). Although each method of solubilization can be used alone, certain combinations show synergistic effects, resulting in greater solubility enhancement. For example the combination of pH modification and complexation has been shown to be effective in increasing solubilization (Li et al., 1998a, Li et al., 1998b). A combination of cosolvent and cyclodextrin has also been used to solubilize non polar drugs (Li et al., 1999). However some combinations of solubilization methods have led to decreased solubility when compared with using one method alone. For example, the combination of sodium lauryl sulphate (SLS) (surfactant) and sulfobuyl ether β- cyclodextrin SBE7M-β-CD) - a complexant resulted in a much lower solubility of NSC-639829 (an investigational anti-cancer drug) than when either method was used alone (Yang et al., 2004). It was suggested by the authors that the reduced solubility when a combination of co-solvent and complexant was used is due to the surfactant
competitively inhibiting the solubilization of the drug by the complexant and the
complexant pulling the surfactant out of solution, hence making it unavailable to
solubilize the drug (Yang et al., 2004).

Solubilizing excipients in oral and injectable formulations have been reviewed
(Strickley, 2004). The different types of solubilizing excipients will only be briefly
discussed here with examples of each type highlighted.

Co-solvents are organic compounds which may be water soluble or water insoluble
(Strickley, 2004). They are particularly useful for solubilizing drugs that do not have
ionisable groups and hence cannot be solubilised using pH adjustment. Co-
solvents increase solubility by disrupting water's self association and hence reducing the ability
of water to squeeze out non-polar, hydrophobic compounds (Millard et al., 2002).

Polyethylene glycol 300 and 400 (PEG 300; PEG 400), propylene glycol, ethanol and
glycerin are examples of water-soluble co-solvents. The FDA specifies the maximum
potency (maximum amount) of inactive ingredient for each route/dosage form
containing that ingredient). The maximum potency of PEG 400 for example is 60 % in
drug solutions and 25 % in suspensions (CDER/FDA, 2005). Amprenavir, a drug used
in the management of HIV for example is solubilized in Vit E TPGS, PEG 400 and
propylene glycol. One millilitre (1 ml) of amprenavir solution consists of 15 mg
amprenavir, 170 mg PEG 400, 550 mg polypropylene glycol and 46 U Vit E TPGS.
Water-insoluble co-solvents include oleic acid, beeswax, d-α-tocopherol (vitamin E), long chain triglycerides e.g. peanut, corn, olive oil, and medium chain triglycerides. They are filled into soft gelatin capsules mainly. Calcitrol used in the treatment of hypocalcaemia is solubilized with coconut oil in soft gelatin capsules. Oleic acid is used to solubilize ritonavir in Norvir and Keleta soft gelatin capsules (Strickley, 2004).

Surfactants solubilize drug molecules by direct cosolvent effects or by uptake into micelles (Strickley, 2004).

Low concentrations of surfactants have been reported to have increased the absorption of drugs and enhanced the activity of antibacterials (Florence, 1981). It was suggested that the effects were due to the adsorption of the surfactant at biological membranes and their penetration into the membrane, causing alteration of fluidity and increasing permeability (Florence, 1981).

Surfactants are particularly useful because of their amphiphilic nature (Yang et al., 2004). They have been used in pharmaceutical preparations and examples include PEG 400 caprylic/capric glycerides (Labrasol), d-alpha-tocopheryl polyethylene glycol 1000 succinate (Vit E-TPGS), peppermint oil and polyoxyl 35 castor oil (Cremphor EL) amongst many others (Strickley, 2004)

Surfactants have been suggested as having three main concentration dependent effects on drug absorption. These are:
• An increase in absorption up to the critical micelle concentration (CMC) of the surfactant followed by a decrease;
• No enhancement in absorption followed by a decrease in absorption above the CMC;
• An increase in absorption not affected by the onset of micelle formation

Complexants are used to enhance the solubility of drugs by forming a complex with that drug to produce a more soluble product. Examples of complexants include cyclodextrins.

The solubility of ionisable molecules may be increased by pH adjustments using pH modifiers. Ionisable molecules generally have higher water solubility than their neutral species (Strickley, 2004). Solubilization of weak acids can be carried out by increasing their pH above their acidic pK\textsubscript{a} whilst the solubility of basic acids is increased by solubilising them at pHs below their basic pK\textsubscript{a} (Strickley, 2004).

### 1.6 Adverse Effects of Excipients on Drug Absorption

In recent years, the notion that excipients are inert/inactive has been challenged by different research groups. Research has shown that although excipients are indeed capable of increasing the bioavailability of drugs by increasing the solubility of a drug (Strickley, 2004), they can also cause detrimental effects on intestinal transit and decrease drug bioavailability (Adkin et al., 1995c, Basit et al., 2002, Rodrigues et al., 2003). A summary of published data on excipients, focusing on those that have
investigated the effects of excipients on gastrointestinal transit and drug bioavailability is shown in Table 1.2

Polyethylene glycol 400 (PEG 400), a well established solubility enhancing excipient is listed as an inactive ingredient in drug monographs. However, recent work has called into question the inert nature of this excipients. For example at doses relevant to pharmaceutical formulations, PEG 400 stimulates gastrointestinal motility and accelerates small intestinal transit (Basit et al., 2001, Basit et al., 2002, Schulze et al., 2003). This effect is attributed to the fact that PEG 400 is poorly absorbed from the gut (Chadwick et al., 1977a). PEG 400 is osmotically active and will "hold" fluid in the lumen of the intestine, leading to an increase in bulk fluid volume, which in turn stimulates peristalsis and hence transit. However, no such effects on transit have been noted with other commonly used solubility enhancing pharmaceutical excipients such as propylene glycol, Vitamin E-TPGS, Labrasol and Capmul®MCM in man or dog (Schulze et al., 2006, Schulze et al., 2005).

A consequence of rapid transit through the small intestine is a reduction in contact time with the primary site of absorption in the gut and the potential reduction in oral bioavailability. A case in point has been observed with the drug ranitidine. Ranitidine is a class III compound (high solubility, poor permeability) according to the Biopharmaceutics Classification System (BCS) and is mainly absorbed in the small intestine (Amidon et al., 1995). PEG 400, sodium acid pyrophosphate and sorbitol have all been shown to reduce the oral bioavailability of ranitidine following acceleration of
intestinal transit (Basit et al. 2002; Schulze et al. 2003; Chen et al., 2007, Koch et al., 1993)
<table>
<thead>
<tr>
<th>Excipient</th>
<th>Amount of Excipient</th>
<th>Drug</th>
<th>Effect on GI tract</th>
<th>Effect on drug bioavailability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Acid Pyrophosphate</td>
<td>1.132 g</td>
<td>Ranitidine</td>
<td>Small intestinal transit time ↓ed by 56 %</td>
<td>↓ed by 50 %</td>
</tr>
<tr>
<td>Sodium Acid Pyrophosphate</td>
<td>1.1 g/200 ml</td>
<td>-</td>
<td>Small intestinal transit time ↓ed by 39 %</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>2.264 g/200 ml</td>
<td>-</td>
<td>Small intestinal transit time ↓ed by 34 %</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>4.08 g/200 ml</td>
<td>-</td>
<td>Small intestinal transit time similar to control</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>0.755 g/200 ml</td>
<td>-</td>
<td>Small intestinal transit time ↓ed by 11 %</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1.509 g/200 ml</td>
<td>-</td>
<td>Small intestinal transit time ↓ed by 23 %</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>2.264 g/200 ml</td>
<td>-</td>
<td>Small intestinal transit time ↓ed by 34 %</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2.264 g</td>
<td>Cimetidine</td>
<td>Small intestinal transit time ↓ed by 23 %</td>
<td>↓ed by 31 %</td>
</tr>
<tr>
<td>Mannitol</td>
<td>10 g</td>
<td>Ranitidine</td>
<td>Small intestinal transit time ↓ed by 37 %</td>
<td>↓ed by 31 %</td>
</tr>
</tbody>
</table>

46
<table>
<thead>
<tr>
<th>Reference</th>
<th>Excipient</th>
<th>Amount of Excipient</th>
<th>Drug</th>
<th>Effect on GI tract</th>
<th>Effect on drug bioavailability</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Schulze et al., 2003)</td>
<td>PEG 400</td>
<td>1 g</td>
<td>Ranitidine</td>
<td>Small intestinal transit time ↓ed by 9 %</td>
<td>↑ed by 41 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5 g</td>
<td></td>
<td>Small intestinal transit time ↓ed by 20 %</td>
<td>↓ed by 38 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 g</td>
<td></td>
<td>Small intestinal transit ↓ed by 23 %</td>
<td>↓ed by 38 %</td>
</tr>
<tr>
<td>(Chen et al., 2007)</td>
<td>Sorbitol</td>
<td>5 g</td>
<td>Ranitidine</td>
<td>-</td>
<td>↓ed by 55 %</td>
</tr>
<tr>
<td>(Hussain et al., 2001)</td>
<td>Sorbitol</td>
<td>5 g</td>
<td>Metoprolol</td>
<td>-</td>
<td>↓ed by 10 %</td>
</tr>
</tbody>
</table>

**Key**

- : information not specified or investigated
- ↑ed : Increased
- ↓ed : Decreased
In an attempt to produce a liquid formulation of ranitidine, sodium acid pyrophosphate was identified as a suitable excipient (Koch et al., 1993). However, instead of the expected increase in bioavailability with an effervescent formulation, the absorption of the effervescent ranitidine was found to be half of that from the conventional ranitidine tablet (Koch et al., 1993). The study by Koch et al (1993) revealed that the decrease in absorption of ranitidine was likely to be due to the excipient causing a more rapid small intestinal transit. Other excipients, xylitol (Wilding et al., 1994) and mannitol (Adkin et al., 1995b) have also been implicated in reducing small intestinal transit time in male subjects.

An oral solution of the non steroidal anti-inflammatory drug, etoricoxib, solubilised in PEG 400 in an attempt to produce a liquid formulation was also found to have reduced bioavailability (83%) compared with that from a conventional tablet formulation (100%) (Rodrigues et al., 2003). Although not investigated by the authors, it is possible that the reduction in absolute bioavailability of etoricoxib when administered as a solution in PEG 400 may be due to the already proven transit effects of PEG 400 (Basit et al., 2001).

Excipients that have been reported to adversely affect drug absorption may not necessarily influence all drugs. For example sorbitol, an excipient commonly used in proprietary pharmaceutical syrup formulations, did not significantly alter the absorption of theophylline (Fassihi et al., 1991) or metoprolol (Hussain et al., 2001) both highly
permeable drugs, but caused a significant reduction in the bioavailability of ranitidine (Hussain et al., 2000).

In the case of PEG 400, human studies with all male volunteers, showed that a 10 g dose of PEG 400 reduced small intestinal transit time by 37% and the oral bioavailability of ranitidine administered in the pellet form by more than 30% (Basit et al., 2001). A subsequent clinical study by Schulze et al (2003) (also in male volunteers), looking at the effects of low doses of PEG 400 (1, 2.5 and 5 g) revealed that PEG 400 had concentration dependent effects on transit and drug absorption (Schulze et al., 2003). Small intestinal transit times were decreased by 9, 20 and 23% respectively. The oral bioavailability of ranitidine was reduced by 38% in the presence of 2.5 and 5 g PEG 400. However, in the presence of 1 g PEG 400 the bioavailability of ranitidine was increased by 41%, despite the reduction in small intestinal transit time (Schulze et al., 2003). It was proposed that the effect noted with 1 g PEG 400 was due to the ability of PEG 400 to modulate intestinal permeability, an absorption enhancing effect that is overshadowed at higher concentrations due to rapid passage through the small intestine.

1.7 Scope and Purpose of Research

Various research groups have suggested that BCS waiver should be extended to rapidly dissolving immediate release products that contain class III drug molecules (e.g. ranitidine and cimetidine), provided they do not contain excipients that might modify GI transit or absorption processes (Blume and Schug, 1999, Kortjejarvi et al., 2005,
Jantratid et al., 2006). Lack of adverse effects is a requirement specified by both The European Medicines Agency and US Food and Drug Agency (Blume and Schug, 1999, CDER/FDA, 2000, EMEA/CPMP, 2001). Although class III drugs are sufficiently soluble in vivo, they have poor permeability (Amidon et al., 1995), it would therefore be important that they are present in the small intestine for a sufficient period of time to allow time for sufficient absorption.

Studying the effects of solubilising excipients on gastrointestinal transit and drug absorption could provide very useful information to identify drug/excipients combinations with potential excipient-induced bioavailability issues. Ultimately this will enable the rationalisation of formulation strategy for poorly water-soluble drugs and may also provide information that can be used in establishing a sound approach to biowaivers for class III drugs.

In light of the intriguing findings of the effects of PEG 400 at the 1 g dose on bioavailability (section 1.6), the purpose of the thesis was to investigate the modulation of intestinal absorption using PEG 400 as an excipient.

The objectives were set to:

- Further challenge the notion that excipients are inert but rather, influence drug absorption and drug transport.
• Determine if the phenomenon noted by Schulze et al (2003) is repeatable in a
different set of volunteers and investigate the effects of low PEG 400 doses on the
bioavailability of ranitidine.

• Determine the optimum dose of PEG 400 for drug bioavailability enhancement.

• Investigate whether there are gender differences in the absorption of ranitidine when
formulated with PEG 400, this was based on the FDA requirements of including both
gender in clinical studies. Previous studies on the effect of PEG 400 on ranitidine
bioavailability were carried out with male volunteers. This study aims to investigate
if the trends seen with male volunteers will hold true for female subjects.

• Investigate if any effects were obtainable with other BCS class III drugs

• Investigate the use of non-human methods (Caco-2 cell model and rats) to determine
excipient effects on modulating drug absorption.
CHAPTER 2: Method Development and Analysis for Quantification of Model Drugs in Human Urine
2.1 Overview

From the objectives the model drugs considered for use in this project included ranitidine and one or more other H$_2$ receptor antagonist (cimetidine, nizatidine or famotidine) along with the excipient polyethylene glycol 400 (PEG 400). Whilst various methods exist in the literature for the analysis of these compounds, it is often impossible to reproduce the results obtained or use the methods for validation. Several attempts were made to use the published methods for the analysis of ranitidine (Carey et al. 1981; Prueksaritanont et al. 1989) and PEG 400 (Carey et al., 1981, Chadwick et al., 1977a, Prueksaritanont et al., 1989), however the results were unsatisfactory (low sensitivity, tedious method preparation). Also these methods were unsuitable for bulk and huge number of samples that were generated in this project. The aim of this chapter is to develop suitable methods for analysing compounds from the volunteer studies.

Developing a selective and sensitive analytical method for quantitative evaluation of the drugs and metabolites used in this project is critical for the success of the volunteer and animal studies to be conducted. Validation of requirements for developed methods is governed by the ICH (The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) and USP guidelines. The FDA has also produced a guidance document for the validation of bioanalytical procedures such as gas chromatography (GC), high-pressure liquid chromatography (HPLC) and mass spectrometry methods. Validation of the proposed method will
demonstrate that the method used for quantititative measurement of the compounds in this project in the biological matrices (urine and rat plasma) are reliable and reproducible.

The ICH validation requirements are defined by Q2A, Text on Validation of Analytical procedures (March 1995) and Q2B, Validation of Analytical Procedures: Methodology (May 1997). The USP validation requirements are defined by the validation of compendia methods (Chapter 1225).

The ICH/USP validation requirements are:

- Specificity/selectivity
- Linearity
- Range
- Accuracy
- Precision
- Limit of Detection
- Limit of Quantification
- Robustness

Following the full validation of a method, partial validations may be carried out in particular circumstances, such as a change in the analytical methodology (e.g. change in detection system), change in matrix within species (e.g. human urine to human plasma), change in species within matrix (e.g. human plasma or urine to rat plasma or urine), where
there is limited sample volume (e.g. paediatric study). Partial validation can range from a simple one intra-assay accuracy and precision determination to a nearly full validation.

A summary of the ICH/USP as well as the FDA’s recommendations for the validation of bioanalytical methods is discussed.

Selectivity of a method is the ability of that method to differentiate and quantify an analyte in a complex mixture without interference from other components of the sample. To determine the specificity of a method, blank samples of the same biological matrix to be used in the studies should be obtained from at least six sources. Each of the blank samples should then be tested for interference and selectivity ensured at the lower limit of quantification (LLOQ).

Linearity obtained from a calibration curve shows the ability of the method to produce test results which are proportional to the concentration of the analyte in samples within a concentration range usually a minimum of six standards within 80 – 120 % of the expected concentration range.

The range is normally derived from linearity studies and is established by confirming that the method provides an acceptable degree of linearity, accuracy and precision. The range required for drug substances is 80 – 120% of the expected concentration.
The **accuracy** of the method shows the closeness of the test results obtained to the true concentration of the analyte. Accuracy is measured using a minimum of five determinations per concentration. The mean value obtained should be within 15% of the actual value except for the lower limit of quantification for which the value calculated must not be more than 20%.

The **precision** of an analytical method demonstrates the closeness (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample. Precision is determined at three levels, repeatability, intermediate precision and reproducibility. Repeatability is also called intra-assay precision and it expresses precision under the same operating conditions over a short interval of time. Intermediate precision is expressed in terms of coefficient of variation (CV) and studies may include data gathered under one or more of the following sets of conditions: on varying days, with different analysts, on different equipment or in different laboratories. The reproducibility of the method may also be determined if appropriate. Reproducibility is the ability of the method to reproduce data within the predefined precision e.g. repeatability test at two different labs.
To obtain the precision of a method, a minimum of three concentrations in the range of concentration level expected should be used and precision measured using a minimum of five determinations per concentration. At each concentration level, the precision should be maximum 15% of the coefficient of variation except for the LLOQ where it should not exceed 20% of the CV. Sub-divisions of precision include within-run, intra batch precision or repeatability.

The stability of the compounds in the volunteer samples to be measured is critical. Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix and the container system. When investigating stability, the stability of the analytes during sample collection, after long term (stored at the intended temperature) and short term (e.g, bench top, room temperature) storage and after going through freeze thaw cycles should be considered.

The limit of detection is the lowest concentration of the analyte in a sample that can be detected and differentiated from the background noise, but not necessarily quantifiable by the method. It is determined as a signal to noise ratio of at least 2:1. Unlike the limit of detection, the limit of quantification is the lowest concentration of analyte in a sample that can be quantified with suitable accuracy and precision; 20% precision and an accuracy of 80-120%. It is estimated by a signal to noise ratio of 10:1. Another validation requirement is robustness which is the capacity of the results to remain unaffected by small but
deliberate variations in the method parameters. The results under differing conditions are compared with the precision under normal conditions. Examples of variations include: variation in pH of the mobile phase, temperature or flow rate.
2.2 SECTION 1: HPLC-UV METHOD DEVELOPMENT AND VALIDATION TO DETERMINE CIMETIDINE, RANITIDINE, FAMOTIDINE AND NIZATIDINE IN HUMAN URINE

2.3 Introduction

A number of HPLC-UV methods have been developed for the analysis of the individual H₂ antagonists in biological samples of urine and plasma or urine; including cimetidine (Iqbal et al., 2004, Strong and Spino, 1987, Kunitani et al., 1981), famotidine (Dowling and Frye, 1999, Cvitkovic et al., 1991, Carlucci et al., 1988, Bologna et al., 1988), ranitidine (Prueksaritanont et al., 1989), nizatidine (Tracqui et al., 1990, Yusuf et al., 2006). More complex or sophisticated liquid chromatography methods have also been reported for the individual analysis of H₂ antagonists in urine, including HPLC-MS (Lant et al., 1985, Martin et al., 1981, Martin et al., 1982), paired-ion HPLC-UV (Imai and Kobayashi, 1992, Carey et al., 1981) post-column florescence derivatisation (Vinas et al., 1997); HPLC-TLC (Shah et al., 2002) and supercritical chromatography (SPC, 2009). Most of these methods require either solid-phase or liquid-phase extraction procedures which are time consuming. Moreover, these methods are only capable of analysing one of the four H₂ antagonists. Two groups have described methods for the determination of all four H₂ antagonists in tablet dosage forms (Gyeresi et al., 2000, Ho et al., 1999). However, there has been no report of a single universal assay capable of analysing all four H₂ antagonists in biological samples such as human urine. For this project, it was important that a single universal method was
developed as one or more of the compounds apart from ranitidine could have be taken forward as model compounds. Studying the excretion of a drug and its metabolites in urine after drug administration provides useful information on the drugs’ absorption, distribution and elimination (Martin, 1967b, Martin, 1967a). Urine is a more readily available biological media than plasma, being easier and less intrusive to obtain. The principal route of ranitidine excretion is the urine (40% recovery of free and metabolized drug in 24 h) (Rxmed, 2006). Also, Sadray et al (2003) concluded that urinary data of ranitidine could be used instead of the plasma data in determination of absorption extent and elimination rate of ranitidine due to its comparability in results and the relative easy of sample collection, extraction procedure, assay and non existence of the double peak phenomenon in urinary data, which in turn can ease bioavailability evaluations (Prueksaritanont et al. 1989). However, to determine the absorption rate constant (K_a), Tmax and Cmax, plasma data are preferred. This phenomenon has been attributed to less frequent sampling in the first hours of urine sampling, since repetitive urine samplings with short time intervals is not practically possible (as opposed to plasma) (Sadray et al., 2003). In the same manner, quantification of PEG 400 in human urine has been used to determine intestinal permeability in man.

In this chapter, a simple, single and universal HPLC-UV method for the analysis of any of the four H_2 antagonists in human urine is described. This method will be used for the analysis of ranitidine, its metabolites (ranitidine N-oxide, ranitidine S-oxide and desmethyl ranitidine) and cimetidine in the volunteer study. The metabolism of ranitidine and
cimetidine are similar, and also it was not possible to obtain the cimetidine metabolites. Hence only the metabolism of ranitidine would be investigated.

2.4 Experimental

2.4.1 Reagents and Chemicals

Ranitidine hydrochloride was obtained from Zhongnuo Pharmaceutical Co., Ltd (Shijiahzuang, China). The metabolites of ranitidine (ranitidine N-oxide, desmethyl ranitidine and ranitidine S-oxide) were obtained from GlaxoSmithKline (Harlow, UK). Cimetidine, nizatidine and famotidine were purchased from Sigma Aldrich (UK). The physicochemical properties of the parent compounds are summarised in table 2.1

HPLC grade acetonitrile and water were obtained from Fisher Scientific (Loughborough, UK). Water used for sample preparation was obtained from an Elga Purelab option purification system. Sodium acetate (Sigma Aldrich, UK) and glacial acetic acid (VWR International, Poole, UK) were of analytical-reagent grade. Blank urine was obtained from laboratory personnel and volunteers during the study days.
Table 2. Physico-chemical properties of cimetidine, famotidine, nizatidine and ranitidine

<table>
<thead>
<tr>
<th></th>
<th>Molecular weight</th>
<th>pKa</th>
<th>Solubility in water (mg/ml) (pH 7.0)</th>
<th>Melting point (°C) (MP)</th>
<th>Molecular formula</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cimetidine</td>
<td>252.34</td>
<td>7.1</td>
<td>6</td>
<td></td>
<td>C_{10}H_{16}N_{6}S</td>
<td></td>
</tr>
<tr>
<td>Famotidine</td>
<td>337.45</td>
<td>7.1</td>
<td>1.1</td>
<td>163-164 °</td>
<td>C_{8}H_{15}N_{7}O_{3}S_{3}</td>
<td></td>
</tr>
<tr>
<td>Nizatidine</td>
<td>331.45</td>
<td>6.8 and 2.1</td>
<td>10-33</td>
<td>203</td>
<td>C_{12}H_{21}N_{5}O_{2}S_{2}</td>
<td></td>
</tr>
<tr>
<td>Ranitidine</td>
<td>350.87</td>
<td>8.2 and 2.7</td>
<td>&gt; 550*</td>
<td>69-70</td>
<td>C_{13}H_{22}N_{4}O_{3}.HCl</td>
<td></td>
</tr>
</tbody>
</table>

(Mummaneni and Dressman, 1994); * (FDA, 1998)
2.4.1.1 Instrumentation and Chromatographic Conditions

The HPLC system consisted of a Hewlett-Packard 1050 Series high-performance LC (HPLC) system, equipped with UV detector set at 230 nm for the determination of the four parent H₂ antagonists. The wavelength was adjusted to 320 nm for the separate analysis of ranitidine and its metabolites. The chromatographic data were collected using PC/Chrom software (H & A Scientific Co, UK). Separation of all molecules was achieved with a Phenomenex Luna SCX column (250 mm x 4.6 mm I.D.) packed with 5 μm strong cation-exchange resin (VWR International, Poole, UK) at 50°C. The mobile phase used for analysis consisted of acetonitrile : 0.1M sodium acetate buffer acidified with glacial acetic acid (pH 5.0; 0.1M) (20:80 v/v). Analysis was complete within 15 min using a flow rate of 2.0 ml/min.

2.4.1.2 Preparation of Standard Solutions, Quality Control Samples and Volunteer Samples

2.4.1.2.1 H₂ Antagonists — cimetidine, famotidine, nizatidine and ranitidine

Standard solution of each drug was individually prepared in mobile phase and urine and injected onto the HPLC column to determine the individual retention times of the molecules. Then a stock solution containing 500 μg/ml of each standard was prepared in diluted blank urine (blank human urine diluted in a 50:50 ratio with a solution of 20:80 acetonitrile:water). Working standard solutions were prepared by serial dilutions of the stock solution with diluted blank urine over the range of 0 – 500 μg/ml. A 10 μl volume
from each solution was injected in the chromatographic system under the conditions
detailed in section 2.4.1.1

2.4.1.2.2 Ranitidine and its Metabolites

A stock solution containing 500 μg/ml ranitidine and 50 μg/ml of each metabolite of
ranitidine (ranitidine N-oxide, ranitidine S-oxide and desmethyl ranitidine were prepared
using blank human urine diluted in a 50:50 ratio with a solution of 20:80 acetonitrile:water
(control urine). A series of standards over the range of 0 – 500 μg/ml ranitidine and 0 – 50
μg/ml of each metabolite were prepared by serial dilution using diluted control urine. For
the human study, each volunteer sample was also diluted in a 50:50 ratio with a solution of
20:80 acetonitrile:water. A 10 μl volume from each solution was injected in the
chromatographic system under the conditions detailed in section 2.4.1.1.

2.4.1.3 Method Validation

Quality control standards of low, medium and high concentrations of the molecules were
prepared in control urine to evaluate the precision and accuracy of the method. Separate
standards of low concentrations were prepared to investigate the limit of detection and
quantification.
2.4.1.4 **Linearity**

The linearity of the method was determined at five different concentrations that ranged from 0.5 to 500 μg/ml for cimetidine, famotidine, nizatidine and ranitidine and 0.1 to 50 μg/ml for the metabolites of ranitidine.

2.4.1.5 **Accuracy and Precision**

The accuracy and precision of the assay were evaluated by calculating the intra- and inter-day coefficient of variation. The quality control samples were investigated for accuracy and precision using five determinations for each quality control concentration at three different time points. The inter-day variation was also evaluated at the three different concentrations on four different non-consecutive days.

2.4.1.6 **Sensitivity**

The limit of detection (LOD) was determined as the lowest concentration of analyte that produced at least twice the baseline noise level and the limit of quantification (LOQ) was determined as the lowest concentration of analyte that could be determined with adequate precision of 20% and accuracy of 80-120%.

2.4.1.7 **Stability**

The stability of ranitidine and its metabolites in urine under different storage conditions was investigated. Spiked samples at two different concentrations of ranitidine (267 and 80 μg/ml) were prepared in duplicate. The spiked samples were analysed after different
storage conditions; immediately after being placed on the auto sampler, 4, 9 and 24 h, after one, two and three freeze/thaw cycles and after three months storage at -20°C. Three freeze-thaw cycles and 48 h room temperature stability was also investigated for the metabolites of ranitidine at two different concentrations (9 and 30 μg/ml).

For the freeze thaw cycle, three aliquots of the samples (9 and 30 μg/ml) were stored at -20°C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. This was then repeated two more times and analysis carried out on the sample on the third cycle.

2.5 Results and Discussion

2.5.1.1 Retention Times

A representative chromatogram of blank urine is shown in Fig. 2.1a. A typical chromatogram produced by the developed HPLC method at 230 nm for the standard solutions of cimetidine, famotidine, nizatidine and ranitidine at their limit of quantification is shown in Fig. 2.1b. The retention times for famotidine, cimetidine, ranitidine and nizatidine were 5.1, 5.6, 9.5 and 9.9 min respectively.

A typical chromatogram using the method at 320 nm for blank urine and spiked standard solutions of ranitidine and its metabolites (ranitidine N-oxide, desmethyl ranitidine,
ranitidine S-oxide) are shown in figures 2.2 a and b respectively. The retention times of ranitidine N-oxide, desmethyl ranitidine, ranitidine and ranitidine S-oxide were 4.1, 7.8, 10.1, 11.6 min respectively. Representative chromatograms pre- and post- administration of ranitidine to a human volunteer is shown in figures 2.3 a and b respectively.
Figure 2.1: Representative chromatograms of (A) blank human urine, (B) blank human urine spiked with H2 antagonists at lower limits of quantification: (1) Famotidine, (2) Cimetidine, (3) Ranitidine, (4) Nizatidine
Figure 2.2: Representative chromatograms of (A) blank human urine, (B) blank human urine spiked with ranitidine and its metabolites: (1) ranitidine N-oxide (0.6 μg/ml), (2) desmethyranitidine (0.6 μg/ml), (3) ranitidine (6μg/ml) and (4) ranitidine S-oxide (0.6 μg/ml)
Figure 2.3: Representative chromatograms of urine from a volunteer (A) pre-dose, (B) post-administration of 150 mg ranitidine: (1) ranitidine N-oxide (2 μg/ml), (2) desmethylranitidine (0.5 μg/ml) (3) ranitidine (10 μg/ml) and (4) ranitidine S-oxide (0.9 μg/ml)
2.5.1.2 Linearity and Correlation Coefficient

The standard curves \((n = 5)\) displayed a linear response up to 500 \(\mu g/ml\) for cimetidine, ranitidine, famotidine, nizatidine and 50 \(\mu g/ml\) for each of the metabolites of ranitidine. Good linearity \((r^2 > 0.9960)\) was obtained for all molecules; cimetidine (0.9999), famotidine (0.9995), ranitidine (0.9987), nizatidine (0.9995), ranitidine N-oxide (0.9983), desmethyl ranitidine (0.9971) and ranitidine S-oxide (0.9966).

2.5.1.3 Precision, Accuracy, Limit of Detection and Quantification

The intra-day and inter-day precision, accuracy, limit of detection and limit of quantification are reported in table 2.2.

The precision for all compounds ranged from 0.1 to 13.6 %. In the analysis of ranitidine and its metabolites, the coefficients of variation for intra- and inter- day variability (Table 2.1) by this method is lower than in previously reported assays (Prueksaritanont et al., 1989).

The separation of ranitidine and its metabolites obtained by this method is an improvement compared to other reported methods (Carey et al., 1981, Prueksaritanont et al., 1989). The resolution obtained by this method for ranitidine and its metabolites was greater than 2.5 for all peaks and tailing was minimal, mean ± S.D was 1.01 ± 0.11. The resolution between cimetidine and famotidine was greater than 1.8 whilst the resolution between ranitidine and nizatidine was low (1.1). Such low resolution between ranitidine and nizatidine has previously been reported (Gyeresi et al., 2000, Ho et al., 1999). In the study by Gyeresi et al. (2000), using the United States Pharmacopeia (USP 23) HPLC method, nizatidine and ranitidine could not be separated as the retention times for both molecules was the same. In
our study, whilst the retention times for ranitidine and nizatidine were close, two clear peaks were observed for the individual drugs. In reality, it is unlikely that all four drugs will be administered concurrently to human subjects and hence there is little need to separate all four molecules simultaneously. The retention times obtained with this new method are comparable to individual assays, but as would be expected, the sensitivity and intra- and inter-day variability are lower than individual assays and other more sophisticated methods such as HPLC-MS. The new method described provides a generic, simple and universal approach for the identification and quantification of any of the four H₂ antagonists. The method utilizes a simplified urine sample processing (dilution of urine with 20% acetonitrile:water) which is cheaper and more time efficient than liquid/solid phase extractions methods used in many of the previous reports.

The detection limits in this study were higher than obtained by previous studies for ranitidine, and the N-oxide and desmethyl metabolites (0.15 µg/ml for each compound) but lower for the S-oxide metabolite (0.2 µg/ml) (Prueksaritanont et al., 1989). The limits of quantification were not reported in the previous HPLC assays for ranitidine and its metabolites (Prueksaritanont et al. 1989; Lant et al. 1985; Carey et al. 1981).
<table>
<thead>
<tr>
<th>QC Sample (µg/ml)</th>
<th>Calculated Conc. (µg/ml)</th>
<th>Accuracy Mean ± SD (%)</th>
<th>Intra-assay CV (%)</th>
<th>Inter-assay CV (%)</th>
<th>LOD (µg/ml)</th>
<th>LOQ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cimetidine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>7.99 ± 0.28</td>
<td>88.8 ± 1.1</td>
<td>8.6</td>
<td>9.5</td>
<td>0.5</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>34.8 ± 0.13</td>
<td>115.9 ±0.4</td>
<td>1.9</td>
<td>3.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>300.2 ± 0.55</td>
<td>100.1 ± 0.1</td>
<td>3.1</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Famotidine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.48 ± 0.49</td>
<td>89.7 ± 3.6</td>
<td>3.4</td>
<td>4.0</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>31.5 ± 0.11</td>
<td>96.4 ± 0.1</td>
<td>2.5</td>
<td>4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>292.6 ± 0.47</td>
<td>97.3 ± 1.0</td>
<td>3.0</td>
<td>3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nizatidine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.27 ± 0.05</td>
<td>89.6 ± 5</td>
<td>13.6</td>
<td>12.0</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>33.2 ± 0.19</td>
<td>110.5 ± 0.6</td>
<td>13.6</td>
<td>12.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>295.3 ± 1.0</td>
<td>98.4 ± 1.0</td>
<td>1.8</td>
<td>2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ranitidine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.9 ± 0.18</td>
<td>97.9 ± 8</td>
<td>2.7</td>
<td>4.3</td>
<td>0.25</td>
<td>2.0</td>
</tr>
<tr>
<td>30</td>
<td>34.3 ± 0.05</td>
<td>114.4 ± 0.3</td>
<td>0.5</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>300.2 ± 0.17</td>
<td>102.4 ± 0.2</td>
<td>0.1</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2 (Continued). Accuracy, intra- and inter- day precision, limit of detection and limit of quantification of H₂ antagonists and the metabolites of ranitidine in human urine (n= 5)

<table>
<thead>
<tr>
<th>QC Sample</th>
<th>Calculated</th>
<th>Accuracy</th>
<th>Intra-assay</th>
<th>Inter-assay</th>
<th>LOD</th>
<th>LOQ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µg/ml)</td>
<td>(µg/ml)</td>
<td>CV (%)</td>
<td>CV (%)</td>
<td>(µg/ml)</td>
<td>(µg/ml)</td>
</tr>
<tr>
<td></td>
<td>Conc.</td>
<td>Mean ± SD</td>
<td>CV (%)</td>
<td>CV (%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| CDH       | Ranitidine N-oxide
| 3         | 3.34 ± 0.02| 111.4 ± 0.6| 0.8         | 0.2         | 0.1  | 0.6  |
| 9         | 9.08 ± 0.01| 100.9 ± 0.3| 0.5         | 0.8         |      |      |
| 30        | 29.6 ± 0.01| 98.7 ± 0.1 | 0.3         | 0.6         |      |      |
|           | Desmethyl ranitidine
| 3         | 3.06 ± 0.04| 102.1 ± 1.4| 4.5         | 1.3         | 0.1  | 0.6  |
| 9         | 8.87 ± 0.06| 98.6 ± 0.1 | 1.6         | 4.8         |      |      |
| 30        | 29.7 ± 0.04| 99.1 ± 0.7 | 0.2         | 1           |      |      |
|           | Ranitidine S-oxide
| 3         | 3.02 ± 0.05| 100.8 ± 2.4| 4.2         | 5.5         | 0.5  | 3    |
| 9         | 9.51 ± 0.06| 105.7 ± 1.2| 0.5         | 2.7         |      |      |
| 30        | 30.2 ± 0.08| 100.7 ± 0.1| 3.1         | 3.7         |      |      |
2.6 Summary

The HPLC-UV method described is a simple, universal, convenient and reproducible method that can be used to determine and quantify any of the four H2 receptor antagonists. The method will be applied to the analysis of ranitidine and its metabolites in the urine of healthy human. This method is useful for bioavailability studies and has the potential of being useful in studying drug interactions in clinical pharmacology trials.

2.7 SECTION 2: QUANTIFICATION OF POLYETHYLENE GLYCOL 400 IN THE URINE OF HUMAN VOLUNTEERS USING FLOW INJECTION ANALYSIS/MASS SPECTROMETRY

2.8 Introduction

Polyethylene glycol 400 (PEG 400) is a highly soluble and hydrophilic polymer which contains nine different molecular weight fractions ranging from 238 to 594, n is the number of ethylene oxide units ranging from 5 to 13. The distribution of the fractions is bell shaped (near Gaussian) with an average molecular weight of 400.

PEG 400 is the most commonly used PEG for gastrointestinal permeability probe (Chadwick et al., 1977a, Chadwick et al., 1977b, Robinson et al., 1981, Ukabam and Cooper, 1984, Jenkins et al., 1986, Ryan et al., 1992, Oliva et al., 1994, Iqbal et al., 1995, Soderholm et al., 1997, Loret et al., 2004). It is a marker for paracellular absorption. Other permeability probes and paracellular markers including 51Cr-EDTA and lactulose. PEGs
are particularly useful as permeability probes because they are not metabolized by intestinal bacteria and are rapidly and almost completely excreted in urine.

![Composition of polyethylene glycol 400 illustrating the Gaussian distribution of the different polymers (adapted from Chadwick et al 1977a).](image)

**Figure 2.4:** Composition of polyethylene glycol 400 illustrating the Gaussian distribution of the different polymers (adapted from Chadwick et al 1977a).

Also their toxicity is very low (Rowe et al. 2006). To determine the intestinal permeability, urinary excretion rate of the orally administered permeability markers such as PEG, lactulose and 51Cr-EDTA (Loret et al., 2004) is measured.

Several methods - gas chromatography (Chadwick et al., 1977a, Jenkins et al., 1986, Sundqvist et al., 1980); GC-LC (Bouska and Phillips, 1980); HPLC (Leister et al., 1995, Rissler et al., 1993) and gel permeation chromatography (GPC) (Sefisko et al., 1993), GC-MS (Fakt and Ervik, 1997) and NMR (Vernooij et al., 1999) have been developed to measure PEGs in biological fluids including urine. The sample preparation for all these
methods are tedious and time consuming; often involving lyophilization of the samples prior to extraction or extensive sample pre-treatment with ion-exchange resins for removal of interfering substances (Fakt and Ervik, 1997). During this project, the gas chromatography approached failed as peaks could not be determined as present despite the use of published methods (Chadwick et al., 1977a, Chadwick et al., 1977b). It was not possible to use HPLC methods since polyethylene glycol does not have a chromophore and a refractive index detector is not available at the School of Pharmacy. Previous methods were used to analyse higher concentrations of PEG 400 than required in this project (> 2 g) [Table 2.3] and do not have appropriate sensitivity to quantify the amount of PEG 400 in the study (Bouska and Phillips, 1980, Chadwick et al., 1977a, Delahunty and Hollander, 1986, Ersoy et al., 1996, Fakt and Ervik, 1997, Loret et al., 2004, Oliva et al., 1994, Parlesak et al., 1994, Ryan et al., 1992, Teahon et al., 1992, Young et al., 1990)

Consequently the aim of this section of the project was to develop a simple, sensitive and fast method to quantify the amount of PEG 400 excreted in the urine of human volunteers (male and females) after oral administration of 0.75 and 1.5 g PEG 400. We describe a flow injection analysis/mass spectrometry (FIA/MS) method. Six of the most intense ions of the PEG 400 oligomer were chosen for selected ion monitoring (SIM) by mass spectrometry.
Table 2.3: Summary of published methods quantifying PEG 400 in human urine

<table>
<thead>
<tr>
<th>Article</th>
<th>Method—HPLC, mass spec etc</th>
<th>Amount of PEG 400 ingested (g)</th>
<th>Collection period (h)</th>
<th>Number of volunteers</th>
<th>Sample Prep</th>
<th>LoD</th>
<th>LoQ</th>
<th>Amount of PEG quantified (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Bouska and Phillips, 1980)</td>
<td>Gas-Liquid chromatography (GC-LC)</td>
<td>5</td>
<td>6</td>
<td>15</td>
<td>SPE</td>
<td>-</td>
<td>1 mg/ml</td>
<td>23</td>
</tr>
<tr>
<td>(Chadwick et al., 1977a)</td>
<td>Gas-liquid chromatography</td>
<td>10</td>
<td>48</td>
<td>5</td>
<td>-</td>
<td>1 µg/ml</td>
<td>50 µg/ml</td>
<td>55.6 ± 5.8</td>
</tr>
<tr>
<td>(Delahunty and Hollander, 1986)</td>
<td>LC</td>
<td>5.6</td>
<td>6</td>
<td>11 males</td>
<td>Lyophilization and extraction with chloroform</td>
<td>-</td>
<td>5 mg/ml</td>
<td>2.9</td>
</tr>
</tbody>
</table>

78
<table>
<thead>
<tr>
<th>Article</th>
<th>Method –HPLC, mass spec etc</th>
<th>Amount of PEG 400 ingested (g)</th>
<th>Collection period (h)</th>
<th>Number of volunteers</th>
<th>Sample Prep</th>
<th>LoD</th>
<th>LoQ</th>
<th>Amount of PEG quantified (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Ersoy et al., 1996)</td>
<td>FT-IR</td>
<td>5.6</td>
<td>6</td>
<td>3</td>
<td>Extraction with dichloromethane and centrifugation</td>
<td>0.8 mg/L</td>
<td>-</td>
<td>26.2</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>5.6</td>
<td>6</td>
<td></td>
<td>Esterification</td>
<td>0.02 mg/L</td>
<td>-</td>
<td>24.9</td>
</tr>
<tr>
<td>(Fakt and Ervik, 1997)</td>
<td>Capillary-gas chromatography-selected ion-monitoring mass spectrometry GC-MS</td>
<td>-</td>
<td>-</td>
<td></td>
<td>SPE; subsequent derivatization with hepafluorobutyric anhydride (HFBA)</td>
<td>0.4 μg/ml</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Article</td>
<td>Method – HPLC, mass spec etc</td>
<td>Amount of PEG 400 ingested (g)</td>
<td>Collection period (hr)</td>
<td>Number of volunteers</td>
<td>Sample Prep</td>
<td>LoD</td>
<td>LoQ</td>
<td>Amount of PEG quantified (%)</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-------------------------------</td>
<td>---------------------------------</td>
<td>------------------------</td>
<td>---------------------</td>
<td>---------------------------------</td>
<td>-----------</td>
<td>--------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>(Loret et al., 2004)</td>
<td>Gel permeation chromatography with refractometric detection</td>
<td>2</td>
<td>8</td>
<td>11</td>
<td>solid phase extraction (SPE)</td>
<td>25 μg/ml</td>
<td>-</td>
<td>24.2 ± 9.3</td>
</tr>
<tr>
<td>(Oliva et al., 1994)</td>
<td>HPLC-RI</td>
<td>10</td>
<td>6</td>
<td>6</td>
<td>SPE</td>
<td>0.75 mg/ml</td>
<td>-</td>
<td>30.1 ± 3.87</td>
</tr>
<tr>
<td>(Parlesak et al., 1994)</td>
<td>HPLC-RI</td>
<td>2</td>
<td>24</td>
<td>21</td>
<td>Centrifugation followed by extraction with chloroform</td>
<td>5 μg/ml</td>
<td>-</td>
<td>33.6 ± 3.2</td>
</tr>
<tr>
<td>Article</td>
<td>Method – hplc, mass spec etc</td>
<td>Amount of PEG 400 ingested (g)</td>
<td>Collection period (h)</td>
<td>Number of volunteers</td>
<td>Sample Prep</td>
<td>LoD</td>
<td>LoQ</td>
<td>Amount of PEG quantified (%)</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------------------------</td>
<td>--------------------------------</td>
<td>------------------------</td>
<td>----------------------</td>
<td>-------------</td>
<td>-----</td>
<td>-----</td>
<td>------------------------------</td>
</tr>
<tr>
<td>(Ryan et al., 1992)</td>
<td>HPLC – Refractive index</td>
<td>5</td>
<td>24</td>
<td>12 males</td>
<td>pH adjustment, centrifugation, filtration, size exclusion membrane separation</td>
<td>0.02 mg/ml</td>
<td>-</td>
<td>26.3 ± 5.1</td>
</tr>
<tr>
<td>(Ruttenberg et al., 1992)</td>
<td>HPLC-RI</td>
<td>5.6</td>
<td>6</td>
<td>31</td>
<td>-</td>
<td>0.2 mg/ml</td>
<td>25 (4.5-39.7)</td>
<td></td>
</tr>
<tr>
<td>(Teahon et al., 1992)</td>
<td>HPLC-RI</td>
<td>5</td>
<td>5</td>
<td>25</td>
<td>SPE</td>
<td>5 µg/ml</td>
<td>-</td>
<td>25 ± 3.3</td>
</tr>
<tr>
<td>(Young et al., 1990)</td>
<td>HPLC</td>
<td>5.6</td>
<td>5</td>
<td>-</td>
<td>50 µg/ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
2.9 Experimental

2.9.1 Reagents and chemicals

Polyethylene glycol 400 (PEG 400) used in this study with molecular formular C\(_{2n}\)H\(_{4n+2}\)O\(_{n+1}\), \(n = 8.2\) to 9.1 and molecular mass 380-420 g/mol was obtained from Sigma Aldrich, Dorset, UK. HPLC grade methanol and water were obtained from Fisher Scientific (Loughborough, UK). Formic acid was obtained from Sigma-Aldrich (UK). Control human urine samples were obtained from the laboratory personnel at the School of Pharmacy.

2.9.2 Direct-infusion ES-MS

Direct infusion ES-MS analysis was performed using a Waters Alliance 2695 Separation Module liquid chromatograph (Waters, UK) and Navigator quadrupole mass spectrometer (Finnigan, UK). Samples, 50 \(\mu\)l (equivalent to 0.01 \(\mu\)L of urine), in 50% methanol 0.1% formic acid in water, were injected via a Waters autosampler coupled through a polyether ether ketone (PEEK) tubing to the mass spectrometer. A PEEK sample loop (100 \(\mu\)L, Upchurch, UK) and a PEEK rotor seal were used in order to minimise carry over of PEG 400 between injections. The isocratic program was maintained with the mobile phase of methanol-water-formic acid (50:50:0.1, v/v/v), at flow rate of 200 \(\mu\)L/min. The total run time was 5 min. The effluent was continuously directed into the ES source of the Navigator mass spectrometer, which was operated in the positive-ion ES mode, under the following conditions: drying gas – 400 L/h, ion spray voltage 3.5 V, cone voltage 26 V, RF voltage 0.3 V, source heater 1200C, LM and HM resolutions 12.5. MS-mode was used for the identification of PEG 400 over the m/z range of 250 – 750 Da (1.0-s scan time, 0.1-s
inter scan time), and a selected ion monitoring (SIM) mode for the quantification of PEG 400 in biological samples. In SIM mode, six channels were recorded for the following characteristic ions of PEG 400: 349.1, 365.1, 409.1, 453.1, 497.1, 541.1. The SIM mode is where very specific ions are monitored. The dwell time of 300 ms, cone voltage of 26 V, and span of 0.2 were chosen for all selected ions during the acquisition. The data collection was performed using a 1-s MS scan, which provides identity information, followed by 1-s SIM scans (0.1-s inter scan time), and the cycle is repeated throughout the total run time. Each sample was analysed three times by direct infusion LC-ES-MS.

2.9.3 Preparation of Standard Solutions, Quality Control Samples and Volunteer Samples

Standard solutions (3 to 100 μg/ml) of PEG 400 were individually prepared in mobile phase and urine and injected. Samples were prepared by dilution of urine samples in a 1:5 ratio with 100% methanol. The diluted solution was then centrifuged at 20,800 x g for 15 min and the supernatant removed for use in analysis. A full scan mass spectrometry analysis of the standard PEG 400 samples in urine was performed and a typical “Gaussian Distribution” mass spectrum of the oligomers was observed (Figure 2.6). Six of the most intense ions were selected from the spectrum for selected ion monitoring (SIM). These were: 349, 365, 409, 453, 497 and 541. The SIM chromatogram showing each of the ion monitored is shown in Fig. 2.8.
2.9.4 Method Validation

Quality control standards of low, medium and high concentrations (3, 30 and 90 μg/ml) of PEG 400 in control urine diluted with methanol were prepared to evaluate the precision, accuracy, limit of detection and quantification of the method.

2.9.5 Linearity

The linearity of the method was determined at ten different concentrations that ranged from 3 to 100 μg/ml. The linearity curve was determined on at least three different occasions.

2.9.6 Accuracy, Precision and Reproducibility

The accuracy and precision of the assay were evaluated by calculating the intra- and inter-day coefficient of variation. The quality control samples were investigated for accuracy and precision using five determinations for each quality control concentration at three different time points. The inter-day variation was also evaluated at the three different concentrations on three different non-consecutive days.

2.9.7 Sensitivity

The limit of detection (LOD) was determined as the lowest concentration of analyte that produced at least twice the baseline noise level and the limit of quantification (LOQ) was determined as the lowest concentration of analyte that could be determined with adequate precision of 20% and accuracy of 80-120%.
2.10 Results and Discussion

The full scan mass spectrum of blank urine and PEG 400 in urine is shown in figures 2.5 and 2.6 respectively. Figure 2.7 shows urine spiked at the lower limit of quantification concentration 2.5 µg/ml. The area under each SIM peak (figure 2.8) was subjected to regression analysis and the actual PEG 400 concentration in each sample was determined by interpolation from the standard curve. The spectrums obtained pre- and post-administration of PEG 400 to a volunteer are shown in figures 2.9 and 2.10 respectively.

The intra-day and inter-day precision, accuracy, limit of detection and limit of quantification are reported in table 2.4. The relative errors in accuracy were within ± 10% for all QC samples. The coefficients of variation for intra- and inter-day variability were less than 12%. The limit of detection of the method was 1 µg/ml and the limit of quantification 2.5 µg/ml. The limit of quantification is lower than many of the published methods except (Fakt and Ervik, 1997) where the limit of quantification was 0.4 µg/ml (Table 2.3). The latter method employed amore advanced and complicated [Capillary-gas chromatography-selected ion-monitoring mass spectrometry (GC-MS)]. The limit of detection of this method (1 µg/ml) is also lower than most of the previously published methods (Table 2.3).
Figure 2.5: Full scan mass spectrum of blank urine
Figure 2.6: Full scan mass spectrum of urine spiked with PEG 400
Figure 2.7: Full scan mass spectrum of urine spiked with PEG 400 at low limit of quantification (2.5 µg/ml)
Figure 2.8: Selected Ion Monitoring chromatogram of PEG 400. Area under the each curve used to calculate concentration of PEG excreted.
Figure 2.9: Representative mass spectrum of urine from a volunteer pre-administration of PEG 400
Figure 2.10: Representative mass spectrum of urine from a volunteer post administration of PEG 400
Table 2.4: Accuracy, intra- and inter-day precision, limit of detection and limit of quantification of PEG 400 in human urine (n = 5)

<table>
<thead>
<tr>
<th>QC Sample (µg/ml)</th>
<th>Calculated Conc. (µg/ml)</th>
<th>Accuracy C.V. (%)</th>
<th>Intra-assay C.V. (%)</th>
<th>Inter-assay C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3.22 ±0.19</td>
<td>107.3</td>
<td>6.0</td>
<td>11.8</td>
</tr>
<tr>
<td>30</td>
<td>31.0 ±3.6</td>
<td>103.2</td>
<td>11.8</td>
<td>9.72</td>
</tr>
<tr>
<td>90</td>
<td>81.7 ±5.9</td>
<td>90.8</td>
<td>3.01</td>
<td>7.22</td>
</tr>
</tbody>
</table>

The limit of detection and limit of quantification were 1 µg/ml and 2.5 µg/ml respectively.

2.11 Conclusion

The method developed is a simple, convenient and reproducible method which is capable of quantifying PEG 400 in human urine and suitable for routine analysis. The method will be applied to samples from the volunteer study and results presented in chapter 3.
CHAPTER 3: An Investigation into the Effects of Polyethylene Glycol 400 on the Bioavailability of BCS Class III Drugs (Ranitidine and Cimetidine)
3.1 Overview

In human studies (male volunteers) a 10g dose of PEG 400 was shown to reduce small intestinal transit time by 37% and the oral bioavailability of ranitidine administered in pellet form by more than 30% (Basit et al., 2001). The plasma profiles in the volunteers in the control leg (ranitidine alone, no PEG 400) showed the classic double peak phenomenon (Figure 3.1) which has also been observed with other BCS class III drugs, cimetidine and famotidine (Kroemer and Klotz, 1987, Walkenstein et al., 1978). However in the presence of PEG 400, the multiple plasma peaks noted in the absence of PEG 400 were abolished (Figure 3.1) and only single plasma peaks present (Basit et al., 2001). The appearance of double peaks following the oral administration of drugs has been attributed to a number of hypotheses including delayed gastric emptying of a portion of the administered dose (Oberle and Amidon, 1987), enterohepatic recycling or secretion of the drug into the lumen of the gut (Roberts, 1984), sequestration of a portion of the drug in the hepatic parenchymal tissue with subsequent bolus release into the systemic circulation (Williams et al., 1992), and the presence of two absorption regions along the gastrointestinal tract (the proximal and distal regions of the small intestine (Mummaneni and Dressman, 1994)

In a subsequent clinical study by Schulze et al (2003) (also in male volunteers), investigating the effects of low doses of PEG 400 (1, 2.5 and 5 g) revealed that PEG 400 had concentration dependent effects on transit and drug absorption (Schulze et al., 2003). Small intestinal transit times were decreased by 9, 20 and 23% respectively. The
oral bioavailability of ranitidine was reduced by 38% in the presence of 2.5 and 5 g PEG 400. However, in the presence of 1 g PEG 400 the bioavailability of ranitidine was increased by 41%, despite the reduction in small intestinal transit time (Schulze et al., 2003). It was proposed that the effect noted with 1 g PEG 400 was due to the ability of PEG 400 to modulate intestinal permeability, an absorption enhancing effect which had been overshadowed at higher concentrations due to rapid passage through the small intestine.

In light of the intriguing result with 1 g PEG 400 by Schulze et al (2003) with male volunteers, coupled with the FDA requirement that the effectiveness and safety data is presented by gender, age and racial subgroups (with gender deemed most important) (FDA, 1998) the objectives of this chapter were to:

- Determine if the phenomenon is repeatable in a different set of male volunteers
- Investigate doses around the peak 1 g dose on the bioavailability of ranitidine
- Determine if the effect seen with male volunteers also occurs with female volunteers
- Investigate if the effect is more general with BCS class III drugs other than ranitidine
3.2 SECTION 1: THE EFFECTS OF PEG 400 ON RANITIDINE BIOAVAILABILITY

3.3 Materials

Ranitidine

Ranitidine hydrochloride was sourced from Zhongnuo Pharmaceutical Co., Ltd (Shijiahzuang, China) and certified as 99.9% pure. It is a pale yellow crystalline powder sensitive to light and moisture. The metabolites of ranitidine (ranitidine N-oxide, desmethyl ranitidine and ranitidine S-oxide) were obtained from GlaxoSmithKline (Harlow, UK).

Polyethylene glycol 400 (PEG 400)

PEG 400 was obtained from Sigma-Aldrich Company (Poole, UK). It is a clear colourless, highly osmotic (2000 mOsm), and viscous liquid.

3.4 Methods

3.4.1 Dosage Forms

3.4.1.1 Preparation of Drug Solutions

168mg ranitidine hydrochloride equivalent to 150 mg ranitidine base, was dissolved in 150 mL of water. Then either 0 (control), 0.5, 0.75, 1.0, 1.25 or 1.5 g PEG 400 were added and the solutions stirred.
3.4.1.2 Preparation and Characterisation of Dosage Forms

The saturated solubility of ranitidine in the different PEG 400 solutions was measured at 37°C using the HPLC method developed in chapter 2. Briefly, excess amount of ranitidine were added to 20 mls of the different PEG 400 concentrations (0.5, 0.75, 1, 1.25 and 1.5 g in 150 ml water). These were placed in shaking water bath at 37°C for 24 h. Samples were taken and analysed by HPLC.

The osmotic pressure of the drug solutions was measured in triplicates using Roebling Digital Micro-osmometer Camb Lab Type 5R (Camlab Ltd., Cambridge).

The pH of the drug solutions was determined in triplicates using a pre-calibrated pH 211 Microprocessor pH Meter (Hanna Instruments).

The viscosity of the drug solutions were measured using microviscometry (Automated Micro Viscometer, AMVn, Anton Paar) using a 1.6-mm capillary tube at 20°C.

3.5 Volunteer Study

3.5.1 Study Protocol

Twelve volunteers (six males and six females) - age range: Males [24-34 years (median 28 years)], Females [24-29 years (median 27 years)]; weight range: Males [66-110 kg (median 74 kg)], Females [50-62 kg (median 53 kg)]; height range: Males [1.63–1.84 m (median 1.75 m)], Females 1.58-1.72 m (median 1.58 m) - participated in a random six-way cross over study after giving informed written consent. All subjects were non-smokers and declared themselves healthy with no history of gastrointestinal disease.
The experimental protocol was approved by The Joint UCL/UCLH Committees on the Ethics of Human Research. The study was conducted in accordance to the Helsinki guidelines for ethics in research (1965) and its subsequent revisions - Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West (SA) (1996), Edinburgh (2000) (WMA, 2008).

The volunteers reported to the study centre after an overnight fast and each received, on six separate occasions, 150 ml of drug solution containing 0, 0.5, 0.75, 1, 1.25 or 1.5 g PEG 400. There was at least a three day washout period between treatments for all volunteers. A standard lunch consisting of two piece cheese sandwich, packet of crisps and a juice drink was provided 4 hours post dose, and water was available ad libitum from this point onwards.

Cumulative urine samples were collected throughout the course of each study day. This involved the collection and measurement of bladder output over the following time periods: 0 (pre-dose), 0 to 2, 2 to 4, 4 to 6, 6 to 12, and 12 to 24 h. For each time point, a 20 mL aliquot was retained and stored at -20°C.

**3.5.2 Urine Analysis**

Urine samples for the study were assayed for the amount of ranitidine, its three metabolites (ranitidine N-oxide, desmethylranitidine, ranitidine S-oxide) and the amount of PEG 400.
3.5.2.1 Analysis of Ranitidine and its Metabolites

Frozen aliquots of urine samples were thawed out at room temperature and 0.65 ml of each sample was mixed with 0.65 ml mixture of 20:80 acetonitrile:water in duplicates. After thorough vortex-mixing, a 10μl aliquot of each solution was injected onto a Luna SCX (Phenomenex, UK) HPLC column using the validated HPLC-UV method described in chapter 2. Briefly, the mobile phase was 20:80 acetonitrile: 0.1M sodium acetate with a flow rate of 2 ml/min at 50°C. Calibration standards were prepared with blank human urine, also diluted (50%) with 20:80 acetonitrile:water.

3.5.2.2 Amount of PEG 400 Excreted in Urine

The frozen urine aliquots from volunteers were thawed out at room temperature and 1.2 ml pure methanol was added to 300 μl of each volunteer sample in duplicates. After vortex-mixing the diluted samples for a few seconds, the samples were centrifuged at 20,800 x g for 15 min at 4°C. Aliquot of 50 μl of each solution was injected onto the mass spectrometer using the method described in chapter 2.

3.6 Statistical Analysis

Using SPSS® software, Kruksal Wallis tests was performed on the solubility, osmolality, viscosity and pH data. The results obtained for the cumulative excretion of ranitidine in urine for males and females were subjected to two-way ANOVA using General Linear Model in SPSS to assess differences between gender. This was followed by one-way ANOVA to assess the effects of the different concentrations of PEG 400 on the bioavailability of ranitidine in males and females separately and then a post hoc Tukey’s
test. Statistical analysis is stated where $p < 0.05$. The results obtained for the excretion of PEG 400 was subjected to one way ANOVA, followed by a post hoc Tukey’s test.

3.7 Results and Discussion

3.7.1 The Effect of Polyethylene Glycol 400 on Ranitidine Bioavailability in Male Volunteers

The oral bioavailability of ranitidine was assessed by the cumulative amounts of unchanged ranitidine excreted in urine over 24 h. The amount of ranitidine excreted by the six male volunteers is shown in Table 3.1.

Table 3.1: Effect of Different Doses of PEG 400 (g) on Ranitidine Bioavailability in Six Male Volunteers

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Control</th>
<th>PEG 400 0.5 g</th>
<th>PEG 400 0.75 g</th>
<th>PEG 400 1.0 g</th>
<th>PEG 400 1.25 g</th>
<th>PEG 400 1.5 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33</td>
<td>37</td>
<td>49</td>
<td>39</td>
<td>51</td>
<td>39</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>53</td>
<td>59</td>
<td>47</td>
<td>39</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>47</td>
<td>59</td>
<td>63</td>
<td>67</td>
<td>62</td>
<td>27</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>44</td>
<td>70</td>
<td>47</td>
<td>53</td>
<td>26</td>
</tr>
<tr>
<td>5</td>
<td>41</td>
<td>61</td>
<td>64</td>
<td>70</td>
<td>63</td>
<td>56</td>
</tr>
<tr>
<td>6</td>
<td>34</td>
<td>25</td>
<td>38</td>
<td>42</td>
<td>32</td>
<td>46</td>
</tr>
</tbody>
</table>

Mean ($\pm$ SD) | 35 (8) | 47 (14) | 57 (12) | 52 (13) | 50 (12) | 37 (12) |

% Diff | +34 | +63 | +49 | +43 | +6
The mean cumulative amount of ranitidine excreted in the absence of PEG 400 (control) in male volunteers was $35 \pm 8$ mg, corresponding to 23% (w/w) of the administered dose. The amount of ranitidine excreted in the absence of PEG 400 (control leg) was consistent with the mean value reported by Schulze et al (2003) where the average amount of ranitidine excreted in normal male subjects was also 23% of the administered dose. This value is also similar to previous studies by (Carey et al. (1981) and van Hecken et al. (1982) where the 24 h urinary recovery of unchanged ranitidine was 27%.

The mean cumulative quantity of ranitidine excretion in the presence of 0.5, 0.75, 1, 1.25 and 1.5 g PEG 400 in the male volunteers were 47, 57, 52, 50 and 37 mg respectively. These correspond to increases in bioavailability of 34, 63, 49, 43 and 6% over the control treatment. Significant optimum bioavailability enhancement was observed with the 0.75 g PEG 400 dose, a 63% increase over the control (p < 0.05).

The enhancement in oral drug bioavailability noted with 1 g PEG in this study of male volunteers (49%) is comparable to that obtained by Schulze et al, where 1 g PEG 400 enhanced the bioavailability of ranitidine by 41% (Schulze et al. 2003). However the data obtained in this thesis clearly show that a lower dose of PEG 400 (0.75 g) has a more potent effect on the bioavailability of ranitidine (increase of 63% over the control). Bioavailability enhancement in the presence of 0.75 g and 1 g PEG 400 occurred in each and every male subject in this study; this was also the case in the Schulze study with 1 g PEG 400 (3), thereby confirming the consistency of the effect in males.
The individual ranitidine excretion profiles for the six male volunteers are shown in Figure 3.2a-f. Figure 3.3 and Figure 3.4 show the excretion rate profiles of ranitidine and mean cumulative excretion over 24 h respectively. The excretion of ranitidine in the presence of PEG 400 is higher compared to the absence of PEG 400. In the absence of PEG 400, peak excretion of ranitidine occurred between 4 and 6 hours, however in the presence of 0.5, 1, 1.25 and 1.5 g PEG 400, peak excretion was between 2 and 4 hours, whilst for 0.75 g PEG 400, peak excretion was between 0 and 2 hours.

The time to reach maximum urinary excretion in the control was between 4-6 hours, this is similar to the value of 4±1.9 hours obtained by Sadray et al. (2003). Other studies which involved urinary excretion did not show cumulative ranitidine excretion or report the time to reach maximum urinary excretion. Also it can be seen that in the presence of 0.75 g PEG 400, there appears to be the presence of multiple peaks. The highest excretion was between 0-2 h, however, there was also increased excretion at 6-12 h. This is particularly interesting because reports with plasma data have shown that double peaks for drugs such as ranitidine are abolished in the presence of high dose PEG 400 (10 g) (Basit, et al. 1999). With the reported plasma data, the first peak usually appears after 1.5 hours and the second after 4 h. Unlike the control where the peak excretion occurs at the time interval of 4-6 h, in the presence of PEG, the peak excretion occurs faster between 2 and 4 h; and surprisingly the presence of double peaks in the presence of 0.75 g PEG 400. The presence of double peaks in the excretion of ranitidine with 0.75 g was observed in five out of the six volunteers. As previously discussed, several mechanisms have been shown to trigger the double peak phenomenon and these include
1) enterohepatic recycling (Roberts, 1984), 2) the presence of two absorption regions along the gastrointestinal tract (the proximal and distal regions of the small intestine (Mummaneni and Dressman, 1994), 3) gastric emptying regulated absorption (Mummaneni and Dressman, 1994, Williams et al., 1992)).

It is difficult to make conclusive decisions as to the reasons why double peaks were not observed in the control study (no PEG) but appears in the presence of 0.75 g PEG 400 as there were no plasma profiles. The absence of double peaks in the control leg suggests that the theory of enterohepatic cycling is not applicable. The faster absorption and presence of double peaks with 0.75 g PEG 400 is also unlikely to be due to gastric emptying as Schulze et al (2003) showed that there were differences in the gastric emptying of ranitidine or ranitidine and PEG 400 solutions. However it should be appreciated that the study was done with 0, 1, 1.5 and 5 g PEG only. Although there is no direct evidence, it is feasible that perhaps 0.75 g PEG 400 emptied faster from the stomach and hence led to the faster absorption noted. Another possible reason for the initial peak of absorption noted at 0-2 hours may be due to PEG 400 at the 0.75 g dose acting on the absorption site to possibly open up tight junctions or affect carrier mediated processes at an earlier stage and hence leading to faster absorption. At the other doses of PEG in this study, this first absorption window is missed.

The absorption of a drug is affected by many parameters, including the characteristics of the drug (solubility, osmolality, pH, viscosity), stability, intestinal wall permeability, transport mechanisms, presence of food and first pass metabolism.
Figure 3.2: Rate of ranitidine excretion over 24 h in the individual male volunteers (A-F)
Figure 3.3: Mean ranitidine excretion over 24 h in the male volunteers

Figure 3.4: Mean cumulative ranitidine excretion in male volunteers
The solubility, osmolality, pH and viscosity of the drug solutions were measured and the results summarised in Table 3.2

Table 3.2: Characteristics of the drug solutions (n = 3)

<table>
<thead>
<tr>
<th>Amount of PEG 400 (g)</th>
<th>Solubility (mg/mL)</th>
<th>Osmolality (m Osm kg⁻¹)</th>
<th>pH</th>
<th>Viscosity (mPa.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>584 (2)</td>
<td>6</td>
<td>7.32</td>
<td>0.88</td>
</tr>
<tr>
<td>0.5</td>
<td>547</td>
<td>15</td>
<td>7.29</td>
<td>9.398</td>
</tr>
<tr>
<td>0.75</td>
<td>559</td>
<td>19</td>
<td>7.26</td>
<td>9.403</td>
</tr>
<tr>
<td>1.0</td>
<td>569</td>
<td>23</td>
<td>7.22</td>
<td>9.438</td>
</tr>
<tr>
<td>1.25</td>
<td>544</td>
<td>28</td>
<td>7.18</td>
<td>9.450</td>
</tr>
<tr>
<td>1.5</td>
<td>517</td>
<td>32</td>
<td>7.18</td>
<td>9.504</td>
</tr>
</tbody>
</table>

The effect of PEG 400 on the solubility of ranitidine was measured (>550 mg/mL) and was in agreement with literature values (FDA, 1998, Martindale, 2004). PEG 400 had no statistically significant effect (p<0.05) on the solubility of ranitidine, thus eliminating it as a reason for the observed differences in absorption in the presence of PEG 400. All solutions were neutral and there was no difference in the viscosity of the different drug solutions containing PEG 400 (Table 3.2).

The osmotic effects of PEG 400 is worthy of consideration. With higher concentrations of PEG 400, osmolality of ranitidine in 2.5, 5 and 10 g PEG 400 in water solutions were 49, 102 and 225 mOsm respectively (Schulze et al., 2003). The osmolality of the drug
solutions used in the current study are lower, they are shown in (Table 3.2). Basit et al (2001) and Schulze et al (2003) explained the reduction in bioavailability of ranitidine in the presence of these concentrations of PEG 400 as being possibly related to the reduction in the concentration gradient of ranitidine across the mucosa because of the diluting effect of the increased fluid load in the gut lumen in the presence of PEG 400. Adverse effects of various compounds in the presence of non-absorbable osmotic load in gut have previously been shown (Riley et al., 1992). Whilst considering the osmotic effects of PEG 400, the phenomenon of solvent drag from the intestinal lumen should also be considered. Administration of hypotonic formulations such as those in this study would mean that water absorption is initiated to equilibrate the osmolality within the gastrointestinal tract to isotonic conditions. The theory of solvent drag suggests that small hydrophilic molecules are dragged across the intestinal mucosa with water and hence lead to enhanced drug absorption (Noach et al., 1994). In the present study, all solutions were hypotonic but with increasing osmolality as PEG 400 concentrations increased. Increase in the osmolality of a solution would result in a decrease in hypotonicity, hence it would be expected that if the solvent drag phenomenon were applicable to explain our results, then the treatments with the lowest amount of PEG 400 would be expected to give the highest solvent drag and hence most enhanced absorption. However this was not the case as the absence of PEG 400 did not lead to highest bioavailability of ranitidine, also the lowest PEG 400 concentration did not cause the highest bioavailability. This phenomenon of solvent drag has mainly been shown in in-situ and in-vitro models and are not often reflected in human studies
especially with molecules with molecular weight greater than 200 Da (Lennernas, 1995).

3.7.2 The Effect of Polyethylene Glycol 400 on Ranitidine Bioavailability in Female Volunteers

Table 3.3 shows the cumulative excretion of unchanged ranitidine in the six female volunteers.

Table 3.3: Effect of different doses of PEG 400 on ranitidine bioavailability in six Female Volunteers

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Cumulative amount of ranitidine excreted in 24 hours (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>PEG 400</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>22</td>
</tr>
<tr>
<td>5</td>
<td>56</td>
</tr>
<tr>
<td>6</td>
<td>43</td>
</tr>
<tr>
<td>Mean (±SD)</td>
<td>38 (12)</td>
</tr>
<tr>
<td>% Diff</td>
<td>-24</td>
</tr>
</tbody>
</table>
The effects of PEG 400 on ranitidine bioavailability in female subjects show a clear contrast to that seen in male subjects. The mean bioavailability of ranitidine was reduced in the presence of PEG 400. The mean amount of ranitidine excreted on the control day was 38 ± 12 mg (25% of the administered dose) and is similar to that measured in males. The mean cumulative ranitidine excretion values with PEG 400 (0.5, 0.75, 1, 1.25 and 1.5 g) were 29, 35, 33, 33, and 33 mg respectively, corresponding to decreases in bioavailability of 24, 8, 13, 13 and 13% compared with the control. The decrease noticed at all concentrations was not significant (p>0.05).

The individual ranitidine excretion profiles for the six female volunteers are shown in Figure 3.5a-f. Figure 3.6 and Figure 3.7 show the excretion rate profiles of ranitidine and cumulative excretion over 24 hours respectively. The excretion of ranitidine in the presence of PEG 400 is lower compared to the absence of PEG 400. In the absence or presence of all concentrations of PEG 400, the mean peak excretion of ranitidine occurred between 2 and 4 hours. This was not consistent in the individual female profiles (Figure 3.5).
Figure 3.5: Rate of ranitidine excretion over 24 h in the individual female volunteers (A-F)
Figure 3.6: Mean ranitidine excretion over 24 h in the female volunteers

Figure 3.7: Cumulative mean ranitidine excretion in female volunteers
3.7.3 Possible Reasons for Differences Noted between Male and Female Volunteers

The results obtained clearly show a difference in the bioavailability of ranitidine in the presence of PEG 400 between males and females. With male volunteers, the bioavailability increased in the presence of all doses of PEG 400 doses whilst in female volunteers, there was a decrease in the bioavailability of ranitidine in the presence of all PEG 400 concentrations. This is presented in Figure 3.8

Figure 3.8: Percentage change in ranitidine excretion over control in male and female volunteers

A previous study involving Caucasian subjects showed that there were no differences between gender in the pharmacokinetics of ranitidine (AbadSantos et al., 1996). However, a study conducted by Flores Perez et al (2003) did show gender dependent
differences in the pharmacokinetics of ranitidine in Mexican subjects with a greater volume of distribution and absorption being determined in males compared to females and a lower clearance in males compared to females (Flores Perez et al., 2003). In our study, subjects were of different ethnic backgrounds including Caucasian, Indian, Chinese and African. There was no difference in the bioavailability of ranitidine when administered to male and female volunteers when the drug solution did not contain PEG. The mean excretion of ranitidine in male and female volunteers urine was 23% and 25% respectively. The value for males is similar to those reported in previous studies (Carey et al., 1981, van Hecken et al., 1982, Schulze et al., 2003). There are no previous studies focused in urinary excretion of ranitidine in females. Our results suggest that whilst there are no differences in the pharmacokinetics of ranitidine between gender, PEG 400 influences on the pharmacokinetics of ranitidine in a different way between gender.

The suggested reasons for the difference in bioavailability between gender in the presence of PEG 400 may be due the possibility that there are differences in the way PEG 400 influences:

1. Metabolism
2. Small intestinal transit
3. Tight junction modulation
4. Active transport of ranitidine - inhibition of transporters e.g. P-gp
3.7.4 The Effect of Polyethylene Glycol 400 on the Metabolism of Ranitidine in Male and Female Volunteers

The bioavailability of ranitidine is being assessed by measurement of excreted ranitidine. The amount of metabolites excreted were measured in order to determine the effect of PEG in metabolites of ranitidine. The major route of elimination of ranitidine is renal (70–80 % of total clearance) with the remainder due to hepatic and intestinal secretions (Roberts, 1984, Lin, 1991, Gramatte et al., 1994). Ranitidine is metabolized in the liver to N-oxide, desmethylranitidine and S-oxide metabolites by cytochrome P450, particularly CYP 2C19, CYP 1A2, CYP 2D6 and CYP 3A4/5 (Martinez et al., 1999, van Hecken et al., 1982). Cytochrome P450 enzymes are present in the gut wall enterocytes, as well as in the liver (Beaumont, 2004), and gender differences are noted with cytochrome P450 which are attributed to the regulation of their expression and activity, probably due to hormonal influences rather than inherent differences based on allele variations (Meibohm et al., 2002, Smith et al., 1998). CYP3A metabolism is higher in women than in men, but this may be due to the lower hepatic P-gp activity previously described. The effect of PEG 400 on the excretion of ranitidine and its metabolites is shown in Figure 3.9. In the absence of PEG 400 the urinary excretion of the three metabolites by the male volunteers was 7.7% of the administered dose (Figure 3.9a) and is in agreement with literature values (Carey et al., 1981). In females the metabolite excretion was 7.3%. The excretion of metabolites in both male and female volunteers follows a similar trend to the parent drug excretion. PEG 400 has been shown to inhibit CYP 3A metabolism (Johnson et al., 2002), hence it would be expected
that if PEG 400 had an effect on the metabolism of ranitidine then the trends seen with the parent drug and its metabolites would be different; for example if PEG 400 inhibits metabolism, then it would be expected that whilst ranitidine excretion peaks at the 0.75 g dose in males, the amount of metabolites excreted would be lowest at this PEG 400 dose. The excretion of metabolites indicated that the observed absorption effects of low dose PEG 400 in both male and female volunteers is unlikely to be due to a PEG 400 mediated inhibition of metabolism. The similar trend noted between males and females also suggest that the gender differences observed are not due to metabolism effects.
Figure 3.9: Mean percentages of (A) all metabolites, (B) ranitidine N-oxide, (C) desmethyl ranitidine, (D) ranitidine S-oxide excreted over 24 h in male and female volunteers
3.7.5 Influence of PEG 400 on Ranitidine’s Transport - Excretion of PEG 400 in the Urine of Volunteers

There are conflicting data on intestinal transit time differences between genders. A number of studies report that there is no difference in small intestinal transit times between gender (Bennink et al., 1999, Degen et al., 2005); however a number of studies have also reported differences between gender (Bennett et al., 2000, Sadik et al., 2003). In the studies where gender differences were observed, small intestinal transit was generally slower in female volunteers compared with the male subjects. Slower transit is likely to lead to greater bioavailability in women compared to men. However in a study investigating the effects of gender on the kinetics of ranitidine in healthy volunteers, ranitidine’s bioavailability was higher in men than women (Flores Perez et al., 2003). A study on the effect of the menstrual cycle on gastrointestinal transit showed that gastrointestinal transit time was significantly prolonged in the luteal phase when progesterone levels are higher than in the follicular phase of normally menstruating women (Wald et al., 1981). Slower transit in females should translate to a higher bioavailability of ranitidine in the control leg. Though not significant, it was indeed observed that the bioavailability of ranitidine in the female volunteers was slightly higher (25 %) than the male volunteers (23 %).

It is possible that there are differences in PEG absorption between gender – perhaps female subjects absorb less PEG, hence there is more PEG left in the lumen such that PEG 400 has greater transit effects in females compared to males even at the low doses studied. And as such, the lower doses of PEG 400 in this project in the female volunteers have similar effects to those noted with higher doses of PEG 400 previously in male volunteers (Basit et al., 2001, Schulze et al., 2003).
Research has already shown that significant decrease in transit times leads to a significant decrease in ranitidine's bioavailability (Basit et al., 2001, Schulze et al., 2003). The previous work by Schulze et al (2003) showed that small intestinal transit of the male volunteers decreased by 9% in the presence of 1 g PEG 400. Low doses may produce stimulating effects in females and hence be the reason for the decreased absorption of ranitidine in females.

There is very little published literature where the absorption of PEG 400 has been reported separately for males and females. It is therefore important to investigate if perhaps differences in PEG absorption might be responsible for the gender differences in bioavailability. The ideal method to investigate whether there are any differences in transit between gender in the presence of PEG 400 would be to use scintigraphy, however this was outside the scope of the project due to cost implications, the objectives set and time constraints of obtaining ethical approval for such work. Instead, the possible effects of PEG 400 on transit would be inferred from the amount of PEG 400 excreted and hence absorbed using the method developed and validated in chapter 2. The amount excreted would be assumed to be the amount absorbed and the remaining the amount of PEG 400 left in the gut lumen to cause shortening of transit time and hence reduction in drug absorption. The ability of compounds to cause a significant difference in transit in one sex over the other is seen for example with Tegaserod, a prokinetic compound which has been shown to accelerate small intestinal transit by 30% in healthy males compared to 37% in females (Degen et al., 2005).
To determine the absorption of PEG 400 by the volunteers, the amount of PEG 400 excreted by all volunteers after consumption of 0.75 g and 1.5 g PEG 400 with ranitidine was determined using mass spectrometry. The two doses of PEG 400 were chosen because the greatest and least change in ranitidine bioavailability in male volunteers were with these particular doses 63 % (0.75 g PEG 400) compared to 6 % (1.5 g PEG 400)

The PEG 400 excretion data, represented by the cumulative amount excreted in the urine over a 24 h period are shown in Table 3.4 and Table 3.5. The corresponding cumulative 24 h ranitidine excretion data were shown previously in Table 3.1 and Table 3.3.

<table>
<thead>
<tr>
<th>Table 3.4: PEG in urine samples gathered over 24 h from healthy male subjects following the ingestion of ranitidine with 0.75 and 1.5 g of PEG 400</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of Ingestion</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Male Subjects</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
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<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Average</td>
</tr>
<tr>
<td>SD</td>
</tr>
</tbody>
</table>
Table 3.5: PEG in urine samples gathered over 24 h from healthy female subjects following the ingestion of ranitidine with 0.75 and 1.5 g of PEG 400

<table>
<thead>
<tr>
<th>Amount of PEG</th>
<th>0.75 g</th>
<th>1.5 g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Excretion (mg)</td>
<td>% dose excreted</td>
</tr>
<tr>
<td>Female Subjects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>255</td>
<td>34</td>
</tr>
<tr>
<td>2</td>
<td>436</td>
<td>58</td>
</tr>
<tr>
<td>3</td>
<td>179</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>234</td>
<td>31</td>
</tr>
<tr>
<td>5</td>
<td>321</td>
<td>43</td>
</tr>
<tr>
<td>6</td>
<td>239</td>
<td>32</td>
</tr>
<tr>
<td>Average</td>
<td>277</td>
<td>37</td>
</tr>
<tr>
<td>SD</td>
<td>90</td>
<td>12</td>
</tr>
</tbody>
</table>

On average 34 and 35 % of the orally administered 0.75 and 1.5 g PEG 400 respectively was recovered in the urine of male volunteers (Table 3.4). In the female volunteers, the recovery was 37 and 42 % respectively (Table 3.5). Chadwick et al (1977) investigated the amount of PEG 400 excreted in urine after human volunteers took 1, 5 or 15 g PEG 400 and found that percentage absorption was independent of dose. The amount of PEG 400 excreted by the volunteers over 24 h in that study was approximately 50 %. Our study also supports the hypothesis that excretion is independent of dose (Chadwick et al. 1977). However in the current study unlike that of Chadwick et al, (1977)the percentage excretion of PEG 400 was lower. The results are however similar to another study which measured PEG 400 excretion after ingestion of 2 g PEG over 24 h (Parlesak et al., 1994). The remainder of the dose is unabsorbed by the GI tract, hence present in the intestinal lumen, where it might have an effect on transit.
The amount of PEG 400 excreted by the female volunteers is higher (though not significant; p>0.05) than the male subjects i.e. more PEG 400 is absorbed in the female volunteers. As a result one would expect less PEG 400 to remain in the lumen to possibly affect transit to a lesser extent which in turn means the bioavailability of ranitidine might be higher. However this was not the case and infact, bioavailability was lower with females, therefore the hypothesis/theory that differences in PEG absorption could be a reason for the gender differences noted can be discounted.

Figure 3.10 and Figure 3.11 represent a comparison between the amount of PEG 400 excreted and the corresponding bioavailability of ranitidine after administration of 0.75 g PEG 400 with ranitidine. The relationship between ranitidine and PEG 400 when ranitidine and 1.5 g PEG 400 was administered to the volunteers is shown in Figure 3.12 and Figure 3.13. A strong negative correlation is noted with the male volunteers in the presence of 0.75 g PEG 400 (correlation coefficient 0.94), but not female volunteers (correlation coefficient 0.0025). Unlike 0.75 g PEG 400, there was no relationship or correlation with ranitidine excretion in the presence of 1.5 g PEG 400 (R² = 0.0324 and R² = 0.0491) respectively.

With 0.75 g PEG 400, a trend to note is that male volunteers who excreted greater quantities of PEG 400 had lower ranitidine excretion and therefore absorption. In the female volunteers, there appears to be no correlation.
Figure 3.10: Correlation between urinary excretion of PEG 400 and absorption of ranitidine based on urinary excretion with orally administered 0.75 g PEG 400 in male volunteers

Figure 3.11: Correlation between urinary excretion of PEG 400 and absorption of ranitidine based on urinary excretion with orally administered 0.75 g PEG 400 in female volunteers
Figure 3.12: Correlation between urinary excretion of PEG 400 and absorption of ranitidine based on urinary excretion with orally administered 1.5 g PEG 400 in male volunteers.

Figure 3.13: Correlation between urinary excretion of PEG 400 and absorption of ranitidine based on urinary excretion with orally administered 1.5 g PEG 400 in female volunteers.
The trend observed with the male volunteers is opposite to that noted by (Basit, 1999) where there was a slight positive correlation between ranitidine and PEG excretion by male volunteers when 10 g PEG 400 was administered with ranitidine. The volunteers who excreted greater quantities of PEG 400 also had a higher ranitidine bioavailability. In the work by Basit and co-workers, ranitidine absorption was reduced by the presence of the 10 g PEG 400 (Basit et al. 2002). In the male volunteers of this study, there was a negative correlation; the volunteers with greater excretion of PEG 400 had lower ranitidine bioavailability (Figure 3.10). Although there were no differences between gender in the excretion of PEG 400, investigating the relationship between the excretion of the two compounds reveals interesting findings. These interesting findings are only noted with 0.75 g. There was no correlation between ranitidine absorption and PEG 400 excretion in both male and female volunteers when 1.5 g PEG 400 was administered (Figure 3.12 and 3.13); once again highlighting the importance of the 0.75 g PEG 400 dose. Since PEG 400 is a very commonly used excipient perhaps it is possible for low doses to be included in formulations of drugs similar to ranitidine which are highly soluble but poorly permeable. Excipients are now being exploited for purposes other than their physiochemical properties. For example, it has been shown that xylitol a commonly used sweetener, inhibits the growth of certain bacteria and helps to prevent otitis media in a significant percentage of children. Hence the combination of xylitol and an appropriate antibiotic in a liquid formulation may provide a synergistic effect against otitis media. Xylitol a commonly used as in pharmaceutical formulations especially chewable and syrups (Kontiokari et al., 1998, Uhari et al., 1998).

3.7.6 Influence of PEG 400 on Ranitidine Permeability

Ranitidine and other H₂ receptor antagonists have been reported to inhibit their own transport across epithelial lining and their poor permeability and limited bioavailability
in man has been attributed to a tightening of the paracellular pathway by modulating interactions among tight junctional proteins (Gan et al., 1998). Perhaps the absorption enhancing effect of PEG 400 in males and absorption inhibition effect in females is due to PEG 400 affecting the interaction between ranitidine and the tight junction proteins and thereby increasing or decreasing both tight junction and paracellular permeability. Contrary to this however, (Rege et al., 2001)) showed that PEG 400 does not alter the integrity of tight junction or the transport of ranitidine.

The influence of low dose PEG 400 on the active transport of ranitidine is considered. It was previously reported that ranitidine is primarily transported by the paracellular route (Collett et al., 1996, Gan et al., 1993). However, recent studies have suggested paracellular transport via tight junctions account for sixty percent of this movement whilst transcellular processes (via absorption transporters such as human organic cation transporter 1 (h-OCT1) account for the other forty percent (Bourdet et al., 2006). OCT transporters (OCT1, OCT2 and OCT3) mediate the entry of organic cations into cells (Koepsell et al., 2007). h-OCT1 is predominantly expressed in the liver, while h-OCT2 is expressed mainly in the kidney (Gorboulev et al., 1997, Zhang et al., 1997). OCTs have been shown to interact with many drugs including ranitidine and other BCS class III compounds: cimetidine, famotidine and metformin (Bourdet et al., 2006, Bourdet et al., 2005, Bourdet and Thakker, 2006, Wang et al., 2002, Thiel-Demby et al., 2009, Choi and Song, 2008).

OCT 1, 2 and 3 play important roles in the biliary and renal excretion of their substrates. Gender differences between these organic cation transporters were
previously only examined in animal models and these suggested that there is greater expression of some members of the OCT family in males compared to females (Groves et al., 2006, Urakami et al., 1999, Urakami et al., 2000). An example of the effect of such difference in expression of OCT transporters is reported with cisplatin, where male rats showed greater renal clearance compared to female rats. More recently, it has been suggested that OCTN2 mRNA levels are comparable between male and female patients (Wojtal et al., 2008).

OCT 1 is present in the epithelial membrane of the intestine whilst OCT2 is primarily expressed in the basolateral membrane of the kidney proximal tubules and OCT3 has a widespread tissue distribution, present in the brain, heart, skeletal muscle, blood vessels, placenta, and liver. More than 30% of clinically available drugs are organic cations, hence it is important to consider the effect of OCT. For example cimetidine, a substrate of OCT1 and OCT2 significantly increased the plasma concentration of metformin and reduced its renal clearance (Wang et al., 2003). The drug interaction between cimetidine and metformin for example has been shown to be caused mainly by OCT transport. Whilst cimetidine has such effects on metformin, metformin does not have an effect on cimetidine disposition, suggesting that the drug interactions between substrates for OCTs can be different. The effects of excipients such as polyethylene glycol 400 on OCT transporters are yet to be investigated. PEG 400 may have some as yet unestablished effect on OCT transporters and subsequently ranitidine absorption.

In addition to drug influx or absorption, carrier mediated efflux can be influential in bioavailability, and the efflux carrier P-glycoprotein (P-gp) has been implicated in intestinal ranitidine transport (Bourdet and Thakker, 2006, Collett et al., 1999). Other
intestinal efflux transporters include multidrug resistance-associated protein 1 and 2 (MRP1, MRP 2) and the more recently discovered breast cancer resistance protein (BCRP). The expression of these efflux transporters along the human intestine have been investigated and showed that the expression of mRNA for P-gp, and MRP2 was highest in the jejunum decreasing towards the ileum and colon, whilst MRP1 was equally distributed in all intestinal regions (Berggren et al., 2007). These transporters expel absorbed drug back into the lumen of the intestine and many drugs are substrates of these transporters; consequently the bioavailability and pharmacokinetics of these drugs are controlled by the expression of these carriers. Perhaps significantly, cimetidine (of the same pharmacological class as ranitidine) has been identified as a both a P-gp and BCRP substrate (Collett et al., 1999, Pavek et al., 2005) and there has emerged extensive evidence that excipients can inhibit these efflux transporters. PEG 300 (a lower molecular weight analogue of PEG 400) was found to inhibit P-glycoprotein in Caco-2 and MDR1-MDCK cell monolayers at very high concentrations (20%) (Hugger et al., 2002b). The impact of PEG 400 on P-glycoprotein efflux has also been investigated in excised rat intestine (Johnson et al., 2002) and the authors reported a dose-dependent inhibition of P-gp. Inhibition of these efflux carriers could theoretically allow an increase in bioavailability of drugs which are substrates for the efflux transporters. For example ranitidine is a substrate for P-gp (Collett et al., 1999, Thiel-Demby et al., 2009). Recent studies have also shown that other non-PEG excipients (Cremophor EL, Tween 20 Span 20, Pluronic P85 and Brij 30) inhibit both P-gp and BCRP and subsequently enhanced the bioavailability of a BCRP substrate (Yamagata et al., 2007b) whilst other excipients such as PEG 300, PEG 400, vitamin E TPGS and Tween 80 have only been studied with P-gp and shown to only inhibit P-gp.
The effects of PEG 400 on BCRP have yet to be studied. However, a recent study showed that PEG 2000 sterate inhibits the effects of BCRP (Zhang et al., 2008). The enhanced bioavailability noted with the low dose PEG 400 in males may be due to this inhibition of efflux transporters, an effect which is negated at high doses due to the previously described transit effects of high dose PEG 400 (Basit et al., 2001, Schulze et al., 2003). Similarly low dose of another excipient labrasol (0.1%) compared to higher doses has been shown to inhibit the function of P-gp in the intestine, hence enhancing the intestinal absorption and bioavailability of P-gp substrates (Lin et al., 2007).

The influence of PEG 400 on the active transport of ranitidine and cimetidine require investigation and will be discussed further in the subsequent chapter.

Interestingly, in light of the gender differences observed here, the expression of efflux transporters is different in males and females. There is greater expression of intestinal BCRP protein in females compared to males (Zamber et al., 2003). This contrasts to hepatic BCRP and P-gp where expression is higher in males than females (Merino et al., 2005, Schuetz et al., 1995). Alonso et al 2007 (Alonso et al., 2007) showed that healthy women had significantly increased intestinal epithelial permeability compared to men in response to incoming stimuli in the jejunum.

While it is in fact not, unusual to have gender differences with drugs, we show for the first time a gender difference when an excipient is added to a drug which does not normally exhibit gender differences. Prior to 1988, women of child bearing age were
rarely included in clinical trials, however with recent guidelines from the regulatory bodies including the FDA, it is now mandatory for women to be included in clinical trials. Both pharmacokinetic and pharmacodynamic differences have been reported between males and females and these have been reviewed by numerous authors (Chen, 2005, Meibohm et al., 2002, Schwartz, 2003b, Franconi et al., 2007). These gender related differences, some of which have been discussed, are often associated with molecular factors (efflux drug transporters especially P-glycoprotein and BCRP, drug metabolizing enzymes especially cytochrome P450s – CYP3A), hormonal factors (menstrual cycle and interactions with oral contraceptives and hormone replacement therapy) and/or physiological factors (body weight, gastrointestinal physiology including gastric acid secretions, gastric emptying and gastrointestinal transit) (Meibohm et al., 2002). More recent literature also suggests that extrinsic factors such as dosing regimen and formulation may impart gender related differences (Chen, 2005). Although a significant gender difference was not observed in the absorption of our parent drug (ranitidine) when administered on its own (0 g PEG 400), many drugs including verapamil, several beta-blockers such as labetalol, metoprolol and selective serotonin reuptake inhibitors have been shown to exhibit gender differences either in their metabolism or bioavailability (Krecic-Shepard et al., 2000, Schuetz et al., 1995, Luzier et al., 1999, Walle et al., 1994, Gilmore et al., 1992, Preskorn, 1997)
3.8 Summary

The results obtained in this study show that PEG 400 even at low concentrations has a dramatic effect on the bioavailability of ranitidine. The absorption enhancing effects observed with PEG 400 in this study in male volunteers are unlikely to be due to its effect on the drug formulation (solubility, osmolality, viscosity) or metabolism. Nor is it due to differences in the absorption of PEG 400 between genders. It may however be related to the effect of PEG 400 on ranitidine permeability via its effects on the epithelial membrane e.g. paracellular or transcellular pathways or efflux transporters. Gender differences may also be attributed to differences in the effects of PEG 400 on the small intestinal transit in males and females. However definitive scintigraphic studies with low dose PEG 400 are needed to elucidate the underlying mechanisms for the intriguing findings. The question remains as to whether this absorption-enhancing effect of PEG 400 in male volunteers at such low doses is specific to ranitidine alone, other H₂ receptor antagonist or even perhaps more generic in nature and applicable to other BCS class III compounds.
3.9 SECTION 2: THE EFFECTS OF PEG 400 ON THE BIOAVAILABILITY OF CIMETIDINE

3.10 Introduction

Cimetidine, like ranitidine is also a BCS Class III drug which is absorbed in the small intestine. Unlike the doses used in the ranitidine study (0 – 1.5 g), the doses to be used in the cimetidine study are 0, 0.5, 0.75, 1.5 and 5 g. The 5 g dose was chosen to allow comparison with the results obtained for ranitidine by Schulze et al 2003 where 5 g PEG 400 was shown to decrease the bioavailability of ranitidine following a decrease in small intestinal transit time.

3.11 Materials and Methods

3.11.1 Preparation of Drug/PEG 400 Solutions

The dosage forms comprised oral solutions consisting of 150 mL of water containing 150 mg cimetidine (GlaxoSmithKline, Harlow, UK) and either 0 (control), 0.5, 0.75, 1.0, 1.5 or 5 g PEG 400 (Sigma Aldrich Company, Poole, UK). The saturated solubility and osmolality of cimetidine in the different PEG 400 solutions was measured at 37°C. The solubility and osmolality of cimetidine in the different solutions were: 0 g PEG (8.0 mg/ml, 12 mOsm kg\(^{-1}\)); 0.5 g (8.23 mg/ml, 19 mOsm kg\(^{-1}\)); 0.75 g (7.60 mg/ml, 25 mOsm kg\(^{-1}\)); 1.0 g (8.2 mg/ml, 29 mOsm kg\(^{-1}\)); 1.5 g (8.52 mg/ml, 39 mOsm kg\(^{-1}\)); 5 g (8.53 mg/ml, 101 mOsm kg\(^{-1}\)).
3.12 Study Protocol

Twelve volunteers (six males and six females) - age range: Males [24-40 years (median 26 years)], Females [23-27 years (median 24 years)]; weight range: Males [55-90 kg (median 62 kg)], Females [50-76 kg (median 60 kg)]; height range: Males [1.66-1.84 m (median 1.73 m)], Females [1.58-1.7 m (median 1.69 m)] participated in a random six-way cross-over study after giving informed written consent. All subjects were non-smokers and declared themselves healthy with no history of gastrointestinal disease. The experimental protocol was approved by The Joint UCL/UCLH Committees on the Ethics of Human Research. The study was conducted in accordance to the Helsinki guidelines for ethics in research (1965) and its subsequent revisions - Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West (SA) (1996), Edinburgh (2000).

The volunteers reported to the study centre after an overnight fast and each received, on six separate occasions, 150 mL of cimetidine solution containing 0, 0.5, 0.75, 1, 1.5 or 5 g PEG 400. A standardised lunch consisting of two piece cheese or egg sandwich, a packet of crisps and a juice drink was provided 4 h post dose, and water was available ad libitum from this point onwards.

Cumulative urine samples were collected throughout the course of each study day. This involved the collection and measurement of bladder output over the following time periods: 0 (pre-dose), 0 to 2, 2 to 4, 4 to 6, 6 to 12, and 12 to 24 h. For each time point, a 20 mL aliquot was retained and stored at -20°C.
3.12.1 Urine Analysis

Urine samples were assayed for the amount of cimetidine. Frozen aliquots from volunteers were thawed out at room temperature and 0.65 ml of each sample was mixed with 0.65 ml mixture of 20:80 acetonitrile:water in duplicate. After thorough vortex-mixing, a 10 μl aliquot of each solution was injected onto a Luna SCX (Phenomenex, UK) HPLC column using a validated HPLC-UV method (chapter 2). Briefly, the mobile phase was 20:80 acetonitrile: 0.1M sodium acetate with a flow rate of 2 ml/min. Calibration standards were prepared with blank human urine, also diluted (50%) with 20:80 acetonitrile:water.

3.13 Statistical Analysis

The results obtained for the cumulative excretion of cimetidine in urine for males and females were subjected to two-way ANOVA using General Linear Model in SPSS to assess differences between gender. This was followed by one-way ANOVA to assess the effects of the difference concentrations of PEG 400 on the bioavailability of cimetidine in males and females separately, and then a post hoc Tukey’s test. Statistical analysis is stated where p < 0.05.

3.14 Results and Discussion

The bioavailability of cimetidine was assessed by the cumulative amounts of unchanged cimetidine excreted in urine over 24 h. The amount of cimetidine excreted by six male volunteers is shown in Table 3.6.
The mean cumulative amount of cimetidine excreted unchanged in the absence of PEG 400 (control) in male volunteers was 72 ± 13mg, corresponding to 48 % (w/w) of the administered dose (Table 3.6). The mean cumulative quantity of cimetidine excretion in the presence of 0.5, 0.75, 1, 1.5 and 5 g PEG 400 in the male volunteers were 94, 111, 102, 62 and 52 mg respectively. These correspond to changes in bioavailability of +29, +53, +41, -16, -29 % compared with the control.

High dose of PEG 400 (5 g) reduced the bioavailability of cimetidine in male volunteers by 29%.

Table 3.6: Cumulative amount of cimetidine excreted by male volunteers in 24 h

| Cumulative amount of cimetidine excreted in 24 h (mg) |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
|                                | 0.5 g          | 0.75 g         | 1.0 g          | 1.5 g          | 5 g            |
| Volunteer                      | Control        | PEG 400        | PEG 400        | PEG 400        | PEG 400        |
| 1                              | 71             | 98             | 111            | 119            | 76             | 50             |
| 2                              | 61             | 124            | 142            | 80             | 54             | 67             |
| 3                              | 73             | 67             | 93             | 100            | 56             | 46             |
| 4                              | 79             | 88             | 90             | 111            | 67             | 67             |
| 5                              | 93             | 96             | 120            | 129            | 62             | 41             |
| 6                              | 57             | 88             | 108            | 75             | 54             | 40             |
| Mean ± SD                      | 72 (13)        | 94 (24)        | 111 (24)       | 102 (21)       | 62 (6)         | 52 (14)        |
| % Diff                         | +29            | +53            | +41            | -16            | -29            |

The trend of the results obtained with the 5 g dose is similar to the study by Schulze et al (2003), where 5 g PEG 400 reduced the bioavailability of ranitidine by 38%.
following a 23% reduction in small intestinal transit. The reduction in bioavailability of
cimeitidine in this study is therefore also likely to be due to such high dose accelerating
transit of the drug through the small intestine. On ingestion of PEG 400, most of the
dose remains unabsorbed in the lumen of the gastrointestinal tract and is excreted
unchanged in feces. At the osmotically active dose, PEG 400 will retain water in the
lumen of the gut to attain iso-osmotic conditions. The retention of water will in turn
lead to an increase in luminal fluid volume which then leads to a stimulation of gut
motility and the subsequent acceleration of drug passage through the small intestine.
Similarly, in another study involving cimetidine, by Adkin et al, 5 g mannitol reduced
the bioavailability of cimetidine by 29 % following a 20 % reduction of small intestinal
transit time.

The rate of cimetidine excretion over 24 h is represented in Figure 3.14. The mean
cumulative excretion of cimetidine over the 24 h collection period is shown in Figure
3.15. The figures show that the peak excretion time for cimetidine in the absence of
PEG 400 (control) and presence of 0.5, 1, 1.5 and 5 g PEG 400 in male volunteers was
2-4 h whilst for 0.75 g PEG 400 it was 0-2 h. The time to peak excretion in the presence
of 0, 0.5, 0.75 and 1.5 g PEG 400 is similar to that obtained with ranitidine.
Figure 3.14: Mean cimetidine excretion rate in male volunteers over different collection intervals

Figure 3.15: Mean cumulative urinary excretion of unchanged cimetidine - male volunteers
Table 3.7 shows the cumulative excretion of ranitidine in the six female volunteers. The mean amount of cimetidine excreted on the control day was $70 \pm 18$ mg (47% of the administered dose) and is similar to that measured in males. The cimetidine excretion values (mean) with PEG 400 (0.5, 0.75, 1, 1.5 and 5 g) were 64, 66, 70, 57, and 53 mg respectively, corresponding to changes in bioavailability of +9, +7, 0 -18 and -24% compared with the control (Figure 3.16). The changes in bioavailability were not significant at any of the doses of PEG 400 ($p > 0.05$).

**Table 3.7: Cumulative amount of cimetidine excreted by female volunteers in 24 hours**

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Control</th>
<th>0.5 g PEG 400</th>
<th>0.75 g PEG 400</th>
<th>1.0 g PEG 400</th>
<th>1.5 g PEG 400</th>
<th>5 g PEG 400</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>60</td>
<td>70</td>
<td>50</td>
<td>50</td>
<td>43</td>
</tr>
<tr>
<td>2</td>
<td>67</td>
<td>67</td>
<td>89</td>
<td>74</td>
<td>95</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>98</td>
<td>98</td>
<td>63</td>
<td>85</td>
<td>84</td>
<td>108</td>
</tr>
<tr>
<td>4</td>
<td>67</td>
<td>67</td>
<td>64</td>
<td>59</td>
<td>62</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>66</td>
<td>66</td>
<td>60</td>
<td>70</td>
<td>75</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>64</td>
<td>64</td>
<td>39</td>
<td>55</td>
<td>56</td>
<td>19</td>
</tr>
</tbody>
</table>

**Mean (±SD)**

<table>
<thead>
<tr>
<th>0.5 g PEG 400</th>
<th>0.75 g PEG 400</th>
<th>1.0 g PEG 400</th>
<th>1.5 g PEG 400</th>
<th>5 g PEG 400</th>
</tr>
</thead>
<tbody>
<tr>
<td>70 (14)</td>
<td>66 (13)</td>
<td>70 (18)</td>
<td>57 (32)</td>
<td>53 (14)</td>
</tr>
</tbody>
</table>

**% Diff**

| -9 | -7 | 0 | -19 | -24 |
Figure 3.16: Change in bioavailability of cimetidine when administered with polyethylene glycol 400

The rate of cimetidine excretion over 24 h is represented Figure 3.17 in and the cumulative excretion of cimetidine in the female volunteers is shown in Figure 3.18, respectively. These show that that the time to peak excretion in the presence of all PEG 400 concentrations was at the 2-4 h interval whilst for the control it was 0-2 h.
Figure 3.17: Mean cimetidine excretion rate in female volunteers over different collection intervals

Figure 3.18: Mean cumulative urinary excretion of unchanged cimetidine – female volunteers
In this study, the amount of cimetidine excreted unchanged by male and female volunteers were 48 and 47% respectively. This is comparable to other studies Albin et al., (1986) where the amount of cimetidine excreted unchanged in the urine of twelve volunteers (10 males, 2 females) was 46 ± 6%. In another study by Berardi et al. (1988), the amount of cimetidine excreted unchanged in male volunteers was 48%.

In male subjects, significant enhancement of bioavailability was seen at each of the low doses of PEG below 1.5 g. In female subjects, there was no change in the bioavailability of cimetidine in the presence of PEG 400. This is similar to the previous study with ranitidine. Cimetidine and ranitidine exhibit different physicochemical properties but their absorption profiles are similar (Table 3.8). Both are absorbed mainly in the small intestine. Although the trends noted is similar to ranitidine, it is worth noting that the increase in cimetidine bioavailability in the presence of 0.75 g PEG is less than that noted in ranitidine (53% compared to 63%). This can be explained by the fact that the oral bioavailability of cimetidine is higher than ranitidine (Table 3.8) hence there is less scope for increase in bioavailability in the presence of excipients such as PEG 400.

The observed trends with cimetidine are similar in a few ways with that seen previously with ranitidine.

- There was no difference in absorption between gender in the absence of PEG 400 (p > 0.05).
- The same general trend of enhanced absorption in the presence of PEG 400 lower than 1.5 g with males, however no changes in bioavailability in female volunteers.
• In male volunteers, time to peak excretion in the presence of 0.5, 1, 1.25 and 1.5 g was between 2-4 h. Whilst time to peak excretion in the presence of 0.75 g PEG 400 was 0-2 h with both compounds.

• In the female volunteers, the time to peak excretion for ranitidine in the presence of all concentrations of PEG 400 except 1.25 g was 2-4 h (4-6 h for 1.25 g). With cimetidine, time to peak excretion for all concentrations of PEG 400 was 2-4 h whilst in the control experiment it was 0-2 h.

However on close inspection of the data, there are some differences that should be noted:

• In male volunteers, whilst there was a 63% increase in the bioavailability of ranitidine in the presence of 0.75 g, the increase in absorption with cimetidine was 53%.

• Unlike ranitidine, in the male volunteers, enhanced bioavailability for cimetidine was only seen up to 1 g PEG after which there was decreased absorption.

• For both ranitidine and cimetidine, the optimum PEG 400 dose to promote bioavailability was 0.75g (63 and 53% significant enhancement respectively)

Unlike ranitidine, cimetidine has been identified as both a P-gp and BCRP substrate (Collett et al., 1999, Pavek et al., 2005). The reason for the difference in the percentage change may be attributed to differences in the transporters involved in the transport of the drugs. Perhaps PEG 400 competes to inhibit both P-gp and BCRP in cimetidine and is hence less effective at inhibiting P-gp, the main intestinal efflux transporter. Possibly causing the smaller increase in absorption noted with cimetidine. This will be investigated in chapter 4.
Table 3.8: Pharmacokinetic parameters of cimetidine and ranitidine

<table>
<thead>
<tr>
<th>Drug</th>
<th>Absorption (% oral bioavailability)</th>
<th>Protein binding</th>
<th>Time to peak concentration after oral dose (hr)</th>
<th>Time to peak effect (hr)</th>
<th>Elimination (% excreted unchanged with oral dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cimetidine</td>
<td>Rapid (60–70)</td>
<td>Low (15–20%)</td>
<td>0.75-1.5</td>
<td>Oral: 1-2</td>
<td>48%</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>Rapid (50–60)</td>
<td>Low (15%)</td>
<td>2–3</td>
<td>Oral: 1–3</td>
<td>30%</td>
</tr>
</tbody>
</table>

(Berardi et al., 1988)

It is significant that gender differences have been reported in the expression of efflux transporters, and may help account for the differences in the effect on bioavailability between males and females. There is greater expression of BCRP protein in females compared to males in the intestine (Zamber et al., 2003) but not in the liver (Merino et al., 2005, Schuetz et al., 1995). It is known that hepatic P-gp is higher in males than in females, but the relative levels in the intestine are not known.

Although the definitive mechanism for the enhancement of bioavailability of ranitidine and cimetidine in male subjects has not yet been elucidated, or the reasons for the gender differences identified, this study serves to confirm that the effect of low dose PEG 400 is not restricted to one drug. The results show that PEG 400 even at low concentrations should not be considered to be an ‘inactive’ excipient. The absorption enhancing effects observed with PEG 400 are suggested to be due to PEG 400’s effects on drug permeability through the gastrointestinal tract via its effects on transporters. This will investigated in the subsequent chapter.
3.15 Summary

The bioavailability of cimetidine alone shows no gender differences but the incorporation of low dose PEG 400 increases cimetidine bioavailability in males, but not in females. This confirms that the PEG 400 is not an "inactive ingredient". The enhancement of bioavailability in the male volunteers was noted with PEG 400 doses below 1.5 g; with a significant increase compared with control (p<0.05). The reasons for the differences between sexes are still unclear, but likely to be an interplay between gastrointestinal transit and efflux transporters.

3.16 Conclusions

The effect of PEG 400 on two BCS class III drugs (ranitidine and cimetidine) was investigated. The results revealed that 0.5, 0.75, 1.25 and 1.5 g PEG 400 enhanced the bioavailability of ranitidine by 34, 63, 49, 43 and 6% over the control treatment, whilst in the female volunteers; there were no changes in bioavailability compared to the control. With cimetidine, a similar trend to that with ranitidine was noted with an enhancement in bioavailability in the male volunteers with PEG 400 doses below 1.5 g and no changes in bioavailability in the female volunteers. However the enhancement in bioavailability in cimetidine was lower than with ranitidine. The differences between gender in the presence of PEG 400 was shown to be unlikely to be due to the actual drug formulation (osmolality, solubility, viscosity), influence of PEG 400 on metabolism, or differences in the absorption of PEG 400 by the male and female volunteers. It was suggested that perhaps the differences may be due to PEG 400's effects on the transport of the drugs. This would be investigated in the following chapter.
CHAPTER 4: 
Use of Animal and Cell Culture Models to Elucidate the Mechanisms Involved in Bioavailability Changes of Ranitidine in the Presence of PEG 400
SECTION 1: THE EFFECTS OF POLYETHYLENE GLYCOL 400 ON DRUG ABSORPTION IN RATS

Given the interesting findings in the human study with PEG 400 doses below 1.5 g enhancing the bioavailability of ranitidine and cimetidine in male subjects but not females, further investigations into the phenomenon will be carried out using animal and cell culture models.

4.1 Introduction

Before a new drug or drug formulation can be used in clinical trials, animal studies are generally used as a predictive indicator of how the drug or formulation will react in humans as well as to gain toxicological information on the drug. There is no single animal model which can replace human studies due to the anatomical, physiological and biochemical variations between human and animals. However rodents (rats, mice, guinea pigs and rabbits) are often used in these pre-clinical studies because of their small size, low cost and due to similar basic gastrointestinal structure to humans. Rats are particularly used more often compared to guinea pigs and rabbits in preference to mice due to being larger and having lower costs than guinea pigs and rabbits. Rodents are also more suitable for determining the mechanism of drug absorption and bioavailability values from powder or solution formulations, whilst larger animals such as dogs, pigs and monkeys are more useful for assessing absorption from drug formulations.
3.16.1 Structure and anatomy of the gastrointestinal tract of humans and rats

The rat was chosen as the animal model to investigate the pharmacokinetic changes with ranitidine in the presence of PEG 400 due to the similarities between the rat’s gastrointestinal tract and that of the human GI tract in that it consists of the oesophagus, stomach, small intestine and large intestine. The main difference between the rat and human GI tracts is the caecum, which is large and well defined in rats however the human caecum is small and continuous with the colon. The rat small intestine is similar to human as it consists of three regions – duodenum, jejunum and ileum and acts as the major absorptive site for ingested materials, including drugs.

The human GI tract was reviewed in Chapter 1. Although animal models, including the rat model, share basic structural similarities with human, variations arising from adaptation, nature of food, frequency of food intake, need for food storage, body size and shape mean that animal models cannot be used as a replacement for human studies (Kararli, 1995). However, having an understanding of the differences between the animal and human models will enable better prediction of drug behaviour in humans. These section focuses on comparing the human and rat GI tract as the rat was chosen as the model in this work to determine if the same trends observed in man would be observed in the rats. A comprehensive review comparing the human and laboratory animal physiology, anatomy and biochemistry was carried out by Kararli (1995).

The stomach in both rat and humans functions mainly as the reservoir for ingested materials. Whilst the human stomach is mainly glandular, the rat stomach has both
glandular and non-glandular sections (Figure 4.1). The glandular stomach is thick-walled compared with the non-glandular stomach which is thin-walled and transparent (Kararli, 1995). The non-glandular section of the stomach is used for storage and digestion for food. The glandular sections contain the gastric glands which contain mucus and pepsinogen cells, hydrochloric acid and secreting parietal cells (Kararli, 1995)

![Diagram of human and rat stomach](image)

**Figure 4.1: Diagram of human and rat stomach (not shown to scale) (Kararli, 1995)**

The small intestine is also similar in both rats and humans as they both consist of three regions, duodenum, jejunum and ileum. In both rats and humans, the small intestine is the major site of absorption for ingested materials including drugs. The surface of both rat and human small intestine is covered with villi and microvilli which increase the surface area for nutrient and drug absorption. Differences in absorption between species are caused by differences in the intestinal absorptive surface area and or cell density between species (Jezyk et al., 1992). A comparison of the lengths and diameters of the small intestine of human and rats are shown in Table 4.1.
Table 4.1: Post mortem length and diameter of parts of the human and rat intestinal tract

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (mm)</td>
<td>Diameter (mm)</td>
</tr>
<tr>
<td>Stomach</td>
<td>200</td>
<td>150</td>
</tr>
<tr>
<td>Duodenum</td>
<td>250</td>
<td>50</td>
</tr>
<tr>
<td>Jejunum</td>
<td>3000</td>
<td>50</td>
</tr>
<tr>
<td>Ileum</td>
<td>3000</td>
<td>50</td>
</tr>
<tr>
<td>Cecum</td>
<td>100-300</td>
<td>7</td>
</tr>
<tr>
<td>Colon</td>
<td>1500</td>
<td>5</td>
</tr>
<tr>
<td>Rectum</td>
<td>150-190</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The large intestine consists of the caecum, colon, rectum and anal canal. The large intestine is similar between human and rat except that the caecum is very large in rats compared to humans, due to rats being herbivores and the caecum which contains microorganisms functions as a site for cellulose digestion. The absorption of water and Na\(^+\) and other minerals takes place in the colon. The largest population of microorganisms is in the colon and the colon is the main site of production and absorption of volatile fatty acids (Sousa et al., 2008).
3.16.2 Physiology of the human and rat gastrointestinal (GI) tract

The pH of the fluids in the GI tract is important in the dissolution, solubilisation and absorption of ionisable drugs. The pH of the contents of different sections of the gastrointestinal tract of humans and rats is shown in Table 4.2. Similar to humans, the lowest pH in rats is also noted in the stomach. The pH of the small intestine is lower in rats compared to humans. This might have implications in vivo testing of drugs in the animals, for example the dissolution of drugs which require a basic pH to dissolve as they may precipitate at the lower pH values in the rat small intestine (McConnell et al., 2008a).

Table 4.2: pH values of human (Gruber et al, 1987; Kararli 1995) and rat GI fluids (McConnell et al 2008) along the GI tract

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>pH; mean (s.d)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fed</td>
<td>1.5-2.5</td>
<td>3.20 (1.0)</td>
</tr>
<tr>
<td>Fasted</td>
<td></td>
<td>3.90 (1.0)</td>
</tr>
<tr>
<td>Stomach</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>5.00 (0.3)</td>
<td>5.89 (0.3)</td>
</tr>
<tr>
<td>Jejunum</td>
<td>6.6</td>
<td>5.10 (0.3)</td>
</tr>
<tr>
<td>Ileum</td>
<td>7.5</td>
<td>5.94 (0.4)</td>
</tr>
<tr>
<td>Caecum</td>
<td>6.1</td>
<td>5.90 (0.4)</td>
</tr>
<tr>
<td>Proximal Colon</td>
<td>6.4</td>
<td>5.51 (0.5)</td>
</tr>
<tr>
<td>Distal colon</td>
<td>7.0</td>
<td>5.77 (0.5)</td>
</tr>
<tr>
<td><strong>Rat</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fed</td>
<td>3.20 (1.0)</td>
<td>3.90 (1.0)</td>
</tr>
<tr>
<td>Fasted</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.89 (0.3)</td>
<td>5.13 (0.3)</td>
</tr>
</tbody>
</table>

Following dissolution and solubilisation, drug permeation through the epithelial surface is essential for a drug to be absorbed. However before permeation can take place at the
epithelial surface, drug molecules encounter several barriers: the mucus layer, unstirred water layer and cellular mechanisms (influx and efflux transporters, metabolic enzymes).

The mucus layer covers the epithelial layer of the GI tract. It is made up of mucin, a water-insoluble, viscous gel. The thickness of the mucus layer varies between species. The thickness and turnover rate of the mucus layer varies along the length of the GI tract plays an important role in the mucosal adhesion of dosage formulations. It can hinder drug diffusion and mucosal adhesion of drug formulations can result in longer GI transit times. The thickness of mucin in the stomach varies among species, the mean (s.d) mucus thickness of the stomach in the rat and human (biopsy) were measured as 184 ± 50 and 144 ± 50 μm respectively (Jordan et al. 1998).

The role of efflux transporters such as P-glycoprotein (P-gp) which expel drug molecules back into the lumen after cellular uptake has been discussed previously. P-gps are encoded by genes called the multiple drug resistance (mdr) genes. Group 1 mdr genes mediate drug transport. Humans only have a single group I gene (called MDR1) whilst rodents such as rats have two group I genes (mdr1a and 1b). Human mdr genes are proteins which are capable of drug transport, whilst rodent P-gps are associated with chemotherapeutic drug resistance and intestinal excretion. In rats, P-gp are also present in the blood-brain barrier, hence they may have additional function to protect cells from naturally occurring toxins (Brady et al., 2002). The trend is also similar for BCRP (Gutmann et al., 2005, MacLean et al., 2008). Unlike P-gp and BCRP, MRP2 expression decreases along the intestine (MacLean et al., 2008).
3.16.3 Gastrointestinal (GI) motility and transit in human and rats

Although absorption from the stomach is limited and not considered significant, gastric emptying is important and influences the uptake of food and drug molecules into the small intestine (Kararli, 1995). The absorption of many drugs is dependent on the small intestinal transit time. The transit time for dosage forms through human and rat small intestine is shown in Table 4.3

**Table 4.3: Transit times (h) in the intestine**

<table>
<thead>
<tr>
<th>Transit times</th>
<th>Human</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small intestine</td>
<td>3 – 4(^1,3)</td>
<td>1.5(^5)</td>
</tr>
<tr>
<td>Colon</td>
<td>6.2 – 54.7(^4)</td>
<td>6 – 7.2(^6)</td>
</tr>
</tbody>
</table>

\(^{1,3}\) Davies et al., 1986, Ibekwe et al., 2008, Fadda et al., 2009

\(^4\) (Degen and Phillips., 1996)

\(^5\) Davis and Morris., 1993

\(^6\) (Sakaguchi et al., 1987)

The pharmacokinetics of ranitidine in rats is well established. Distinct double peaks have been observed in the plasma concentration-time profile in both humans and rats in many studies (Li et al., 2006, Schaiquevich. et al., 2002, Suttle and Brouwer., 1994, Tang et al., 2008). Possible mechanisms for the double peak phenomenon include enterohepatic recirculation, variable absorption sites along the GI tract, discontinuous absorption, delayed gastric emptying (Oberle and Amidon, 1987, Pedersen, 1981, Suttle et al. 1992, Suverkrup, 1979).
Using the rat as a model to predict human oral absorption is common practice as rat and human appear to show similar drug absorption and transporter expression profiles in the small intestine. In both humans (Johnson et al., 2006, Trezise et al., 1992, Valenzuela et al., 2004) and rats (Englund et al., 2006, Mouly and Paine, 2003, Zimmermann et al., 2005), the expression of P-gp has been shown to increase from the upper to lower small intestine, but decrease in the colon. Also, good correlation has been noted between the bioavailability of many compounds and drugs (PEG, atenolol, cimetidine, ranitidine and hydrochlorothiazide) in humans and rats, with the rat tending to slightly under predict bioavailability in humans (Chiou., 1998; Jezyk., 1992). In contrast, permeability is very low in certain species (particularly the dog) with no correlation to that in humans. These data suggest that the rat is a good model for predicting human bioavailability especially for paracellular and carrier mediated absorbed compounds (He, 1998).

The aims of this section are to:

(i) Determine if the results obtained in man are also be observed in rats and,

(ii) Determine if rats can be used as a model to investigate the effects of excipients on drug absorption.

3.17 Methods

3.17.1 Animal Housing

The rats were caged in groups of five and were allowed to move freely before and during the experimental period. They were kept on a light-dark cycle of 12 h at a room temperature of 25 °C, and provided with food and water ad libitum. All animals were allowed to acclimatise for a minimum of 7 days prior to experimentation.
3.17.2 Bioavailability Studies

Each rat was weighed on the day of the experiment and administered the appropriate amount of drug solution using a oral gavage syringe.

Wistar (male and female) rats weighing between 180-220 g (Harlan, UK) were used for the in vivo experiments and were allowed free access to both food and water prior to and during the study.

Rats were divided into two groups of three each and administered with ranitidine solution or PEG and ranitidine solution by oral gavage. Before dosing, 20 mg/ml ranitidine and 100 mg/ml PEG 400 solutions were prepared. The doses administered to the rats were calculated on the basis of the human dose. For example 150 mg ranitidine and 750 mg (0.75g) PEG 400 in an average weight human (70 kg) is equivalent to 2.1 mg/kg and 10 mg/kg respectively. This is therefore a 1:5 ratio of ranitidine to PEG 400 in humans. Therefore for the rats, the same ratio of ranitidine:PEG 400 was maintained.

The rats were administered with 50 mg/kg ranitidine, an amount previously used in literature and also quantifiable using the HPLC method. On the basis that the approximate weight of the rats in the study was 200 g, the amount of ranitidine would be approximately 10 mg and the amount of PEG 400 was calculated to be 50 mg, to maintain the 1:5 ratio of ranitidine and PEG 400 used in man. For solution of ranitidine and PEG, the appropriate amount of each solution was measured separately initially and then mixed together in another syringe which was then attached to the oral gavage for administration. The maximum amount of liquid that can be administered to a rat is 5 ml. The actual volume given to the rat was between 0.9 and 1.2 ml depending on weight of
the rat. After dosing, the rats were placed in a cage where they were allowed to move freely. Approximately 100-200 μL of blood was collected from the tail vein of the rats into heparinised tubes (Microvette CB300, Sarstedt, UK) at times 0.30, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0 h post-administration. At 8 h, the animals were sacrificed by a Schedule 1 method and 2 ml of blood was immediately obtained via cardiac puncture.

The blood samples were centrifuged at 3 000 rpm (750 x g) for 10 min (Eppendorf Centrifuge 5415D, Eppendorf AG, Hamburg, Germany fitted with a fixed angle rotor 45°; 24 X 1.5-2.0 ml with polypropylene lid) within 24 h of sampling. 50 μL of the plasma (supernatant) was measured accurately and placed into a 1.5 ml Eppendorf tube and immediately frozen (-20°C) prior to analysis.

3.17.3 Blood Sampling

To obtain blood samples from the rats, the rats were placed in a plastic restraining device and a small incision was made in the tail vein with a scalpel blade. Approximately 100 - 150 μl of blood was collected into anticoagulant (EDTA) coated centrifuge tubes, (Microvette CB300, Sarstedt, UK) which were stored on ice. In between sampling, the rats were placed in a holding temperature controlled device. Further bleeding was stopped by the application of light finger pressure on the blood vessel. Subsequent samples were taken from the same site by removal of the clot with a damp cloth to re-open the wound. If bleeding would not recommence, a fresh incision was made in the tail either in a different vein or at a more proximal site to the body along the same vein.
After sampling, the capillary tubes containing the blood samples were centrifuged for 10 minutes at 3000 rpm (750 x g) (Eppendorf Centrifuge 5415D, Eppendorf AG, Hamburg, Germany), and 50 μL of the plasma (supernatant) was then transferred to a 1.5 mL Eppendorf tube and frozen (ca -20 °C) until analysis. For collection of the final sample, animals were sacrificed by a Schedule 1 method (CO₂ euthanasia chamber) and blood was obtained by cardiac puncture.

Blood volumes taken were in accordance with the project license criteria that for multiple blood samples, not more than 10% and 15% of the circulating blood volume can be removed in any 24 h or 28 day period respectively.

3.17.4 Method Validation

Following the full method validation in chapter 2, only partial validation was determined here in accordance to the recommendations by the FDA. The intra accuracy and precision was determined.

3.17.5 Analysis of Plasma Samples

The frozen plasma samples were thawed by standing them at room temperature for 30 min. Ranitidine was extracted from the plasma by protein precipitation. 50 μL acetonitrile was added to 50 μL plasma and the mixture was vortex mixed. The samples were then centrifuged at room temperature for 10 min at 10 000 rpm (9 300 x g). 50 μL of the supernatant was recovered and transferred a glass insert placed inside a HPLC sample tube.
The plasma samples were analyzed using the high performance liquid chromatographic (HPLC) method developed in chapter 2 following partial validation.

3.18 Results and Discussion

3.18.1 Method validation

According to the FDA recommendations, a partial validation may be used where the matrix is being changed, in these cases simple intra- and inter-day precisions are sufficient. Following the full method validation in chapter 2, only partial validation was determined here. The intra-assay accuracy and precision at all the QC levels were less than 8 and 10%, respectively. These results show the validity of the method developed in chapter 2 for the analyses of ranitidine in plasma.

<table>
<thead>
<tr>
<th>QC Sample (µg/ml)</th>
<th>Calculated Conc. (µg/ml)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.64 ± 0.05</td>
<td>129.4 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>4.89 ± 0.05</td>
<td>97.9 ± 3.1</td>
</tr>
<tr>
<td>10</td>
<td>10.03 ± 0.17</td>
<td>100.4 ± 1.5</td>
</tr>
</tbody>
</table>

3.18.2 Bioavailability studies

The individual and mean plasma profiles obtained for rats administered with oral ranitidine and ranitidine and PEG 400 are shown in Figure 4.2 to Figure 4.7.
Figure 4.2: Individual plasma profiles of male rats with ranitidine control

Figure 4.3: Individual plasma profiles of male rats with ranitidine and PEG 400
Figure 4.4: Individual plasma profiles of female rats with ranitidine control

Figure 4.5: Individual plasma profiles of female rats with ranitidine and PEG 400
Figure 4.6: Plasma profiles (mean) of male rats with (a) ranitidine and (b) ranitidine and PEG 400

Figure 4.7: Plasma profiles (mean) of female rats with (a) ranitidine and (b) ranitidine and PEG 400
The pharmacokinetic data are shown in Tables 4.4 and 4.5. The AUC of ranitidine in the absence of PEG 400 for male rats was 112.69, whilst for the female rats it was 337.7. The difference was not statistically significant. The AUC obtained in this study is similar to AUC in reported studies of ranitidine pharmacokinetics in rat plasma 255.6 ± 67.6 (Suttle and Brower (1993)) ; and 298 ± 57 (Huang et al., 2005). In male rats, the AUC of ranitidine is somewhat enhanced by 14.5 % in the presence of PEG 400 whilst in the female rats, bioavailability was reduced (a 22 % difference). These differences in the bioavailability of ranitidine was not significant in both gender (p>0.05).

Table 4.4: Summary of pharmacokinetic data for male rats orally administered ranitidine and ranitidine combined with PEG 400

<table>
<thead>
<tr>
<th>Male Rats</th>
<th>AUC (µg ml⁻¹)</th>
<th>C_max (µg ml⁻¹)</th>
<th>T_max (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ranitidine</td>
<td>112.69 ±50.9</td>
<td>0.69 ±0.3</td>
<td>90</td>
</tr>
<tr>
<td>Ranitidine (plus</td>
<td>127.76 ±45.3</td>
<td>1.27 ±0.5</td>
<td>60</td>
</tr>
<tr>
<td>0.75 g PEG 400)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.5: Summary of pharmacokinetic data for female rats orally administered ranitidine and ranitidine & PEG 400

<table>
<thead>
<tr>
<th>Female Rats</th>
<th>AUC (µg ml⁻¹)</th>
<th>C_max (µg ml⁻¹)</th>
<th>T_max (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ranitidine</td>
<td>337.7 ±191.8</td>
<td>1.8 ± 0.4</td>
<td>15</td>
</tr>
<tr>
<td>Ranitidine (plus</td>
<td>263.5 ±90</td>
<td>1.9 ±0.1</td>
<td>15</td>
</tr>
<tr>
<td>0.75 g PEG 400)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

160
In the plasma profile of the male rats, only one peak is noted in both the presence and absence of PEG 400. The number of animals used in this work is a limitation of the study. Another limitation of using rats is the fact that the study was not a cross over study. The plasma profile of the female rat showed the presence of multiple peaks. This phenomenon has previously been reported in animals such as rats (Schaiquevich et al., 2002) and also in humans (Shim and Hong, 1989, van Hecken et al., 1982, Basit et al., 2002). Various hypotheses have been proposed to explain the presence of double peaks following the oral administration of ranitidine and other $H_2$ antagonists (cimetidine and famotidine) in humans. These hypotheses include, delayed gastric emptying of a portion of the administered dose (Oberle and Amidon, 1987), enterohepatic recycling or the drug being secreted into the lumen of the gut (Roberts, 1984) and discontinuous absorption from the gut (Funaki et al., 1986). The most common hypothesis for the presence of double peaks in human is that involving enterohepatic recycling. However it is thought that this is unlikely to be the reason for the double peak appearance in rats as less than 0.2% of the oral dose is recovered in bile as unchanged drug after oral administration of the drug (Schaiquevich et al., 2002). It is believed that the regional differences in the absorption of ranitidine in rats, with the terminal ileum being the optimal site of absorption is a more suitable hypothesis for the plasma profiles noted in rats (Mummaneni et al., 1994).

Similar to human studies, the results here show that in the absence of PEG 400, there was no difference in the bioavailability of ranitidine between the male and female rats. A very interesting observation is the presence of multiple peaks in the plasma profiles of the female rats and only single peaks in those of male rats. As previously discussed, the appearance of double peaks has been reported in the literature (Li et al., 2006,
Schaiquevich et al., 2002, Suttle and Brouwer, 1994, Tang et al., 2008). For most of these studies, the sex of the rats used are not disclosed and where disclosed they are usually male rats. Unlike man, it is known that in rat there is higher expression of the influx transporter organic cation transport (OCT) in male rats compared to female rats (Urakami, 1999; Urakami, 2000). OCT has reported to be important in ranitidine transport (Bourdet, 2005) hOCT1, which is expressed in the intestine and liver, is reported to play a major role in the intestinal absorption and hepatic disposition of ranitidine.

Although there were observed differences in the bioavailability of ranitidine between gender in the presence of PEG 400, these were not significant, besides the small sample size makes it very difficult to make conclusions. Further studies involving a larger sample size and more concentrations of PEG 400 are required to elucidate if the rat would be a useful model for investigating excipient effects on drug bioavailability and any gender differences.
SECTION 2: THE EFFECTS OF POLYETHYLENE GLYCOL POLYMERS 200, 300 AND 400 ON EFFLUX TRANSPORT ACTIVITY IN CACO-2 MONOLAYERS

3.19 Introduction

Following the studies to investigate the suitability of the rat model for investigating the effects of excipients such as PEG 400 on the absorption of drugs, it was determined to investigate the results obtained in humans mechanistically in order to have an improved understanding of the phenomenon. Whilst there is an obvious advantage of using the rat to predict oral absorption in humans, it is difficult to use the rat model to screen potential drug and excipient candidate in the early phase of drug discovery and development. In-vitro methods are more useful for screening and gaining mechanistic understandings.

Many drugs undergo absorption in the small intestine via permeation through the intestinal mucosa. Although the small intestine has a large surface area of around 120 m², the residence time can be short here. A dosage will spend an average 3 to 4 h in the human small intestine, but the this can be as low as 30 min (Davis et al., 1986). As discussed in chapter 1, the intestinal mucosa serves as both a physical and biochemical barrier to drugs administered via the oral route. Drug permeation occurs across the intestinal mucosa by passive diffusion, or by active mechanisms, and can be transcellular or paracellular. The paracellular route is controlled by tight junctions and on the basis of the molecular size and charge of the drug molecules. Hydrophilic compounds can permeate the intestinal mucosa via the transcellular route if they are
substrates for a nutrient transporter or they are been modified to be more lipophilic (Gangwar et al, 1997). The presence of metabolic enzymes and efflux transporters within the intestinal mucosa also enables it to serve as a major biochemical barrier to the oral delivery of lipophilic drugs and drug compounds (Ambudkar et al., 1999, Gupta, 1995, Wacher et al., 1996). Efflux transporters such as P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP) are known to be over expressed in tumor cells and are widely distributed throughout normal tissues in the human body including the kidney, intestinal mucosa and the endothelial cells of the brain and the liver (Merino et al., 2005, Schwartz, 2003a, Seithel et al., 2006, Taipalensuu et al., 2001, Takano et al., 2006). P-gp is the most important transporter that controls the efflux transport of drugs and drug candidates. It is a protein present on the apical (AP) membrane of mucosal cells and is encoded by the MDR1 gene in humans. Ranitidine is a drug that has its absorption window in the small intestine, with poor absorption in the colon (Basit et al., 2001). The bioavailability of ranitidine has been shown to be improved in male subjects by the administration of low dose PEG 400 and this may be due to an effect of PEG 400 on ranitidine permeability. At high doses, the improvement in bioavailability was not observed and this was attributed to the tendency for high-dose PEG to accelerate small intestinal transit (Basit et al., 2001, Schulze et al., 2003).

It was reported that ranitidine is transported through the intestinal wall via the paracellular route (Collett et al., 1996, Gan et al., 1993). However, more recent studies have suggested paracellular transport via tight junctions account for just sixty percent of this movement whilst transcellular processes (via absorption transporters such as human organic cation transporter 1 [OCT]) account for the other forty percent (Bourdet et al., 2006, Bourdet and Thakker, 2006). The absorption of ranitidine is also affected by
efflux transporters. Carrier mediated transporters [P-gp, MRP 1, MRP 2 and BCRP] expel drugs and drug candidates which are substrates for them back to the apical side (lumen) of the intestinal mucosal cells via an ATP-dependent process and this greatly reduces the overall permeability and oral bioavailability of these drugs. Many drugs are substrates of these transporters; consequently the bioavailability and pharmacokinetics of these drugs are controlled by the expression of these carriers. The efflux carrier P-gp has been implicated in intestinal ranitidine transport (Collett et al., 1999) whilst cimetidine (of the same pharmacological class as ranitidine) has been identified as a both a P-gp and BCRP substrate (Collett et al., 1999, Pavek et al., 2005).

It is not possible to use human beings to understand mechanistically, the findings observed in humans. Nor can they be used in early phase drug discovery and development. Various models have been proposed and used over many years to gain a better understanding to membrane permeation of a drug through the intestinal mucosa, the most important step in drug absorption.

3.19.1 Methods Used to Study Permeation in the Small Intestine

The main methods for studying permeation in the small intestine are in-vitro methods. However other models include in-situ models and in-vivo models.

3.19.1.1 In-vitro methods:

There are a variety of cell culture monolayer models which mimic the intestinal epithelium in humans. These include:
Caco 2 cells

Caco 2 cells are an immortalized human male colorectal carcinoma-derived cell line. They are the most widely and extensively characterized and used of all the cell types to model drug behaviour in the human intestine for mechanistic studies (Balimane and Chong, 2005). They are particularly useful because the permeation characteristics of drugs across Caco-2 cell monolayers correlate with their human intestinal mucosa permeation characteristics and hence Caco-2 cells are often used to predict oral absorption of drugs in humans. They are particularly used for mechanistic studies and rapid in-vitro screening tool during drug discovery. On putting Caco-2 cells into culture and especially plating the cells on porous polycarbonate membranes, they undergo spontaneous differentiation into mature enterocytes and express several biochemical and anatomical features common to normal human enterocytes. They also display a fully defined brush border membrane on the apical domain which contain several transporters, metabolic enzymes and efflux pumps such as MDR1 P-gp which are stable and functional (Artursson et al., 2001, Bourdet and Thakker, 2006, Collett et al., 2005, Hilgendorf et al., 2000, Koljonen et al., 2006, Pontier et al., 2001, Shah et al., 2006, Usansky and Sinko, 2005, Takano et al., 2006, Hunter et al., 1993).

Caco-2 cells (2 to 5 week old) have been shown to express similar transporters and at similar levels to the human jejunum and hence their usefulness for obtaining small intestinal transport data assured (Seithel et al 2006), however it is worth noting that whilst levels of 9 of 10 ATP-binding Cassette (ABC) transporters (MRP2, MDR1, MRP3, MRP6, MRP5, MRP1 and MRP4, MDR3) correlated well between jejunum and Caco-2 cells (r2 = 0.90), BCRP exhibited a 100-fold lower transcript level in Caco-2 cells compared with the jejunum (Taipalensuu et al 2001).

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Madine-Darby Canine Kidney cell mono layers

MDCK cells are derived from dog kidney cells. They are gaining popularity for use of permeability measurements because of their shorter culture time (three days). Despite similarities to Caco-2 cells and having a membrane composition similar to that of the intestine, there is a high probability that the expression levels of some transporters in MDCK would be different from Caco-2 cells lines which are derived from human colon carcinoma cells. As a solution to this disadvantage in a useful cell line, the human MDR 1 gene has been successfully transfected into MDCK cells (Pastan et al., 1988). Other human efflux transporters can also be transfected into the MDCK cells. Unlike the Caco-2 cells derived in the 1970s, the use of MDCK cell line as a model to evaluate intestinal permeation characteristics of compounds was first discussed 20 years ago (Cho et al., 1989). Recently researchers have investigated the use of MDCK cells as a tool for assessing membrane permeability properties of compounds during early drug discovery; however more studies are currently needed to correlate permeability in MDCK cells to human absorption values.

Isolated intestinal segments and everted gut sacs

These models are specific to animal studies. In this model, sections of the intestine are resected and irrigated with saline to clear the luminal content, they are then either mounted in a diffusion apparatus (Ussing chamber) or everted with the ends closed to form closed sacs (everted sac). Tissue accumulation of a compound in vitro is used to determine drug uptake rate. They provide a relatively quick and inexpensive technique for measuring drug uptake into tissue as they do not require the time and expense of start-up and maintenance of caco-2 cells and also do not require live animals along with
the associated husbandary costs which are needed for statistical power with perfusion methods.

Studies have shown that intestinal membrane permeability determined by intestinal segments correlate well with human oral bioavailability for compounds that are absorbed by passive and carrier-mediated mechanisms (Amidon et al., 1988, Levet-Trafit et al., 1996, Stewart et al., 1995)

**Brush membrane border vesicles**

Membrane vesicles are formed from intact cells. They require some skill in their preparation and hence for many years this limited their use as rapid screen for P-gp efflux. However in recent years, ready made assay kits that include membrane vesicles have been commercially produced and hence their use is gaining some popularity (Polli et al., 2001, Sarkadi et al., 1992, Konya et al., 2006). The assays commercially developed by companies such as Solvo Biotechnology (Budapest, Hungary) available via Tebu-bio (Peterborough, UK) and GENTEST (Woburn, MA) are performed using purified membrane vesicles from insect cells or mammalian cells transfected to express high levels of a specific selected human ABC transporter protein.

### 3.19.1.2 In-situ models

In-situ models perfusion studies are typically performed with anesthetized live animals. A perfusion loop is inserted into the intestine and the transport of compounds is measured in a physiologically relevant environment. A number of workers have made use of this method to study the effect of P-gp on its substrates (Lennernas, 1997, Shen et al., 2006). Although this method offers a valuable method of studying drug transport, it
has a number of limitations: The method requires significant surgical skills, also the anesthetizing agent can affect the results with respect to altered membrane fluidity and possible inhibitory effects on P-gp mediated activity.

3.19.1.3 In-vivo models

In-vivo models used to study drug transport include the use of mice with disruption of individual mdr1a, mdr1b or mdr2 genes (Mauad et al., 1994, Groen et al., 1995). Although the mouse mdr1 P-gp is not totally homologous to the human P-gp, mice that are negative for the mdr1a gene provide useful in-vivo information on the role P-gp-mediated efflux activity plays in the absorption, distribution, metabolism and elimination of its substrates (vanAsperen et al., 1996, Schinkel, 1998, van Waterschoot et al., 2008)

3.19.2 Effect of PEG 400 on Drug Transport

PEG 400 is thought not to have an effect on paracellular transport in Caco 2 cells (Rege et al., 2001, Johnson et al., 2002) and although its effects on OCT transporters is yet to be established there is extensive emerging evidence that excipients such as PEG can inhibit efflux transporters and could be instrumental in the increased bioavailability witnessed in these previous studies. One group report a dose-dependent inhibition of P-glycoprotein efflux transporter in excised rat intestine in the presence of PEG 400 (Johnson et al., 2002). PEG 300, PEG 400, vitamin E TPGS and Tween 80 have been shown to inhibit P-gp in Caco 2 cells without affecting the integrity of the cells tight junctions (Hugger et al. 2002a, (Johnson et al., 2002, Yamagata et al., 2007a) whilst other non-PEG excipients (Cremophor EL, Tween 20 Span 20, Pluronic P85 and Brij 30) inhibit both P-gp and BCRP and subsequently enhanced the absorption of a BCRP
substrate (Yamagata et al., 2007b). The effect of PEG 400 on P-gp activity in Caco-2 cells has not previously been studied.

In the previous study, in male volunteers, the mean cumulative amount of unchanged ranitidine excreted in the presence of 0, 0.5, 0.75, 1, 1.25 and 1.5 g (0, 0.3%, 0.5%, 0.7%, 0.8% and 1% w/v) PEG 400 were 35, 47, 57, 52, 50 and 37 mg respectively. These correspond to increases in bioavailability of 34%, 63%, 49%, 43% and 6% over the control treatment. In the female subjects, there were no differences in the mean cumulative quantity of ranitidine excretion in the absence and presence PEG 400.

Hugger et al. 2002a have previously shown that PEG 300 (20% v/v) inhibited P-gp mediated efflux without affecting paracellular or transcellular pathway.

Following the review of the different methods of mechanistic studies of influence of drugs on intestinal permeability, it was determined that the in vitro methods (brush-membrane border vesicles and Caco-2 cells) would be applied to this study based on the ease of availability and usefulness of data that would be obtained. Although these models will not explain the gender difference; it may explain the results observed in male volunteers as the cell line (Caco-2) is male derived.

The aims of this section are to:

1. Determine the interactions between cimetidine, ranitidine and PEG analogues on ATPase activity; screen for P-gp interaction using commercially available ATPase assays prepared with human P-gp membranes from Sf9 insect cells.

2. Establish the conditions for Caco-2 cells growth and culture.
3. Investigate the effect of PEG 400 and its lower molecular weight analogues (PEG 200 and 300) at 20% on Caco-2 cell efflux transporter activity with specific focus on ranitidine transport.

4. Investigate the effects of various concentrations of PEG 400 (0.3, 0.5 and 1 % v/v) on the permeability of ranitidine through Caco-2 cell layers.

5. Investigate the effect of PEG 400 on other efflux transported drugs – digoxin and cimetidine to determine the if PEG 400 has greater effects on one efflux transporter over another (digoxin is classic P-gp substrate, whilst cimetidine is also a BCRP substrate)

3.19.3 Materials

3.19.3.1 P-gp ATPase activity
PREDEASY ATPase Kit containing human P-gp membranes from Sf9 insect cells, phosphate, sodium-orthovandate, verapamil, cyclosporine and MgATP solution was obtained from Tebu-bio (Peterborough, UK).

3.19.3.2 Caco-2 Cell Culture
Caco-2 cells human adenocarcinoma cell line, were obtained from European Collection of Cell Cultures (ECACC; Wiltshire, UK) were used from passage 35 to 58. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, non-essential amino acids, L-glutamine, 0.25% trypsin-EDTA, gentamicin (50mg/ml), Hanks’ Balanced Salt Solution (HBSS), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich (Dorset, UK). Transwell® Corning Costar Corporation (12-well, 1.13cm² surface area, 0.4 μm pore size) and 162 cm² flasks were obtained from Fisher (Leicestershire, UK).
$^{14}$C-mannitol (specific activity = 61 mCi/mmol) and $[^3]$H Cimetidine (specific activity = 20 Ci/mmol) were purchased from Amersham Biosciences (Buckinghamshire, UK). Scintillation Cocktail (Emulsifier) from Perkin Elmer (Buckinghamshire, UK). $[^3]$H Ranitidine (specific activity = 2.5 Ci/mmol) and $[^3]$H Digoxin (specific activity = 40 Ci/mmol) were purchased from Moravek, Ca, USA.

3.20 Methods

3.20.1 P-gp ATPase activity

ATPase activity of cimetidine, ranitidine and PEG 400 and its two lower analogues (200 and 300) were measured in the presence and absence of verapml a P-gp substrate. The two lower analogues of PEG 400 were chosen for comparison and especially to validate the results of these studies as Hugger et al 2002 has previously shown that PEG 300 inhibits P-gp and PEG 200 has previously not been studied. A modified version of a high-throughput screening assay developed by (Sarkadi et al. (1992) was used with commercially available human P-gp membranes from Sf9 insect cells. The ATPase activity was estimated by measuring inorganic phosphate released from ATP according to the manufacturer’s protocol. Briefly, the membrane was diluted with assay mix (mix (50 mM 2-(N-morpholino)-propanesulfonic acid (MOPS) and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) (Mops–Tris), pH 7.0; 50 mM KCl; 5 mM Na-azide; 2 mM Dithiothreitol (DTT); 0.1 mM ethylene glycol tetraacetic acid (EGTA)–Tris, pH 7.0; 1 mM Ouabain in distilled water). Forty microlitres of diluted membrane solution was pipetted into the wells of a 96-well microplate. One microlitre of each of
the test compounds (PEG 200, 300 and 400) dissolved in DMSO and/or 1 µl of
verapamil dissolved in DMSO were also added to the membrane suspension. DMSO at
the same volume was added to the control wells. The reaction mixtures were pre­
incubated at 37°C for 20 min, then stopped by the addition of 10 µl ATP-Mg solution.
The amount of liberated inorganic phosphate was determined by colorimetric reaction.
After incubating at 37°C, the absorbance was read at 610 nm in a microplate reader and
the concentration of the liberated phosphate was calculated from calibration curve
generated following the method of the assay. The drug stimulated ATPase activity
(nmol/min/mg of protein) was determined as the difference between the amounts of
inorganic phosphate released from ATP in the absence and presence of vanadate.
Phosphate standards were prepared in each plate and verapamil served as a positive
control. Drug-stimulated Pgp ATPase activity was reported as a relative in comparison
to the basal Pgp ATPase activity in the absence of drug (DMSO control). A compound
was classified as an activator if the increase obtained was greater than 2-fold over that
obtained with the DMSO control.

3.20.2 Caco-2 Cell Culture

3.20.2.1 Cell maintenance
Caco-2 cells were grown and maintained in culture as previously described (Hidalgo et
al. 1989). Briefly, cells were grown in 162 cm² cell culture flasks and subcultured
weekly on achieving 80-90% confluence. Cell culture growth medium was DMEM,
supplemented with 10% v/v fetal bovine serum, 1% v/v non-essential amino acids, 1%
v/v L-glutamine, 0.1%v/v gentamicin (50 mg/ml). Cells were stored in an incubator at
37°C with humidified environment of 95% and 5% CO₂. Medium was changed every 2
to 3 days. All processes were carried out using trained techniques and precautions relative to cell culture in a Class II Flow Cabinet.

3.20.2.2 Subculture

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

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

1. Using a sterile pipette, old culture medium was removed and discarded
2. The cell monolayer was rinsed with 5 mL of calcium- and—magnesium-free phosphate buffered saline (CMF-PBS) to remove all traces of FBS.
3. 3 mL prewarmed trypsin solution was then added to the flask, cells incubated for at least one minute on the bench and then the flasks were transferred to a shaking (100 rpm; 37°C) incubator for detachment of cells.

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

3.20.2.3 Cell Freezing

At regular intervals, cells that had reached confluency in 162 cm² flasks were prepared for cryopreservation. Prior to freezing as with subculturing, the cells were checked under an inverted microscope to check the general appearance of the culture looking for signs of microbial contamination. The culture was also observed with an unaided eye to look for fungal colonies that could be floating at the medium-air interface. The cells were harvested in the same manner as described above for subculture. However prior to centrifugation, a sample was taken for counting. Whilst the cells were spinning, a viable cell count was carried out as previously discussed and the number of cells/mL was calculated as well as the total cell number. After centrifugation, the supernatant was removed from the centrifuged cells and the cell pellet resuspended in enough cryoprotective medium to give a final cell concentration of 1 to 2 x 10⁶ cells/mL. The cryopreservation medium was prepared by mixing 45% (v/v) of maintenance medium with 45% (v/v) FBS and 10% v/v cryoprotectant DMSO.

Cyrogenic vials were labeled with the cell line, passage number and date and 1.8 mL of the DMSO containing cell suspension was added to each of the vials and sealed. The cells were frozen in a -80°C freezer using a ‘Mr Frosty’ cell freezer containing room temperature isopropanol in the bottom compartment to ensure a gradual freezing of the
cells. After 24 h, the vials were transferred to a liquid nitrogen storage vessel until required.

3.20.2.4 Cell revival

The required vial was transferred from the liquid nitrogen storage and rapidly thawed in a 37°C water bath within 60 to 90 s. The contents of the vial were then transferred to a flask containing 15 mL of cell culture medium and incubated at 37°C. After attachment of majority of the cells to the surface of the flask, after 4 h, the medium containing the diluted cryoprotective agent was removed and replaced with fresh medium.

3.20.2.5 Permeability studies

For permeability studies, cells were seeded at a density of 60,000 cells/cm² (1.2 x 10⁵ cells/ well) onto Transwell® polycarbonate membranes with 12 mm diameter, pore size 0.4 μm and a surface area of 1.13 cm². Cells growing on Transwell® membranes were provided with fresh complete medium three times a week until the time of use. For feeding the cells, 0.5 mL of complete medium was added to the top [apical (AP) compartment] of the cell layer and 1.5 mL was added to the bottom [basolateral (BL) compartment] of each Transwell®. All cells used in this study were between passages 35 and 58.

The filters were used between 21 and 28 day of culture. The growth media was removed and replaced with preheated transport media (HBSS supplemented with 10 mM HEPES) at 37°C; 0.5 mL to the apical side and 1.5 mL to the basolateral side. This was repeated three times to wash the cell monolayer.
For absorptive permeability (Apical-to-Basolateral; AP-to-BL), the experiment was initiated by adding 0.5 mL of the appropriate test solution to the apical (donor) chamber of inserts bathed with 1.5 mL basolateral solution (receiver chamber) containing HBSS or PEG in HBSS. For the secretory permeability (BL-to-AP), the experiment was initiated by adding 1.5 mL of the appropriate test solution to the basolateral (donor) chamber of wells with 0.5 mL in the apical chamber. At 30 min intervals (0, 30, 60, 90, 120, 150 and 180 min), 100 μL samples were withdrawn from the receiver chamber and at 0 and 180 min after administration from the donor chamber. At each time interval, the volume withdrawn was replaced with fresh transport media of HBSS or PEG in HBSS which was corrected for in calculations. During experimentation, the plates were agitated on a Gyrotory® Shaker-Model G2 (New Brunswick Scientific Co., Hertfordshire, UK).

3.20.3 Measuring integrity to cell monolayers

The integrity of the monolayer was confirmed by measuring the permeability of the paracellular marker compound ^14C-mannitol and taking transepithelial electrical readings (TER) before and after the experiments. Mannitol permeability was simultaneously measured in all samples, providing an opportunity to weigh any potential excipient effect against the general integrity of the monolayer.

3.20.3.1 ^14C-mannitol measurement

The 100 μL radiolabelled samples removed at the 30 min intervals and added to 5 mL scintillation cocktail. The amount of ^14C isotope was detected using a Beckman Coulter LS6500 (Buckinghamshire, UK), counted for 5 min per sample
3.20.3.2 Transepithelial electrical readings (TEER)

The integrity of the monolayers was confirmed by taking TEER readings before (0 min) and after the experiment (180 min) using an EVOMTM epithelial voltohmmeter (World Precision Instruments, Hertfordshire, UK). The cells were maintained in the temperature controlled incubator at all times except during measurements. The shorter of the chopstick silver chloride electrodes was placed inside the insert (apical chamber) and the longer electrode in the bathing solution (basolateral well). The resistance reading (Ohms) was then obtained from the EVOM. The resistance of the monolayer was determined by subtracting the resistance of the solution and the membrane support from the total resistance.

\[
\text{Resistance (cell monolayer)} = \text{resistance (membrane support + cell monolayer)} - \text{resistance(membrane support)}
\]

**Equation 1: Calculation of cell monolayer resistance**
The resistance (membrane support) was the TEER of the membrane taken before cells were added the transwells.

3.20.4 Transport Studies

All transport studies were performed on Transwell® grown Caco 2 cells maintained in culture for 21 to 28 ds During the transport studies, the AP-to-BL transport of D-[\(^{14}\)C] mannitol was also determined and was typically <1 x 10⁶ cm/s. Transepithelial resistance measurement before and at the end of each transport experiment was also used to determine the integrity of the cells used in experiments.
Bidirectional (AP-to-BL and BL-to-AP) transport experiments were performed with ranitidine, cimetidine and digoxin for 3 h (30-min sampling intervals) in a 37°C shaking (55 rpm) incubator. Prior to the transport studies, the cell culture medium was removed and replaced with pre-warmed transport buffer (HBSS with HEPES, pH 7.4). The monolayers were washed three times with HBSS prior to the start of the experiment. In all bidirectional transport studies, either HBSS or polyoxyethylene polymers were present on both sides of the Caco-2 cell monolayers. This was done to maintain the sink conditions for the duration of the study (Hugger et al., 2002a, Rege et al., 2001) as the polyoxyethylene polymers used for in the studies are hyperosmotic. The osmotic pressure of HBSS and the different PEGs used were:

In AP-to-BL transport studies, 1.5 ml of incubation buffer (containing HBSS or PEGs) was added to each receiver (BL) compartment and 0.5 ml of a radioactive donor solution (HBSS or PEGs in HBSS with mannitol or drug solution – cimetidine, ranitidine or digoxin) was added to each donor (AP) compartment. Similarly for the BL-to-AP transport studies, 0.5 mL incubation buffer was added to each AP compartment and 1.5 mL of radioactive solution containing HBSS or PEGs in HBSS with drug solutions was subsequently added to each BL compartment. At 30 min intervals, 100 µL samples were removed from each receiver compartment and each compartment was appropriately replenished with HBSS or HBSS containing PEGs. The amount of radiolabeled solute (¹⁴C or ³H isotope) transported across the Caco 2 cell monolayers was determined by counting the samples in a Beckman Coulter LS6500 Liquid scintillation counter (Buckinghamshire, UK), counted for 5 min per sample. The apparent permeability coefficients (Papp) for the radiolabelled solute were determined
in the AP-BL (Papp AP-to-BL) and BL-to-AP (Papp BL-toAP) direction using the equation:

\[ \text{Papp} = \frac{1}{AC_0} \frac{dQ}{dt} \]

**Equation 2: Calculating permeability**
Where: \( \frac{dQ}{dt} \) is the flux across the monolayer (dpm/min), \( A \) is the surface area of the Transwell membrane (4.71 cm\(^2\)), and \( C_0 \) is the original donor concentration (dpm/mL) of the radiolabeled solute.

### 3.21 Results and Discussion
#### 3.21.1 Effect of ranitidine and PEG analogues on P-gp ATPase activity
The interaction between cimetidine, ranitidine and the PEG analogues on P-glycoprotein (P-gp; an efflux transporter) was investigated using a P-gp ATPase activity kit. The results (Table 4.6) show that all the compounds, except PEG 200 and cimetidine, stimulated P-gp ATPase activity.

The ATPase assay obtained for cimetidine is similar to that observed by Polli et al (2001); ATPase ratio [(nmol/min/mg of protein) was determined as the difference between the amounts of inorganic phosphate released from ATP in the absence and presence of vanadate.] was 1.33 in their assay (Polli et al., 2001). However the ATPase assay ratio ranitidine is different between this study and the investigations by Polli et al (2001); 4.15 in this study compared to 1.23 in the Polli et al study. Both ranitidine and cimetidine have however been shown to be substrates of P-gp in various studies (Bourdet et al., 2006, Gan et al., 1993, Takamatsu et al., 2001). Perhaps the low activity of cimetidine in this P-gp specific ATPase assay may be due to the fact that cimetidine is also a known BCRP substrate (Merino et al., 2005, Pavek et al., 2005). It is also
possible that the lack of stimulation is due to cimetidine binding tightly to the drug-binding site of the P-gp without eliciting ATP hydrolysis (Scarborough, 1995).

The ATPase assay provides a useful method for screening compounds and determining if they are P-gp substrates. The stimulation of P-gp ATPase activity by ranitidine, PEG 300 and PEG 400 suggests that there may be some mechanism interaction between these PEG molecules and ranitidine.

**Table 4.6: Effect of ranitidine and PEG analogues on ATPase activity; screen for P-gp interaction**

<table>
<thead>
<tr>
<th>Compound</th>
<th>ATPase Assay Ratio</th>
<th>ATPase Activator/Interaction with P-gp (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cimetidine</td>
<td>1.44</td>
<td>Yes</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>4.15</td>
<td>Yes</td>
</tr>
<tr>
<td>PEG 200</td>
<td>0.53</td>
<td>No</td>
</tr>
<tr>
<td>PEG 300</td>
<td>3.71</td>
<td>Yes</td>
</tr>
<tr>
<td>PEG 400</td>
<td>3.06</td>
<td>Yes</td>
</tr>
</tbody>
</table>

"The drug-stimulated Pgp ATPase activity is reported as fold-stimulation relative to the basal Pgp ATPase activity in the absence of drug (DMSO control). A compound is classified as an activator if the fold-stimulation was greater than 2-fold over the DMSO control" (Polli et al., 2001)

**3.21.2 Transepithelial electrical readings (TEER) during culture**

After subculture, cells were seeded onto Transwell® filters and the monolayer development was monitored by TEER measurements (figure 4.1). The TEER of the cells increased with number of days in culture. The greatest rate of increase occurred
within day 3 and 7. The maximum TEER obtained for the cells was \(1983 \pm 4 \ \Omega \ cm^2\) (n=18). The blank TEER used for calculating resistance was measured from several plates over many weeks at \(123 \pm 5 \ \Omega \ cm^2\) (n=18).

![Graph showing TEER over time](image)

**Figure 4.8: Transepithelial electrical readings (TEER) during culture**

3.21.3 Integrity of monolayers – effects of PEG 400 on \([^{14}C]\)-mannitol flux and Transepithelial Electrical Resistance (TEER)

Before investigating the effects of PEG 400 on the transport of P-gp substrates across Caco-2 cell monolayers, it was important to determine its effects on the integrity of the cell monolayer. Two methods are commonly used to test cellular monolayer integrity and these are to measure a mannitol flux (Chen et al., 2002) or to measure TEER (Hidalgo et al., 1989).
In these studies, both TEER measurements and mannitol flux were used to test cellular integrity in the presence of the highest concentration (20% v/v) of PEG 400 used in these studies. The results (Table 4.7) show that the average transport of mannitol in the control monolayers and in the monolayers treated with the highest concentration of the different PEG polymers PEG 200, 300 and 400 in a typical 3 h experiment was not significantly different (t-test, p>0.05). The permeability of mannitol in the control monolayers is similar to that observed by Rege et al (2001) and was <1 x 10^6 cm/s x 10^6 hence the results are considered acceptable (Rege et al., 2001). Also, the change in TEER in the control monolayers and the monolayers treated with PEG 400 was not significantly different (t-test, p>0.05).

The results show that the highest concentration of the different PEG polymers used did not affect the integrity of Caco-2 cell monolayers (t-test; p>0.05). This also shows that PEG 400 at the high concentration used does not affect the passive permeation of solutes across the paracellular pathway of Caco-2 cell monolayers, an in-vitro model of the intestinal mucosa.

In all bidirectional transport studies, either HBSS or polyoxyethylene polymers were present on both sides of the Caco-2 cell monolayers. This was done to maintain the sink conditions for the duration of the study (Hugger et al., 2002a, Rege et al., 2001) as the polyoxyethylene polymers used for in the studies are hyperosmotic. The osmotic pressures: HBSS (324 mOsm/kg); 20% PEG 200 (2400 mOsm/kg); PEG 300 (2365 mOsm/kg); PEG 400 (2250 mOsm/kg).
Table 4.7: The apparent permeability, % recovered and transepithelial electrical resistance (TEER) values for $^{14}$C-mannitol determined by permeability experiments in apical to basolateral (n=9 Three non consecutive experiments with three vials per compound)

<table>
<thead>
<tr>
<th>Excipient</th>
<th>Mannitol</th>
<th>% Recovery</th>
<th>Initial</th>
<th>Final</th>
<th>Change in TEER (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papp (cm/s x 10^-6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>0.75 ± 0.05</td>
<td>91.4</td>
<td>790</td>
<td>649</td>
<td>-17.8 (2.1)</td>
</tr>
<tr>
<td>PEG 200 (20%)</td>
<td>0.86 ± 0.05</td>
<td>93.1</td>
<td>1326</td>
<td>873</td>
<td>-34.2 (5)</td>
</tr>
<tr>
<td>PEG 300 (20%)</td>
<td>0.93 ± 0.01</td>
<td>92.1</td>
<td>1385</td>
<td>1181</td>
<td>-14.7 (0.75)</td>
</tr>
<tr>
<td>PEG 400 (20%)</td>
<td>0.95 ± 0.06</td>
<td>91.5</td>
<td>725</td>
<td>616</td>
<td>-15.0 (1.2)</td>
</tr>
</tbody>
</table>

Previous studies have shown that the presence of PEGs on the AP side only and HBSS containing sugars or salts to the BL side still led to disruption in cellular tight junction integrity (Hugger et al., 2002a) however whilst this design does not mimic physiological conditions, it has been successfully used in transport studies containing hyperosmotic solutions (Hugger et al., 2002a, Rege et al., 2001)

In terms of human studies, this suggests that the results observed in man is unlikely to be due to the PEG 400 affecting the paracellular transport of ranitidine. Schulze et al
2003 had previously suggested that perhaps the effect of lower doses of PEG 400 was due to PEG 400 influencing the modulation of tight junctions.

3.21.4 Transport Studies

Once established that PEG 400 does not affect the paracellular pathway of Caco-2 cell monolayers, its potential effects on efflux transporters especially P-gp was then investigated.

3.21.4.1 Effects of Polyoxyethylene polymers on the Bidirectional Transport of Ranitidine

The Papp values for the permeation of $^3$H-ranitidine across Caco-2 cell monolayers in the AP-to-BL (Papp, AP-to-BL) and BL-to-AP (Papp, BL-to-AP) directions in the absence and presence of 20% v/v each of PEG 200, 300 and 400 is shown in Table 4.3. The results show that in the absence of PEG (control monolayers), ranitidine exhibits polarised secretory transport (an efflux ratio significantly above 1). This is consistent with ranitidine being a substrate for P-gp (Collett et al., 1999).

The polyoxyethylene polymers did not affect the paracellular pathway, however, in the presence of the PEG 300 and 400 (but not PEG 200), the efflux ratio decreased compared to control (Table 4.3). Although efflux was not abolished entirely in the presence of PEG 300 and 400, the results suggest that PEG 300 and 400 but not PEG 200 are capable of inhibiting P-gp activity in Caco-2 cell monolayers. Also, the results noted with PEG 300 and 400 in the transport studies support those obtained from the specific P-gp screen; suggesting that the higher molecular weight polymers may exert their effect through an interaction with P-gp. The inhibition of P-gp may account for the
enhanced bioavailability of ranitidine noted in males in the previously conducted human studies.

Table 4.3: Effects of PEG 200, 300 and 400 on [3H]ranitidine Transport Across Caco-2 Cell Monolayers

<table>
<thead>
<tr>
<th>Excipient</th>
<th>Papp (cm/s x 10^-6)</th>
<th>Papp (cm/s x 10^-6)</th>
<th>Papp(BL-to-AP)/Papp(AP-to-BL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AP-to-BL</td>
<td>BL-to-AP</td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>1.06 ± 0.48</td>
<td>5.72 ± 3.44</td>
<td>5.38</td>
</tr>
<tr>
<td>PEG 200 (20%)</td>
<td>1.07 ± 0.48</td>
<td>5.40 ± 2.65</td>
<td>5.05</td>
</tr>
<tr>
<td>PEG 300 (20%)</td>
<td>2.26 ± 0.99</td>
<td>5.63 ± 3.70</td>
<td>2.49</td>
</tr>
<tr>
<td>PEG 400 (20%)</td>
<td>2.88 ± 1.23</td>
<td>7.12 ± 1.71</td>
<td>2.47</td>
</tr>
</tbody>
</table>

*The bidirectional transport of [3H]ranitidine (concentration 0.1 mM; specific activity – 2.5Ci/mmol) was examined across Caco-2 cell monolayers in the absence (no PEG, only HBSS) and presence of 20% v/v of the different polyoxyethylene polymers (200, 300 and 400) on both sides of the Caco-2 cell monolayers (grown 21–28 days; n=3); experiment performed in triplicate with 3 replicates per variable on each occasion. Samples (100 μL) were taken from the receiver compartments every 30 min for 3 h and each receiver compartment was replenished with the appropriate transport buffer solution (HBSS or PEG in HBSS). The apparent permeability coefficients (Papp) for [3H]ranitidine were calculated as described in the Materials and Methods section.
3.21.4.2 Effects of Various Concentrations of PEG 400 on the Bidirectional Transport of Ranitidine

Following the effects noted with the highest concentration of the polyoxyethylene polymers, it was necessary to further investigate the effects of different concentrations of PEG 400 in order to have some understanding of the effects of more realistic concentrations of PEG 400 (especially concentrations around those studied in the human volunteers) on P-gp activity. The concentrations, 0.3, 0.5 and 1% are equivalent to 0.5, 0.75 and 1.5 g PEG 400 used in the human studies.

The Papp values for the permeation of $^3$H-ranitidine across Caco-2 cell monolayers in the AP-to-BL (Papp, AP-to-BL) and BL-to-AP (Papp, BL-to-AP) directions in the absence and presence of various concentrations of PEG 400 is shown in Table 4.4. There was a reduction in efflux ratio in the presence of lower concentrations of PEG 400 up to 1% suggesting increased inhibition of P-gp. However none of the results obtained for the efflux ratios were significantly different from control.
Table 4.4: Effects of different concentrations of PEG 400 on $[^3]$Hranitidine Transport Across Caco-2 Cell Monolayers

<table>
<thead>
<tr>
<th>Excipient % (v/v)</th>
<th>Papp (cm/s $\times 10^6$) AP-to-BL</th>
<th>Papp (cm/s $\times 10^6$) BL-to-AP</th>
<th>Papp(BL-to-AP)/Papp(AP-to-BL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.06 ± 0.48</td>
<td>5.72 ± 3.44</td>
<td>5.38</td>
</tr>
<tr>
<td>0.3</td>
<td>1.95 ± 0.08</td>
<td>6.47 ± 0.14</td>
<td>3.3</td>
</tr>
<tr>
<td>0.5</td>
<td>1.55 ± 0.02</td>
<td>3.23 ± 0.2</td>
<td>2.1</td>
</tr>
<tr>
<td>1.0</td>
<td>2.54 ± 0.02</td>
<td>2.94 ± 0.14</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>1.08 ± 0.35</td>
<td>4.15 ± 1.09</td>
<td>3.85</td>
</tr>
<tr>
<td>10</td>
<td>1.29 ± 0.30</td>
<td>5.16 ± 1.13</td>
<td>4.01</td>
</tr>
<tr>
<td>20</td>
<td>2.88 ± 1.23</td>
<td>7.12 ± 1.71</td>
<td>2.47</td>
</tr>
</tbody>
</table>

The bidirectional transport of $[^3]$Hranitidine (concentration 0.1 mM; specific activity = 2.5Ci/mmol) was examined across Caco-2 cell monolayers in the absence (no PEG, only HBSS) and presence of various concentrations of PEG 400 on both sides of the Caco-2 cell monolayers (grown 21–28 days; n=3); experiment performed in triplicate with 3 replicates per variable on each occasion. Samples (100 µL) were taken from the receiver compartments every 30 min for 3 h and each receiver compartment was replenished with the appropriate transport buffer solution (HBSS or PEG in HBSS). The apparent permeability coefficients (Papp) for $[^3]$Hranitidine were calculated as described in the Materials and Methods section.

3.21.5 Influence of PEG 400 on Other Efflux Substrates (BCRP and P-gp)

In order to determine if PEG 400 induced inhibition of P-gp activity was a ranitidine-specific phenomenon, the bidirectional transport of $[^3]$H digoxin (P-gp substrate) (Eichelbaum et al., 2002, Gold et al., 1953, Greiner et al., 1999, Greiner et al., 1998) and $[^3]$H cimetidine (BCRP and P-gp substrate) (Merino et al., 2005) was performed.
across Caco-2 cell monolayers in the presence and absence of the highest concentration (20%) of PEG 400 used in these studies. As shown in Tables 4.5 and 4.6, a polarized efflux of both digoxin and cimetidine occurred in the control monolayers (no PEG 400). However in the presence of PEG 400, there was a partial inhibition of the efflux transport of digoxin but no effect on cimetidine transport. This suggests that PEG 400 is most likely influencing P-gp. This links back to the human study where PEG 400 had more of an influence on the bioavailability of ranitidine compared with cimetidine.

Table 4.5: Effects of PEG 400 on $[^3H]$ Digoxin Transport Across Caco-2 Cell Monolayers

<table>
<thead>
<tr>
<th>PEG 400 Concentration, (%)</th>
<th>Papp (cm/s x 10$^{-6}$) AP-to-BL</th>
<th>Papp (cm/s x 10$^{-6}$) BL-to-AP</th>
<th>Papp(BL-to-AP)/</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.72 ± 0.13</td>
<td>3.04 ± 0.13</td>
<td>4.25</td>
</tr>
<tr>
<td>20</td>
<td>1.56 ± 0.15</td>
<td>3.72 ± 0.3</td>
<td>2.38</td>
</tr>
</tbody>
</table>

*The bidirectional transport of $[^3H]$ digoxin (concentration 0.1 mM; specific activity – 2.5 Ci/mmol) was examined across Caco-2 cell monolayers in the absence (no PEG-300 and 400, only HBSS) and presence of varying concentrations of PEG-300 and 400 on both sides of the Caco-2 cell monolayers (grown 21–28 days; n=3); experiment performed in triplicate with 3 replicates per variable on each occasion. Samples (100 µL) were taken from the receiver compartments every 30 min for 3 h and each receiver compartment was replenished with the appropriate transport buffer solution (HBSS or PEG in HBSS). The apparent permeability coefficients (Papp) for $[^3H]$ digoxin were calculated as described in the Materials and Methods section.*
Table 4.6: Effects of PEG 400 on [3H] Cimetidine Transport Across Caco-2 Cell Monolayers

<table>
<thead>
<tr>
<th>PEG 400 Concentration, (%)</th>
<th>Papp (cm/s x 10⁻⁶) AP-to-BL</th>
<th>Papp (cm/s x 10⁻⁶) BL-to-AP</th>
<th>Papp(BL-to-AP)/Papp(AP-to-BL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.44 ± 0.008</td>
<td>0.90 ± 0.03</td>
<td>2.06</td>
</tr>
<tr>
<td>20</td>
<td>0.44 ± 0.02</td>
<td>1.20 ± 0.17</td>
<td>1.75</td>
</tr>
</tbody>
</table>

*The bidirectional transport of [³H] cimetidine (concentration 0.1 mM; specific activity – 2.5Ci/mmol) was examined across Caco-2 cell monolayers in the absence (no PEG-300 and 400, only HBSS) and presence of varying concentrations of PEG-300 and 400 on both sides of the Caco-2 cell monolayers (grown 21–28 days; n=3); experiment performed in triplicate with 3 replicates per variable on each occasion. Samples (100 μL) were taken from the receiver compartments every 30 min for 3 h and each receiver compartment was replenished with the appropriate transport buffer solution (HBSS or PEG in HBSS). The apparent permeability coefficients (Papp) for [³H] cimetidine were calculated as described in the Materials and Methods section.

Influence of excipients on efflux transport has been investigated in vitro and changes in cellular membrane fluidity have been linked to the inhibition of P-gp activity in vitro for example (Hugger et al. 2002a). However, little work has been done to investigate the effects of these excipients in vivo in order to establish possible in vitro/ in vivo correlations. Enhancement of absorption when drugs which are P-gp substrates (e.g. daunorubicin) are co-administered with drugs that are P-gp inhibitors (e.g. verapamil) have been shown in vivo (Krishna & Mayer, 2000; Nooter et al 1987). The question remains as to whether the addition of excipients such as PEG 300 and 400 and non-ionic surfactants (e.g. Cremophor EL) to pharmaceutical formulations or exploratory formulations for animal studies produce similar inhibitory effects in the intestinal mucosa and hence enhance the permeation of drugs.
The results from the investigations in this study suggest that PEG 400 at lower concentrations (around 1%) has some effect on P-gp activity, however the effects are not significant. PEG 300, a lower analogue of PEG 400 has previously been shown to significantly reduce the P-gp activity in Caco-2 cell monolayers (Hugger et al., 2002a). However it has been established that Caco-2 cells differ depending on their source and passage number. Hence this might have an influence on the results noted in this study.

The results from the in-vivo study show that PEG 400 at lower doses, enhance the absorption of ranitidine in male volunteers. Although the results from the present study give some explanation for the results obtained for the male volunteers in the human studies, it does not categorically explain the phenomenon of gender differences noted in the human studies as Caco-2 cells are male derived cell lines. There are no known specific female cell lines available. Availability of female derived intestinal cell lines may be useful to explain the female effect. However it has increased the knowledge of the influence of PEG 400 of transporters, showing that whilst high concentrations may inhibit efflux, lower concentrations may have even greater effects. The results suggest that PEG 400 most likely has more of an effect of P-gp compared with BCRP.
3.22 Summary

The PEG polymers even at high concentrations (20%) did not affect Caco-2 cell layer permeability (i.e. paracellular transport), thus alternative explanations for the effects of PEG on ranitidine absorption observed in the human studies were investigated.

Ranitidine, PEG 300 and PEG 400 were shown to increase P-gp ATPase activity.

Polarised secretory transport of ranitidine was observed in Caco-2 cell monolayers, with an indication that this may be modified by PEG 300 and 400.

P-gp inhibition may explain the in vitro transport data for ranitidine and the enhanced bioavailability of ranitidine in the presence of PEG 400 in vivo.

PEG 400 was shown to influences the efflux transport of digoxin a classic P-gp substrate with no influence from BCRP on its efflux transport. However, PEG 400 was shown not to affect the efflux transport of cimetidine, the later being both a P-gp and BCRP substrate (Pavek et al., 2005)
CHAPTER 5:
General Discussion and Conclusion
The objective of this project was to determine the effects of polyethylene glycol 400 (PEG 400) on the intestinal absorption of BCS Class III drugs with high solubility, low permeability and to elucidate any gender effects.

Excipients are listed as inert compounds in formulations. However recent research has shown that many excipients exert not only physicochemical effects in drug formulations, but may also exert pharmacological effects. For example, PEG 400 at high doses (> 2.5 g) has been shown to accelerate intestinal transit and subsequently reduce the bioavailability of ranitidine, a drug with high solubility but poor permeability in male subjects (Basit et al. 2001, Schulze et al. 2003). In this thesis, the effects of lower doses of PEG 400 (0.5, 0.75, 1.25 and 1.5 g) on the bioavailability of ranitidine were investigated in both male and female subjects. All doses of PEG 400 enhanced the absorption of ranitidine in male subjects but not females, with the most pronounced effect in males noted with the 0.75 g dose of PEG 400 (63% increase in cumulative ranitidine excretion compared to control, p<0.05). The mechanism behind this effect was investigated by determining the effects of these lower doses on the metabolism of ranitidine in male and female subjects and also investigating the effects of PEG 400 on efflux transport in Caco-2 cell monolayers. The amount of PEG 400 excreted by the male and female volunteers was also determined. The results revealed that differences noted between males and females were not due to differences in metabolism or PEG 400 excretion in urine. The results of the effects of PEG 400 on the efflux transport in Caco-2 cells revealed that whilst PEG 400 does not affect the paracellular transport of ranitidine it may have some influence on its efflux transport.
Following the effects noted with ranitidine, the effects of PEG 400 on the bioavailability of another BCS class III drug (cimetidine) was also investigated in male and female subjects. The results followed a similar trend to those noted with ranitidine. The absorption of cimetidine was enhanced with PEG 400 doses below 1.5 g in male subjects. In the female volunteers, there was no difference in the bioavailability of cimetidine in the presence of all concentrations of PEG 400. This confirms that the phenomenon is not restricted to ranitidine. Although the trends were similar for both ranitidine and cimetidine, PEG 400 had less of an effect in enhancing the absorption of cimetidine compared to ranitidine. This may be due to the fact that cimetidine is a substrate for two transporters P-gp and BCRP compared with P-gp only for ranitidine. The effects of PEG 400 on influx transporters OCT which are involved in the transport of ranitidine and cimetidine are yet to be determined.

The unexpected findings in this research are expected to have considerable regulatory implications for the use of PEG 400 in pharmaceutical formulations, especially the gender differences observed.

Another primary objective of this research was to establish whether the rat would comprise a good model to investigate the effects of PEG 400 on ranitidine bioavailability, to determine if the results in man are reproduced in the rat and more importantly to be able to obtain plasma profiles. The results showed there was no difference in the bioavailability of ranitidine in the absence of PEG 400. However unlike the human studies, no gender
differences were observed in the presence of PEG 400. This may be due to the low sample number (n=3 for each gender).

The importance of this type of research is widely recognised:

- The Biopharmaceutics Classification Systems Working Group of the Product Quality Research Institute (PQRI) which identifies biopharmaceutics research areas to facilitate the regulation of oral drug products suggested three proposals to the FDA as being of great importance in evaluating the risks in potentially expanding the BCS classification (Polli, 2002). The proposals include studying:
  1) Influence of common excipients on intestinal transit and absorption of class 3 drugs
  2) Influence of common excipients on drug intestinal permeability.
  3) Criteria for dissolution profile similarity for class 1 and class 3 drugs.

- From 1998, the Final Rule on the investigational new drug applications and new drug applications (FDA, 1998) requires that effectiveness and safety data are presented by gender, age, and racial subgroups and any modifications of dose or dose interval needed for specific subgroups should be identified. This document allows the FDA to refuse the filing of any NDA that does not analyse efficacy and safety data information by gender, age and racial subgroups. Gender is deemed to be most important and essential.

The findings of this thesis contribute to the knowledge and research the PQRI have suggested to the FDA as being important areas of research required in the biopharmaceutics
field. This research also contributes to the knowledge required in the debate of extending biowaivers to BCS class III drugs. Also the FDA currently has a database of excipients in FDA approved products and includes the maximum potency of excipients that can be contained in a particular route or dosage form a drug product containing the excipient (CDER/FDA, 2005). Excipients used in these dosage forms should not have significant effect on the rate and extent of absorption of the drug (CDER/FDA, 2000, EMEA/CPMP, 2001). This work suggests that perhaps some of the excipients in this list may require further investigations
CHAPTER 6:
Future Work
The following suggestions for future work are proposed to complete the picture and gain a better understanding of the effects of excipients on modulating intestinal absorption:

- Studies by Bourdet and Thakker (2006) have suggested that the absorptive transport of ranitidine in Caco-2 cells is partially mediated via a pH dependent uptake transporter for organic cations and that it is subject to attenuation by P-gp. The uptake carrier was shown to exhibit similar properties to cloned human organic cation transporters. Hence investigations into the effects of excipients such as PEG 400 on influx transporters such as organic cation transporters may elucidate if perhaps the effects observed in man may also be due to influx transporters and not limited to efflux transport by P-gp alone. Further studies to investigate the effects of PEG 400 on other efflux transporters such as BCRP and MRP may elucidate if the effects of PEG 400 are more general to efflux transporters.

- Investigation into the effects of PEG 400 on other drugs: cimetidine and ranitidine are both H₂ receptor antagonists and BCS Class III compounds. It would important to determine if the enhanced bioavailability observed with these two drugs would also occur in other BCS class III drugs. It would also be important to determine if the PEG 400 effects are limited to drugs affected by efflux transporters or if perhaps drugs transported predominantly via the paracellular route such as atenolol may also be influenced by PEG 400.

- Previous studies have shown the influence of high amounts of excipients such as PEG 400 (2.5 – 10 g) and mannitol (5 g) on shortening intestinal transit using
scintigraphy and the subsequent reduction in drug bioavailability. These studies were all in male volunteers. In light of the results obtained in this thesis, it would be important to investigate if there are gender differences in the influence of excipients such as PEG 400 on intestinal transit.

- The use of rat in-situ perfusion studies may provide more realistic mechanistic understanding of findings from the human studies. The in-situ rat intestinal perfusion method has been shown to correlate well with in vivo-human data (Fagerholm et al. 1996; Salphati et al. 2001)

- The main aim of future work would be to provide additional data to compile a comprehensive library on the effects of pharmaceutical excipients on gastrointestinal transit and drug bioavailability. This would allow pharmaceutical companies to use such information as a screening tool to identify drug excipient combinations with potential excipient induced bioavailability issues or added advantage and therefore help rationalise future formulation strategy for poorly water-soluble drugs.
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