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Sex, Drugs and Excipients:
PEG 400 enhances the bioavailability of BCS class III drugs via P-glycoprotein inhibition

Tese de doutoramento em Ciências Farmacêuticas na especialidade de Tecnologia Farmacêutica
Orientada pelos Professores Doutores Francisco Veiga, Joao Sousa e Abdul Basit
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Thank you all for these amazing years!

Sex, age, genetics, hormones, diet and disease can alter the activity of drugs through either or both their pharmacokinetic and pharmacodynamic parameters. Excipients can significantly affect the oral bioavailability of several drugs and their identification is crucial for the drug formulation process. Polyethylene glycol 400 (PEG 400) is widely used excipient in the pharmaceutical industry.

It was previously found in humans that PEG 400 can change the oral bioavailability of ranitidine in a sex dependent way. At high doses, PEG 400, acts as an osmotic laxative reducing oral bioavailability of drugs in the sexes. However, at lower doses, PEG 400 significantly increased the bioavailability of ranitidine (BCS class III drug) in men but not women.

This PhD thesis describes the experimental work which has been undertaken to further our understanding and elucidate the potential effects of low doses of PEG 400 in oral drug delivery and whether sex plays an important role. Ideally, such studies should be conducted in humans; however, this approach would be costly and impractical. A more efficient alternative was to conduct the *in vivo* studies in animals. An animal model was developed. The gastrointestinal tract of different animals was characterised (rat, rabbit and pig) and the rat was selected due to being relatively easy to handle and inexpensive (Chapter 1). Sex differences were subsequently identified in the rat gastrointestinal tract because the effect of PEG 400 on the oral bioavailability of ranitidine was found to be sex dependent (Chapter 2). Male and female rats were then administered ranitidine (Chapter 3) and cimetidine (Chapter 4) and different doses of PEG 400, to validate the rat as a suitable model. Both ranitidine and cimetidine followed the same trends in humans and in the proposed model: PEG 400 increased the oral bioavailability of ranitidine and cimetidine, in a dose dependent manner, in males but not females. A dose of PEG 400 was identified as yielding the highest increase in the oral bioavailability of ranitidine and cimetidine in male rats and was used in the subsequent work.

The mechanism behind the bioavailability enhancing effect of PEG 400 was unclear and as such further studies were conducted. We found that PEG 400 could increase the oral bioavailability of ranitidine and cimetidine by increasing the solubility of the active substances and/or by inhibiting the metabolism and/or excretion and/or increasing their absorption. From literature data and the work conducted it was considered that the interaction of PEG 400 with membrane transporters was more likely to affect the oral bioavailability of ranitidine and cimetidine as these two substances suffer extensive efflux at the small intestine.

Other BCS class III compounds were selected based on their affinity to different intestinal transporters: ranitidine, cimetidine, ampicillin and metformin (Chapter 5). These were orally administered to male and female rats in the absence and presence of PEG 400. We found that PEG 400 increased the $AUC_{0\text{-}480}$ of ranitidine, cimetidine and ampicillin in male but not female rats. Metformin oral bioavailability was not affected by dosing with PEG 400 in either sex. A major transporter was identified as interacting with ranitidine, cimetidine and ampicillin, but not metformin, whose inhibition would lead to an increase of the bioavailability of these drugs: P-glycoprotein (P-gp). P-gp is an efflux pump at the intestinal lumen that prevents substances

from being absorbed. PEGs are known to inhibit P-gp in a concentration-dependent manner. By inhibiting P-gp, the oral bioavailability of our compounds would predictably be increased. This was confirmed in the subsequent work (Chapter 6) where a mechanistic study was undertaken by using a well-established P-gp blocker, verapamil.

We found that verapamil increased the oral bioavailability of ranitidine in both sexes. However, the increase was much higher in male (+252%) than in female rats (+140%), suggesting a higher sensitivity to P-gp inhibition in males. PEG 400 did not affect the oral bioavailability of ranitidine in the sexes if P-gp was blocked by verapamil. These results support the hypothesis that the sex-specific effect of PEG 400 on the bioavailability of certain drugs is due to the interaction of PEG 400 with the efflux transporter P-gp. If ranitidine was administered intravenously the route of administration of PEG 400 (oral or IV) did not bear an impact on the bioavailability of ranitidine. This showed us that the effect of PEG 400 is predominantly located at the intestine.

Excipients are capable of changing drug disposition and their role should not be underestimated. The degree to which excipients modulate drug bioavailability may be modified by sex. It is important that regulatory authorities and pharmaceutical industry take this knowledge into account during the pharmaceutical development stages of pharmaceutical products.

A farmacocinética e farmacodinâmica dos fármacos pode ser afectada por diversos factores como sexo, idade, genética, dieta e estado de saúde. Os excipientes podem ter um efeito significativo na biodisponibilidade oral. Entender esses efeitos é fundamental para o processo de formulação. O Polietileno glicol (PEG) 400 é um excipiente comumente usado na indústria farmacêutica.

Em experiencias anteriores descobriu-se que o PEG 400 era capaz de modular a biodisponibilidade oral da ranitidina dependendo do sexo dos voluntários. O PEG 400 é osmoticamente activo e como tal, em doses elevadas, funciona como um laxante reduzindo a biodisponibilidade oral de fármacos. No entanto, em doses baixas, o PEG 400 aumentou significativamente a biodisponibilidade oral da ranitidina em homens mas não em mulheres.

Nesta tese, procura-se obter uma melhor compreensão dos efeitos de doses baixas de PEG 400 na administração oral de fármacos, e se o sexo do individuo é um factor importante.

Idealmente estes estudos seriam conduzidos em humanos. No entanto seria demasiado caro e complexo. Como alternativa decidiu-se desenvolver um modelo animal para realizar o trabalho *in vivo*. O tracto gastrointestinal de vários animais (rato, coelho, porco) foi caracterizado (osmolalidade, pH, tensão superficial, capacidade tampão, solubilidade de dois fármacos modelo – prednisolona e 5-ASA) e optou-se pelo rato para continuar os trabalhos (Capitulo 1). Tendo em conta que estamos também a investigar efeitos relacionados com o sexo, o tracto gastrointestinal de ratos machos e fêmeas foi analisado (Capitulo 2). Para confirmar o modelo animal, foi administrada ranitidina (Capitulo 3) e cimetidina (Capitulo 4) a ratos machos e fêmeas na presença e ausência de PEG 400. Ambos os fármacos seguiram a mesma tendência em ratos e humanos: o PEG 400 aumentou a biodisponibilidade oral da ranitidina e da cimetidina, de um modo proporcional a dose administrada, em machos mas não em fêmeas. A dose de PEG 400 que levou ao maior aumento da biodisponibilidade oral destes fármacos foi selecionada para os estudos seguintes.

Nos estudos seguintes preocupámo-nos com o esclarecimento do mecanismo pelo qual o PEG 400 aumenta a biodisponibilidade destes fármacos em machos mas não em fêmeas. Várias explicações seriam possíveis mas a nossa hipótese suportada por dados acima e por literatura publicada prende-se com a capacidade do PEG 400 de interagir com transportadores membranares já que a ranitidina e a cimetidina sofrem efluxo a nível da mucosa intestinal, dificultando a sua absorção.

Outros fármacos cuja absorção é igualmente limitada por efluxo intestinal (Capitulo 5), ampicilina e metformina, foram administrados oralmente a machos e fêmeas na presença ausência de PEG 400. Descobrimos que o PEG 400 aumenta a $AUC_{0\text{-}480}$ da ranitidina, cimetidina e ampicilina em machos e não em fêmeas. No entanto aparenta não ter efeito na biodisponibilidade da metformina, independentemente do sexo. Um transportador intestinal responsável pelo efluxo dos três primeiros fármacos mas não da metformina foi identificado: Glicoproteína P, P-gp. Esta, e responsável pelo efluxo de fármacos do epitélio intestinal para o lúmen, dificultando a absorção. Sabe-se que o PEG inibe a P-gp. A inibição da P-gp levaria a

um aumento da absorção dos seus substratos, uma vez que não seriam secretados de volta para o lúmen intestinal. Este facto foi confirmado no Capitulo 6, onde os efeitos de um bloqueador da P-gp na biodisponibilidade da ranitidina foram estudados.

O PEG 400 não alterou a biodisponibilidade oral da ranitidina em ambos os sexos se a P-gp já tivesse sido bloqueada pelo verapamilo. Ora, isto significa que o efeito do PEG 400 está de facto relacionado com a P-gp e a sua inibição. Os nossos resultados demonstram que o verapamilo oral aumenta a biodisponibilidade oral da ranitidina em ambos os sexos. No entanto este aumento e substancialmente mais significativo nos machos (+252%) que nas fêmeas (+140%), sugerindo uma maior sensibilidade dos machos a inibição da P-gp que as fêmeas. Importa salientar ainda que quando a ranitidina foi administrada por via intravenosa o PEG 400 não afectou a sua biodisponibilidade, independentemente da via de administração do PEG (oral ou IV). Isto demonstra que o efeito do PEG se deve essencialmente a um efeito localizado a nível intestinal.

Com este trabalho, foi demonstrado que os excipientes podem alterar a biodisponibilidade de vários fármacos, afectando a sua absorção. Importa salientar que o grau de modulação da absorção oral de fármacos mediada por excipientes pode ser dependente do sexo do individuo. Como tal, as autoridades regulamentares e a indústria farmacêutica devem ter especial cuidado aquando da formulação e avaliação de medicamentos.

List of Publications

Published papers

Mai, Yang; Afonso-Pereira, Francisco; Murdan, Sudaxshina; Basit, Abdul W. Excipient-mediated alteration in drug bioavailability in the rat depends on the sex of the animal. European Journal of Pharmaceutical Sciences, v. 107, n. 1, p. 249-255, 2017.

Afonso-Pereira, Francisco; Murdan, Sudaxshina; Sousa, Joao; Veiga, Francisco; Basit, Abdul W. Sex differences in excipient effects: Enhancement in ranitidine bioavailability in the presence of polyethylene glycol in male, but not female, rats. International Journal of Pharmaceutics, v. 506, n. 1-2, p.237-241, 2016

Merchant, Hamid A.; Afonso-Pereira, Francisco; Rabbie, Sarit C.; Youssef, Sandy A.; Basit, Abdul W. Gastrointestinal characterisation and drug solubility determination in animals. Journal of Pharmacy and Pharmacology, v. 67, n. 5, p. 630-639, 2015.

Merchant, Hamid A.; Rabbie, Sarit C.; Varum, Felipe J.O.; Afonso-Pereira, Francisco; Basit, Abdul W. Influence of ageing on the gastrointestinal environment of the rat and its implications for drug delivery. European Journal of Pharmaceutical Sciences, v. 62, n. 1, p. 76-85, 2014.

Prepared manuscripts

Afonso-Pereira F, Murdan S, Sousa J, Veiga F, Basit AW; Sex differences in the gastrointestinal tract of adult Wistar rats.

Afonso-Pereira F Ashiru D, Patel R, Murdan S, Basit AW; Sex differences in the gut: influence of an excipient on cimetidine bioavailability in humans and rats.

Afonso-Pereira F, Yang M, Murdan S, Basit AW; Establishing Bioequivalence: Can sex play a role in the effect of excipients in the bioavailability of BCS III drugs?

Afonso-Pereira F, Murdan S, Sousa J, Veiga F, Basit AW; *In vivo* inhibition of P-glycoprotein enhances the bioavailability of ranitidine: a mechanistic study.

Plagiarism statement

I, Francisco de Matos Afonso Pereira confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

The work presented hereafter also had the contribution of other members of the research group at the UCL School of Pharmacy, London, UK. This is clearly stated in each experimental chapter.

The following should be highlighted:

- Chapter 1 - *Gastrointestinal characterisation and drug solubility determination in animals*

  This chapter has already been published. There is data corresponding to three different animal species: rat, rabbit and pig. In this work I characterised the GI tract of the rabbit and approximately of half the rats. I also performed determination of the solubility of the model drugs, ran the HPLC analysis and performed the statistical analysis of the entirety of the data. The Pig data is included as it is relevant to the discussion.

- Chapter 4 – *Sex differences in the gut: influence of an excipient on cimetidine bioavailability in humans and rats*

  In this chapter there is rat and human data. The rat data was generated by me. The Human data was generated by Diane Ashiru in her PhD Thesis from August 2009 in the School of Pharmacy, London, UK. The Human data has not been published but is relevant to my thesis. As such this data was integrated to support the discussion of the rat results.

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the mean AUC_{0-480} with the control groups are also represented. *Denotes statistically significant difference compared to the control group ($p<0.05$).

178 Figure 10 Individual plasma profiles for male and female rats dosed with ranitidine intravenously or orally.

179 Figure 11 Mean AUC_{0-480} of male rats dosed with ranitidine, verapamil and/or PEG 400 through different routes of administration. The percentage differences in the mean AUC_{0-480} with the control group are also represented.

Introduction

Sex, age, genetics, hormones, diet and disease can alter the activity of drugs through either or both its pharmacokinetic and pharmacodynamic parameters (Jamei et al., 2009, Nicolas et al., 2009). Sex-related differences have been shown in areas ranging from the efficacy of selective serotonin reuptake inhibitors (Berlanga and Flores-Ramos, 2006) to cardiovascular pharmacology (Oertelt-Prigione and Regitz-Zagrosek, 2009, Ueno and Sato, 2012), pain management (Fillingim et al., 2009, Pleym et al., 2003), incidence of adverse drug effect, and drug toxicity (Bigos et al., 2009, Nicolas et al., 2009, Makkar et al., 1993, Aichhorn et al., 2005). It is commonly accepted that active substances cannot, for most of the times, be directly administered to patients. Generally, different chemical substances (excipients) are combined with the active substance to produce a final medicinal product that is safe, practical to administer and stable. This process is called *"Pharmaceutical formulation*" and it yields a pharmaceutical form. The excipients used in any given formulation are carefully chosen to optimise the stability, deliverability and manufacturability of pharmaceutical products. Different excipients have been reported to significantly affect the oral bioavailability of several drugs (Garcia-Arieta, 2014). Some excipients can change the residence time of active substances in their respective absorption sites, while others can affect the permeation of compounds across biological membranes (Goole et al., 2010). Identifying excipient effects in the bioavailability of drugs is crucial for the formulation process.

One such excipient is polyethylene glycol 400 (PEG 400). It is widely used as a solubilizer in liquid pharmaceutical forms, and as a binder or plasticizer for solid oral dosage forms (Raymond, 2009). PEGs, for example, can be used to improve solubility of active substances, preservatives, stabilizers, antioxidants, flavourings and colourings within syrups, lotions, oral solutions, among others.

Interestingly, it was previously found that, PEG 400 can modulate the bioavailability of different drugs in a sex dependent way. At high doses, PEG 400, acts as an osmotic laxative, reducing gastrointestinal transit time, thus reducing bioavailability, of drugs in the sexes (Basit et al., 2002, Schulze et al., 2003). However, at lower doses, PEG 400 significantly increases the bioavailability of ranitidine (BCS class III drug) in men but not women (Afonso-Pereira et al., 2017, Ashiru et al., 2008). Following these findings in humans, it was unclear if this effect was specific to ranitidine or if PEG 400 could also increase the oral bioavailability of other drugs. There was little understanding of the mechanism behind the sex dependent increase in oral bioavailability of ranitidine.

This PhD thesis describes the experimental work which has been undertaken to further our understanding and elucidate the potential effects of low doses of PEG 400 in oral drug delivery and whether sex plays an important role.

Ideally, such studies should be conducted in humans; however, this approach is prohibitively costly and impractical. A more efficient alternative was to conduct the studies in animals.

Pre-clinical testing has been paramount for the improved understanding of the behaviour of orally administered drugs. Various animal models are routinely used with the aim to mimic as closely as possible the conditions of the human gastrointestinal tract (Flaisher-Grinberg et al., 2010, Insel, 2007, Hannah-Poquette et al., 2011), however their relationships to humans is often underappreciated. There are known advantages of using animal models, however the lack of direct correlation or physiological compatibility between animals and humans remains, centrally, limiting. This is the case of adjustments for size and species. Results obtained between species are usually not typically transposable due to inter-subject variability (Calabrese, 1984). Other studies have demonstrated interspecies differences at the expression of membrane transporter in the gastrointestinal (GI) tract, such as efflux and influx pumps (Li et al., 2008, Dresser et al., 2000, Lai, 2009). However, it is important to understand that the usefulness of animal models lies in the estimation of drug behaviour, rather than in providing a physiologically identical paradigm immediately preceding human testing.

In oral drug delivery, understanding the physiology of the gastrointestinal tract is essential. So that an appropriate animal model could be chosen, the environment of the gastrointestinal tract of different candidate species was characterised.

In Chapter 1, "*Gastrointestinal characterisation and drug solubility determination in animals"*, the GI environment in the rat, rabbit and pig was characterised for osmolality, surface tension, pH and buffer capacity. The solubility of two model drugs, mesalazine (ionisable) and prednisolone (unionisable), were also measured and the results were correlated to the physicochemical fluid data. The results suggested that solubility of ionisable drugs or pH-responsive formulations were significantly influenced by differences in pH, depending on the animal species and the location of the GI tract. It was also found that the data on the GI solubility of prednisolone (a neutral compound) in rats might overestimate its true value in humans.

Because rats are a commonly used laboratory species, relatively easy to handle and inexpensive, they were chosen as the animal in which to develop the model.

Sex differences in the response of ranitidine bioavailability to PEG 400 were also identified thus, potential sex differences at the GI level of the rat were investigated.

In Chapter 2, *"Sex differences in the gastrointestinal tract of adult Wistar rats",* we aimed to characterise and identify potential sex related differences in the gastrointestinal luminal environment of male and female wistar rats. The pH, buffer capacity, surface tension and osmolality of the fluids collected from each GI tract section, stomach, duodenum, jejunum, ileum, caecum and colon were analysed. The solubility of two model drugs, prednisolone and 5-ASA was also quantified in the same fluids of male and female rats. The trends followed by each parameter were similar in the sexes, however differences in the values were observed. These were mainly identified in the stomach and in the distal portion of the gastrointestinal tract: ileum, caecum and colon. The differences identified in this chapter would predictably

affect the solubility of active substances, however, the ones used in the subsequent studies that compose this Thesis are all very soluble and are not considered to be significantly affected by these parameters.

After choosing the rat as an animal model and identifying physiological sex differences at the GI level, it was important to investigate whether the enhancing effect of PEG 400 in the oral bioavailability of ranitidine would be reproducible in the rat.

In Chapter 3, *"Sex differences in excipient effects: Enhancement in ranitidine bioavailability in the presence of polyethylene glycol in male, but not female, rats"*, ranitidine was orally administered with increasing doses of PEG 400 in an oral solution. Blood samples were withdrawn and assayed via HPLC-UV. Individual ranitidine plasma profiles were plotted for each rat, and standard pharmacokinetic parameters were determined. It was found that the effect of PEG 400 was dose dependent in the male rat. A dose of PEG 400, 26 mg/kg, was identified as causing the highest significant increase (+49%) in the mean area under the plasma concentration *versus* time curve ($AUC_{0\text{-}480}$) of ranitidine compared to the control group where no PEG was administered. On the other hand, females showed no statistically significant difference between the different groups with various PEG 400 concentrations. In conclusion, low doses of PEG 400 enhanced the bioavailability of ranitidine in male, but not female, rats. These findings are in agreement with previously published human data, therefore supporting the rat model.

Data on ranitidine alone, would not be sufficient to establish the rat as a suitable model. It could be argued that the effects observed were singular to ranitidine. It was decided to repeat the experiments but this time with cimetidine, in humans and rats, males and females.

In Chapter 4, *"Sex differences in the gut: influence of an excipient on cimetidine bioavailability in humans and rats",* the aim of the study was to understand if the sex dependent influence of PEG 400 was more universal (*i.e.* applied to other drugs) and if the rat is a robust predictor of bioavailability trends in humans. Cimetidine was co-administered with different doses of PEG 400 to fasted men and women. The cimetidine amount excreted in urine over 24 hours was quantified as an indication of oral bioavailability. Fasted male and female Wistar rats were also dosed with cimetidine and different doses of PEG 400. The changes in the bioavailability of cimetidine in humans and rats by PEG 400 followed the same trend as for ranitidine. It was found that low doses of PEG 400 increased the oral bioavailability of cimetidine in men and male rats. The degree at which PEG 400 modified the oral bioavailability of cimetidine was dose dependent. Women and female rats were not affected by low doses of PEG 400. A sex difference was clear.

Several mechanisms could be hypothesized to explain why PEG 400 increases the bioavailability of ranitidine and cimetidine:

- PEG 400 increases the solubility of the active substances;
- PEG 400 inhibits the metabolism and / or excretion of the active substances;

- PEG 400 stimulates the absorption of the active substances by:
    - Increasing the intrinsic permeability of the intestinal membrane;
    - Inducing absorptive transporters of the intestinal membrane;
    - Inhibiting secretive transporters at the intestinal membrane.

These hypotheses were further tested in Chapters 5 and 6. The dose of PEG 400 that led to a highest increase in the oral bioavailability of ranitidine and cimetidine in male rats was selected to be carried forward in the subsequent studies (26 mg/kg).

In Chapter 5, *"Establishing Bioequivalence: Can sex play a role in the effect of excipients in the bioavailability of BCS III drugs?"*, we attempted to understand if different BCS class III compounds were affected by low doses of PEG 400; if the effect of PEG 400 is sex dependent and, in doing so, to gain a better understanding of the mechanisms underlying the bioavailability enhancing effect of PEG 400. The potential impact of sex dependent excipient effects on establishing bioequivalence between medicinal products was also considered.

In this study male and female wistar rats were administered ranitidine, cimetidine, ampicillin or metformin in the presence and absence (control) of 26 mg/kg of PEG 400. The pharmacokinetic profiles of the active substances were evaluated. It was found that PEG 400 increased the $AUC_{0\text{-}480}$ of ranitidine, cimetidine and ampicillin. This effect was only observed in male rats but not female rats. Metformin oral bioavailability was not affected by dosing with PEG 400 in either sex.

These active substances are all very soluble so it was considered unlikely that PEG would further affect their solubilisation in the GI fluids.

It was reported that PEG 400 is not very well absorbed (Lin and Hayton, 1983). It was unlikely that metabolism played an important role in the metabolism and excretion of these drugs. However, this possibility should to be further investigated.

Concentrations of PEG 400 up to 15% w/w were reported not to have had an effect in the passive permeability of some drugs (Ma et al., 2017). In our study, the concentration of PEG 400 in the oral solution administered to the rats was approximately of 1.2%, well below the concentration identified as interfering with intestinal tight junctions. It is improbable that PEG 400 enhances the bioavailability of ranitidine, cimetidine and ampicillin by increasing membrane permeability. Furthermore, metformin was not affected by PEG 400 on either sex and is also substantially absorbed by passive diffusion.

Ranitidine, cimetidine, ampicillin and metformin are BCS – III drugs. This means that they are classified in the biopharmaceutical classification system as being class III. Class III drugs are known to be very water soluble but of limited absorption. BCS class III drugs can be grouped based on the mechanism underlying their poor permeability (Estudante et al., 2013):

- Intrinsically poorly permeable molecules – ionized, polarized or simply too large to effectively cross the phospholipid bi-layer;

- Substrates for efflux transporters – Molecules that can cross the cell membrane with relative ease but are subjected to efflux transport mechanisms at the apical end of the enterocytes. Once having reached the cytosol, the active substance will be actively transported back into the intestinal lumen, delaying the absorption.

Having ruled out the effect of PEG 400 in these active substances solubility and passive membrane permeability we hypothesised that PEG 400 could interact with GI transporters, thus influencing drug bioavailability.

A major transporter was identified as interacting with ranitidine, cimetidine and ampicillin, but not metformin, whose inhibition would lead to an increase of the bioavailability of these drugs: P-glycoprotein (P-gp). PEGs are known to inhibit P-gp in a concentration-dependent manner (Hugger et al., 2002, Shen et al., 2006). PEG 400 at concentrations of 1% (w/v) and 2% (w/v) is capable of blocking P-gp in a caco-2 cell line model (Hodaei et al., 2015).The presence of PEG at 1%, 5%, and 20% (w/v) reduced efflux of digoxin (by 47%, 57%, and 64%, respectively, when compared to control (Johnson et al., 2002). Also, the P-gp inhibitor cyclosporine A, when orally administered to male and female rats led to an increase of the bioavailability of ranitidine and ampicillin but not metformin (Mai et al., 2017). In our oral solution PEG 400 was used in a concentration approaching 1.2%, which would lead to an inhibition of approximately 50% on the activity of P-gp. It is thus plausible to assume that P-gp plays a major role in the effect of PEG 400 in BCS-III drugs subjected to efflux transporters at the intestinal lumen.

PEG 400, in small amounts, was capable of modifying the oral bioavailability of some BCS-III drugs in a sex dependent manner. This mechanism is most likely related to its inhibitory effects over P-gp.

In Chapter 6, *"In vivo inhibition of P-glycoprotein enhances the bioavailability of ranitidine: a mechanistic study"*, a mechanistic study was conducted with a well-established P-gp inhibitor, verapamil. The aims were: to understand if the P-gp inhibitor, verapamil, affected the oral bioavailability of ranitidine in a similar manner to PEG 400 and if there were similar sex differences; to understand if food played a role in the bioavailability enhancing effect of PEG 400 in male rats; to determine the absolute bioavailability of ranitidine in male and female rats; to understand if PEG 400 exerts its action by influencing drug absorption, metabolism and/or excretion of ranitidine.

We found that PEG 400 increased the oral bioavailability of ranitidine in male but not female rats. Verapamil increased the oral bioavailability of ranitidine in both sexes. However, the increase was much higher in male (+252%) than in female rats (+140%). PEG 400 did not affect the oral bioavailability of ranitidine in the sexes if P-gp was blocked by verapamil. These

results prove the hypothesis that the sex-specific effect of PEG 400 on the bioavailability of certain drugs is due to the interaction of PEG 400 with the efflux transporter P-gp.

A double plasma concentration peak was observed when ranitidine was administered orally in both sexes but disappeared if ranitidine was given intravenously. If ranitidine was administered intravenously the route of administration of PEG 400 (oral or IV) did not bear an impact on the bioavailability of ranitidine. This showed us that the effect of PEG 400 is predominantly located at the intestine.

Influx and efflux transporters, GI luminal fluid volumes, and GI transit time may play a role in these sex differences. Furthermore, PEG 400 effects may be applicable to even more BCS III drugs which are substrates for P-gp. Consequently, the potential for an "active" role of excipients in pharmaceutical formulations should not be underestimated, and particularly in the case of formulations for poorly permeable and/or soluble compounds belonging to BCS classes III and IV. This also applies to the potential for previously unrecognized gender-specific effects, which may prove to be dangerous should these excipient effects be widened to other excipients and active substances. For example, considering generic or hybrid products, bioequivalence studies may have been conducted only in male subjects that deemed the products bioequivalent when in reality they would not be so in females.

Excipients are capable of changing drug disposition. The degree to which excipients modulate drug bioavailability may be modified by sex. It is important that regulatory authorities and pharmaceutical industry take this knowledge into account during the pharmaceutical development stages of their products.

1. REFERENCES

AFONSO-PEREIRA, F., ASHIRU, D., PATEL, R., MURDAN, S. & BASIT, A. W. 2017. Sex differences in the gut: influence of an excipient on cimetidine bioavailability in humans and rats. UCL School of Pharmacy, University College London.

AICHHORN, W., GASSER, M., WEISS, E. M., ADLASSNIG, C. & MARKSTEINER, J. 2005. Gender differences in pharmacokinetics and side effects of second generation antipsychotic drugs. *Current Neuropharmacology,* 3**,** 73-85.

ASHIRU, D. A., PATEL, R. & BASIT, A. W. 2008. Polyethylene glycol 400 enhances the bioavailability of a BCS class III drug (ranitidine) in male subjects but not females. *Pharm Res,* 25**,** 2327-33.

BASIT, A. W., PODCZECK, F., NEWTON, J. M., WADDINGTON, W. A., ELL, P. J. & LACEY, L. F. 2002. Influence of polyethylene glycol 400 on the gastrointestinal absorption of ranitidine. *Pharmaceutical Research,* 19**,** 1368-74.

BERLANGA, C. & FLORES-RAMOS, M. 2006. Different gender response to serotonergic and noradrenergic antidepressants. A comparative study of the efficacy of citalopram and reboxetine. *Journal of Affective Disorders,* 95**,** 119-123.

BIGOS, K. L., POLLOCK, B. G., STANKEVICH, B. A. & BIES, R. R. 2009. Sex Differences in the Pharmacokinetics and Pharmacodynamics of Antidepressants: An Updated Review. *Gender Medicine,* 6**,** 522-543.

CALABRESE, E. J. 1984. GASTROINTESTINAL AND DERMAL ABSORPTION - INTERSPECIES DIFFERENCES. *Drug Metabolism Reviews,* 15**,** 1013-1032.

DRESSER, M. J., GRAY, A. T. & GIACOMINI, K. M. 2000. Kinetic and selectivity differences between rodent, rabbit, and human organic cation transporters (OCT1). *Journal of Pharmacology and Experimental Therapeutics,* 292**,** 1146-1152.

FILLINGIM, R. B., KING, C. D., RIBEIRO-DASILVA, M. C., RAHIM-WILLIAMS, B. & RILEY, J. L., III 2009. Sex, Gender, and Pain: A Review of Recent Clinical and Experimental Findings. *Journal of Pain,* 10**,** 447-485.

FLAISHER-GRINBERG, S., KRONFELD-SCHOR, N. & EINAT, H. 2010. Models of mania: from facets to domains and from animal models to model animals. *Journal of Psychopharmacology,* 24**,** 437-438.

GARCIA-ARIETA, A. 2014. Interactions between active pharmaceutical ingredients and excipients affecting bioavailability: impact on bioequivalence. *Eur J Pharm Sci,* 65**,** 89-97.

GOOLE, J., LINDLEY, D. J., ROTH, W., CARL, S. M., AMIGHI, K., KAUFFMANN, J.-M. & KNIPP, G. T. 2010. The effects of excipients on transporter mediated absorption. *International Journal of Pharmaceutics,* 393**,** 17-31.

HANNAH-POQUETTE, C., ANDERSON, G. W., FLAISHER-GRINBERG, S., WANG, J., MEINERDING, T. M. & EINAT, H. 2011. Modeling mania: Further validation for Black Swiss mice as model animals. *Behavioural Brain Research,* 223**,** 222-226.

HODAEI, D., BARADARAN, B., VALIZADEH, H. & ZAKERI-MILANI, P. 2015. Effects of polyethylene glycols on intestinal efflux pump expression and activity in Caco-2 cells. *Brazilian Journal of Pharmaceutical Sciences,* 51**,** 745-753.

HUGGER, E. D., AUDUS, K. L. & BORCHARDT, R. T. 2002. Effects of poly(ethylene glycol) on efflux transporter activity in Caco-2 cell monolayers. *Journal of Pharmaceutical Sciences,* 91**,** 1980-1990.

INSEL, T. R. 2007. From animal models to model animals. *Biological Psychiatry,* 62**,** 1337-1339.

JAMEI, M., DICKINSON, G. L. & ROSTAMI-HODJEGAN, A. 2009. A Framework for Assessing Inter-individual Variability in Pharmacokinetics Using Virtual Human Populations and Integrating General Knowledge of Physical Chemistry, Biology, Anatomy, Physiology and Genetics: A Tale of 'Bottom-Up' vs 'Top-Down' Recognition of Covariates. *Drug Metabolism and Pharmacokinetics,* 24**,** 53-75.

JOHNSON, B. M., CHARMAN, W. N. & PORTER, C. J. 2002. An in vitro examination of the impact of polyethylene glycol 400, Pluronic P85, and vitamin E d-alpha-tocopheryl polyethylene glycol 1000 succinate on P-glycoprotein efflux and enterocyte-based metabolism in excised rat intestine. *AAPS PharmSci,* 4**,** E40.

LAI, Y. 2009. Identification of interspecies difference in hepatobiliary transporters to improve extrapolation of human biliary secretion. *Expert Opinion on Drug Metabolism & Toxicology,* 5**,** 1175-1187.

LI, M., YUAN, H. D., LI, N., SONG, G. T., ZHENG, Y., BARATTA, M., HUA, F. M., THURSTON, A., WANG, J. & LAI, Y. R. 2008. Identification of interspecies difference in efflux transporters of hepatocytes from dog, rat, monkey and human. *European Journal of Pharmaceutical Sciences,* 35**,** 114-126.

MAI, Y., AFONSO-PEREIRA, F., MURDAN, S. & BASIT, A. W. 2017. Excipient-mediated alteration in drug bioavailability in the rat depends on the sex of the animal. *European Journal of Pharmaceutical Sciences,* 107**,** 249-255.

MAKKAR, R. R., FROMM, B. S., STEINMAN, R. T., MEISSNER, M. D. & LEHMANN, M. H. 1993. FEMALE GENDER AS A RISK FACTOR FOR TORSADES-DE-POINTES ASSOCIATED WITH CARDIOVASCULAR DRUGS. *Jama-Journal of the American Medical Association,* 270**,** 2590-2597.

NICOLAS, J.-M., ESPIE, P. & MOLIMARD, M. 2009. Gender and interindividual variability in pharmacokinetics. *Drug Metabolism Reviews,* 41**,** 408-421.

OERTELT-PRIGIONE, S. & REGITZ-ZAGROSEK, V. 2009. Gender Aspects in Cardiovascular Pharmacology. *Journal of Cardiovascular Translational Research,* 2**,** 258-266.

PLEYM, H., SPIGSET, O., KHARASCH, E. D. & DALE, O. 2003. Gender differences in drug effects: implications for anesthesiologists. *Acta Anaesthesiologica Scandinavica,* 47**,** 241-259.

RAYMOND, C. R. P. J. S. M. E. Q. 2009. *Handbook of Pharmaceutical Excipients*, Pharmaceutical Press.

SCHULZE, J. D., WADDINGTON, W. A., ELI, P. J., PARSONS, G. E., COFFIN, M. D. & BASIT, A. W. 2003. Concentration-dependent effects of polyethylene glycol 400 on gastrointestinal transit and drug absorption. *Pharmaceutical Research,* 20**,** 1984-8.

SHEN, Q., LIN, Y., HANDA, T., DOI, M., SUGIE, M., WAKAYAMA, K., OKADA, N., FUJITA, T. & YAMAMOTO, A. 2006. Modulation of intestinal P-glycoprotein

function by polyethylene glycols and their derivatives by in vitro transport and in situ absorption studies. *International Journal of Pharmaceutics,* 313**,** 49-56.

UENO, K. & SATO, H. 2012. Sex-related differences in pharmacokinetics and pharmacodynamics of anti-hypertensive drugs. *Hypertension Research,* 35**,** 245-250.

Chapter 1 - Gastrointestinal characterisation and drug solubility determination in animals*

* This chapter has been published, please refer to the List of Publications.

ABSTRACT

To characterise the gastrointestinal (GI) environment in rat, rabbit and pig for the purpose of determining their utility as animal models for drug delivery in humans.

GI fluid samples were characterised for osmolality, surface tension, pH and buffer capacity. The solubility of two model drugs, mesalazine (ionisable) and prednisolone (unionisable), were also measured and the results were correlated to the physicochemical fluid data.

The solubility of the ionisable drug mesalazine was positively correlated to the GI pH in all three species and was significantly influenced by the pH difference. In contrast, the solubility of the unionisable compound prednisolone was not correlated significantly to the changes in pH, buffer capacity, osmolality or surface tension. In general, the solubility of prednisolone was constant irrespective of the location of the sample in the gut from rabbit and pig; however, an unusual trend was observed for the solubility of prednisolone in rats.

The results suggest that solubility of ionisable drugs or pH-responsive formulations is significantly influenced by the differences in pH along the GI tract and inter-species differences. It was also found that the data on the GI solubility of prednisolone (a neutral compound) in rats might overestimate its true value in humans.

1. INTRODUCTION

Pre-clinical testing has been paramount for the improved understanding of the behaviour of orally administered drugs. Various animal models are routinely used with the aim to mimic as closely as possible the conditions of the human gastrointestinal tract (Flaisher-Grinberg et al., 2010, Insel, 2007, Hannah-Poquette et al., 2011), however their relationship to human gastrointestinal tract is often underappreciated.

The characteristics of the gastrointestinal milieu have a significant influence on orally administered drugs. The luminal environment is complex, leading to drug solubility and orally administered dosage form behaviour being affected by several factors: pH, fluid volume, buffer capacity, surface tension and osmolality.

Gastrointestinal pH and fluid volumes are especially important consideration; the volume of free fluid in the gut influence the dispersion of dosage forms as well as the drug solubility, while pH influences drug or compound ionisation, which in turn influences its solubility, stability, absorption and bioavailability. In addition, the change in gastrointestinal pH along the gastrointestinal tract affects the behaviour of modified release dosage forms, such as pH responsive enteric coated formulations.

Osmolality and fluid volume have been investigated in relation to buffer capacity and pH, given that the alteration of salt concentration and ingested fluids can alter both parameters through stimulating the secretion of gastric acid, bile and pancreatic juices (Hörter and Dressman, 2001, Fordtran and Locklear, 1966). This also bares direct relation to drug solubility, where a drug may be sparingly soluble in the gut fluids, or where fluids may be sporadically located along the length of the gut, as demonstrated by Schiller et al (2005) in the case of fluid 'pocket' presence in the small intestine. Alteration of fluid ionic content may furthermore influence drug ionisation, and hence limit absorption. Surface tension has previously been shown to be near-independent of pH in human gastric fluids, but is also known to affect dosage form kinetics following oral administration where a decrease in surface tension is associated with an increase in dissolution rate (Finholt and Solvang, 1968).

Despite the known advantages of using animal models, the lack of direct correlation or physiological compatibility between animals and humans remains, centrally, limiting. This is typical in the case of adjustments for size and species. Results obtained between species are also not typically transposable due to inter-subject variability (Calabrese, 1984). Other studies have additionally demonstrated interspecies differences at the membrane transporter expression in the gastrointestinal tract, such as efflux and influx pumps (Li et al., 2008, Dresser et al., 2000, Lai, 2009).

Nevertheless, the underlying principle of using animal models lies in the estimation of drug behaviour, rather than in providing a physiologically identical paradigm immediately preceding human testing. As such, a profound knowledge of different animals GI tract conditions should be made available. Although attempts have been made to elucidate these

features and critically reviewed across a wider range of species (Kararli, 1995), conflict between measurements have been apparent, namely in the case of pH of GI fluids (McConnell et al., 2008a, Ward and Coates, 1987, Smith, 1965). As far as we managed to assert, there is currently no animal model that directly relates to *in vivo* human behaviour in the whole extension of the gastrointestinal tract. It is thus important to elucidate how the GI luminal environment changes along the GI tract of different potential animal models, particularly for modified release formulations, as this will have a bearing on drug bioavailability.

As in the past we have investigated the pH, fluid volumes and lymphoid tissue distribution along the gastrointestinal tract in rat, mice (McConnell et al., 2008b) and guinea pig, rabbit and pig (Merchant et al., 2011), in the present study, we have expanded our investigations to cover physiological data of the rabbit and the pig in addition to the rat, including measurements of fluid volume, osmolality, surface tension and buffer capacity.

This study also aims to elucidate how the solubility of two model drugs, 5-Aminosalicylic acid and prednisolone changes in luminal fluids at different regions of the GI tract of the different species. Solubility, a major determinant of dissolution rate from a dosage form, is also one of the two factors used by the Biopharmaceutics Classification System (BCS). It is appreciated that *in-vivo* solubility is dependent upon the physicochemical properties of the drug and the composition of the dissolution medium it is exposed to and information on drug solubility in human small intestinal fluids is reported in the literature (Clarysse et al., 2009, Kalantzi et al., 2006, Fadda et al., 2010). 5-Aminosalicylic acid (5-ASA, mesalamine) and prednisolone were chosen as models for ionisable and non-ionisable drugs, respectively. These were selected as they are predominantly administered as enteric, delayed/modified release formulations, therefore regional solubility of such compounds along the gastrointestinal tract and interspecies differences, if any, becomes paramount.

2. MATERIALS AND METHODS

2.1 Materials

Mesalazine (mesalamine, 5-ASA) was obtained from Sigma Aldrich and prednisolone was obtained from Severn Biotech Ltd (Worcestershire, UK). Water, methanol, tetrahydrofuran and trifluoroacetic acid were purchased from Fisher Scientific (Loughborough, UK), and were of HPLC grade, volumetric standards for NaOH and HCl were used for buffer capacity determinations and were procured from Sigma Aldrich (Dorset, UK).

2.2 Gastrointestinal tissues

All procedures were approved by the Home Office (PPL No.70/6421) and were conducted in accordance with the Animals (Scientific Procedures) Act 1986, UK. The gastrointestinal tract from pig was obtained from freshly killed male animals (cross-breed of large white and landrace, 95-110 kg, 6 months) at Cheale Meats Ltd. (Essex, UK). Adult New Zealand white rabbits (n=6, 2.1-2.3 kg, 9-10 weeks) and Wistar rats (n=8, 220-255 g, 8 weeks) were purchased from Harlan UK Ltd (Oxfordshire, UK). All animals were fed *ad libitum*, rabbits: laboratory diet 5322 with added Vitamin C (IPS, Nottinghamshire, UK), pigs: Eltabreed Bingle Sow Cake – compound feed for pigs (ABN Ltd., Peterborough, UK), rats: Teklad Global 18% Protein Rodent Diet (Harlan Ltd., Oxfordshire, UK).

2.3 Gastrointestinal fluids

All measurements were performed on supernatant obtained from the gastrointestinal (GI) fluids from the laboratory animals. The animals were killed and the gastrointestinal tract was divided into stomach, small intestine, caecum and colon. Small intestine and colon were further divided. The gastrointestinal sections were emptied into appropriate containers, labelled and stored at -80 °C freezer until used. The GI fluids collected from rats, rabbits and pigs were centrifuged (Centrifuge 5415D, Eppendorf AG, Hamburg, Germany) at 13200 rpm (~16,110 x g rfc) for 20 minutes. The supernatant obtained were kept in the freezer (-80 °C) until analysed.

2.4 Osmolality

Osmolality of the supernatants from the gastrointestinal fluids was determined with use of a Digital Micro-Osmometer (Type 5R), (Hermann Roebling MESSTECHNIK, Berlin, Germany), where osmolality is measured exploiting the theory of freezing point depression. Samples were thawed to room temperate before measurements were conducted, and subsequently centrifuged (at 4472 rcf/5000 rpm for 10 minutes) to obtain the supernatants. A volume of 100 µL was used for each measurement.

2.5 Surface tension

Surface tension was measured using a Delta 8 Tensiometer (Kibron Inc) controlled by Delta-8 manager software (version 3.8). The measurement was performed using a DynePlates (96-well plate designed for tensiometer), with 50 µL supernatant (as obtained in section 2.4.6) in each well.

2.6 pH and Buffer capacity

The pH and buffer capacity of gastrointestinal supernatants was measured using a pH meter (HI99161) equipped with an FC202 electrode designed for measurements in viscous and semi-solid materials (Hannah Instruments, Bedfordshire, UK). Buffer capacity was measured at a pH change of 0.5 and 1.0 units by adding aliquots of HCl (for intestinal fluids) or NaOH (for gastric fluids) to a 300 µL supernatant sample from GI fluid to achieve the desired pH change. Buffer capacity was then calculated using following equation:

$$\beta\ (mmol/L/\Delta\mathrm{pH}) = \frac{M_a \times V_a}{\Delta pH} \times \frac{1000}{V_b}$$

Equation 1.

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

2.7 Solubility Studies

The solubility of an ionisable drug, mesalazine (5-ASA), and a neutral drug, prednisolone, were measured in supernatants from gastrointestinal fluids from rats, rabbits and pigs. The solubility data was compared with published data in human fluids, and correlated with investigated gastrointestinal features, where applicable.

a) *Sample handling and solubility measurements*

An excess of drug (20 mg prednisolone or 15 mg mesalamine), was added to microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) containing 200 µL of the supernatants from GI fluid. The samples were then placed in a shaking bath (Gallenkamp, Loughborough, UK) maintained at 37 °C at 170 rpm. After 24 hours, the samples were centrifuged at 5000 rpm (~4472g rcf) for 10 minutes (Centrifuge 5804R, Eppendorf AG, Hamburg, Germany) at 37 °C. The supernatant was transferred successively to a centrifuge filter tube (Corning® Spin-X®, 0.22 µm cellulose acetate membrane, obtained from Fisher Scientific, Leicestershire, UK) and then centrifuged at 5000 rpm (~4472g rcf) for 20 minutes (Centrifuge 5804R, Eppendorf AG, Hamburg, Germany) at 37 °C. Aliquots of the filtrate (50 µL) were removed and diluted with 1,950 µL of the mobile phase before analysis.

b) Analytical methods

The amount of prednisolone or mesalamine dissolved in the sample was assayed by a reversed-phase HPLC equipped with a UV detector (Agilent Technologies 1200 Series) using a method reported previously (Fadda et al., 2010). Blank gastrointestinal fluid samples (that is, without drug) were also analysed like solubility samples to rule out the possibility of interfering peaks being present at desired retention times. The blank samples were diluted in the same fashion as the sample, filtered and subsequently analyzed. The chromatographic conditions were: mobile phase (95% water, 5% methanol, 0.05% trifluoroacetic, 68.8% water for mesalazine and 25% tetrahydrofuran, 6.2% methanol for prednisolone); reverse phase columns, Water Symmetry C8 5 µm (Waters, Massachusetts, USA) for prednisolone and LiChrospher 100, C18, 5 µm (Merck, Darmstadt, Germany) for mesalamine. Drugs were detected at 228 nm (mesalazine) and 254 nm (prednisolone) at retention times 7.0 and 9.9 minutes for mesalazine and prednisolone respectively. The other assay conditions were same for both drugs (i.e., 20 µL injection volume, 1mL/min flow rate at 124 bar, and 40 °C column temperature).

2.8 Statistical analysis

The overall data was analysed by one-way ANOVA, followed by a Tukey post-hoc analysis with a 95 % confidence interval using IBM SPSS Statistics 19 (SPSS Inc., Illinois, USA). A Univariate General Linear Model tool was used with Tukey post-hoc analysis taking species and locations as fixed factors and statistical significance is stated at $P < 0.05$ on post-hoc results.

3. RESULTS AND DISCUSSION

Table 1. pH Buffer capacity, osmolality, surface tension of the gastrointestinal (GI) fluids (supernatants) from rat, rabbit and pig, and solubility of mesalazine and prednisolone in these fluids.

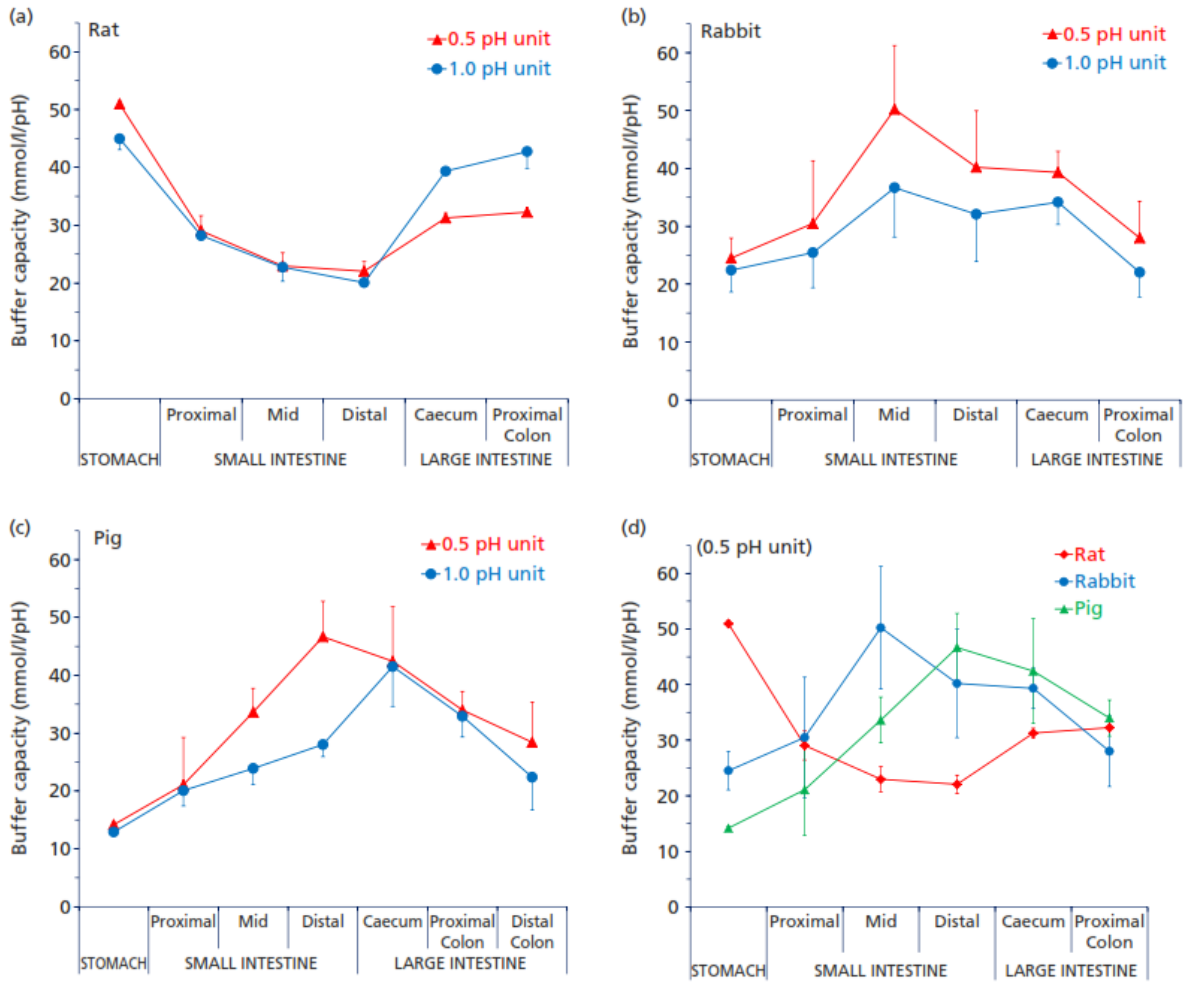
	Stomach	***Small Intestine***			***Caecum***	***Appendix***	***Colon***	
		Proximal	*Mid*	*Distal*			*Proximal*	*Distal*
pH*								
Rat	4.16 ± 0.01	5.99 ± 0.01	6.34 ± 0.01	6.76 ±0.04	6.08 ± 0.01	-	5.84 ± 0.01	-
Rabbit	2.22 ± 0.52	6.11 ± 0.66	7.01 ± 0.09	7.07 ± 0.22	6.23 ± 0.16	-	6.67 ± 0.28	-
Pig	2.38 ± 0.31	5.25 ± 1.00	6.79 ± 0.11	7.09 ± 0.09	5.71 ± 0.20	-	6.11 ± 0.20	6.70 ± 0.62
Human	1.0 – 2.5[5]	3.1 – 6.7[6]	7.1 ± 0.5[d]	7.2 ± 0.9[d]	-	-	6.0 – 7.7[d]	-
Osmolality (mOsm.Kg^{-1})								
Rat	794 ± 260	896 ± 104	640 ± 73	546 ± 62	540 ± 63	-	545 ± 89	-
Rabbit	285 ± 24	475 ± 36	527 ± 60	541 ± 37	419 ± 29	365 ± 51	372 ± 51	-
Pig	159 ± 38	540 ± 19	488 ± 57	439 ± 32	442 ± 30	-	470 ± 19	496 ± 32
Human	559/217 [a]	~400/287 [a]	200 ± 68 [b]	-	-	-	224 ± 125 [c]	-
Surface tension (mN.m^{-1})								
Rat	38 ± 2	33 ± 1	5 ± 1	39 ± 5	51 ± 8	-	52 ± 8	-
Rabbit	49 ± 3	39 ± 4	42 ± 8	39 ± 7	50 ± 1	49 ± 3	51 ± 5	-
Pig	49 ± 1	33 ± 5	35 ± 3	44 ± 6	48 ± 2	-	50 ± 2	58 ± 4
Human	30/31 [a]	28.1/28.8 [a]	28 ± 1 [5]	-	-	-	32.9 [c]	-
Buffer capacity (mmol.L^{-1}.⊿pH^{-1})								
Rat (0.5 unit)	51 ± 0.6	29.1 ± 2.6	23 ± 2.3	22.1 ± 1.7	31.3 ± 0.9	-	32.3 ± 0.4	-
(1.0 unit)	45 ± 1.9	28.2 ± 0.8	22.7 ± 2.4	20.1 ± 0.7	39.4 ± 0.6	-	42.7 ± 2.9	-
Rabbit (0.5unit)	24.5 ± 3.4	30.5 ± 10.8	50.2 ± 11.0	40.2 ± 9.7	39.3 ± 3.6	-	28.0 ± 6.3	-
(1.0 uni)	22.4 ± 3.8	25.4 ± 6.1	36.7 ± 8.5	32.1 ± 8.2	34.2 ± 3.7	-	22.0 ± 4.2	-
Pig (0.5 unit)	14.2 ± 0.4	29.8 ± 1.6	36.7 ± 9.0	44.3 ± 11	42.4 ± 9.4	-	34 ± 3.2	28.4 ± 7
(1.0 unit)	12.9 ± 0.3	21.3 ± 1.3	24.3 ± 4.2	27.3 ± 6	41.5 ± 6.9	-	33 ± 3.6	22.4 ± 6
Human	14/28 [a]	18-30 [a]	3.23 ± 1.27 [d]	6.4 [d] (22.9±17.3)[6]	-	-	~18.9 [c]	44.4 [d]
Prednisolone solubility (mg/mL) †								
Rat	0.67 ± 0.02	0.85 ± 0.03	1.31 ± 0.06	0.93 ± 0.05	0.85 ± 0.14	-	0.81 ± 0.28	-
Rabbit	0.42 ± 0.01	0.40 ± 0.04	0.43 ± 0.05	0.44 ± 0.06	0.39 ± 0.05	-	0.43 ± 0.05	-
Pig	0.59 ± 0.03	0.55 ± 0.06	0.61 ± 0.02	0.65 ± 0.08	0.57 ± 0.04	-	0.44 ± 0.03	0.46±0.07
Human	-	-	0.52 ± 0.02 [d]	0.46 [d]	-	-	0.41-0.55 [d]	0.54 [d]
Mesalamine solubility (mg/mL) †								
Rat	1.95 ± 0.09	3.31 ± 0.32	3.4 ± 0.21	3.41 ± 0.17	2.49 ± 0.1	-	2.16 ± 0.01	-
Rabbit	1.66 ± 0.20	4.06 ± 0.96	5.19 ± 0.84	5.28 ± 1.03	2.62 ± 0.58	-	3.25 ± 0.86	-
Pig	1.62 ± 0.14	3.32 ± 1.14	5.36 ± 0.5	4.41 ± 1.65	1.83 ± 0.43	-	2.68 ± 0.3	3.43±1.06
Human	-	-	1.98 ± 0.26 [d]	3.3 [d]	-	-	5.4-7.0 [d]	7.9 [d]

* pH measured from thawed gastrointestinal fluids during buffer capacity determination and does not relate to the *in situ* pH in the gut. In situ results can be found elsewhere (Freire et al., 2011, McConnell et al., 2008b).

[a] gastric or duodenal aspirates from 220 subjects (16M, 4F), figures represent 30/210 minutes post ingestion of Ensure® Plus, (β at 1.0 unit change in pH)(Kalantzi et al., 2006)
[b] supernatants from jejunal aspirates from six healthy subjects (2M, 3F) after an overnight *fast(Perez de la Cruz Moreno, 2006)*
[c] supernatants from 12 healthy subjects (6M, 6F) under *fed* state, (β at 1.0 unit change in pH)(Diakidou et al., 2009)
[d] average of four batches of jejunal aspirates, single batch of ileal and transverse/descending colonic fluid, (β at 0.5 unit change in pH)(Fadda et al., 2010)
[†] aqueous solubility at 37 °C: 1.32 mg/mL (prednisolone) and 0.223 mg/mL (mesalazine)
[5] (McConnell et al., 2008b)
[6] (Mudie et al., 2010)

3.1 Buffer capacity of gastrointestinal fluids

Figure 1. Buffer capacity (mean ± standard deviation (SD)) of gastrointestinal fluids (supernatants) from (a) pooled samples from rats (n = 3), (b) rabbits (n = 6), (c) pigs (n = 3), measured at 0.5 and 1.0 change in pH unit and (d) inter-species trend in buffer capacity across the gastrointestinal tract.



It is generally seen that the buffer capacity measured at a 1.0 pH unit change is lower than when measured with 0.5 unit change, which is due to a proportionally lower amount of acid or base needed to bring a 1.0 unit ΔpH compared to only 0.5 unit ΔpH. However, it was interesting to note that in rat caecum and colon, a reverse situation was found where buffer capacity at 1.0 unit change was higher; this can be attributed to the presence of multiple

buffer species in fermentation products, such as short chain fatty acids, having different pk_a hence a positive contribution to the buffer capacity at a higher pH change. It is also interesting to note that this behaviour was not seen in pig fluids, perhaps due to caecal fermentation being more important in rat nutrition (coprophagia) than in pig. As for the rabbit's buffer capacity, on average it was higher at 0.5 ΔpH units than at 1.0 ΔpH, nevertheless the difference was only statistically significant at the jejunum and ceacum ($p < 0.05$).

When measured at 0.5 unit change, the buffer capacity in the pig stomach was low which then increased in the small intestine ($p < 0.05$) peaking at the distal small intestine. Buffer capacity then decreased in the colon particularly achieving statistical significance in the distal colon ($p < 0.05$). Buffer capacity measured at 1.0 pH unit change showed a similar trend as with 0.5 pH units. Buffer capacity in the stomach was low which then increased in the small intestine ($p < 0.05$) but peaked in the caecum, unlike 0.5 pH unit where it peaked in the distal small intestine. Buffer capacity then decreased in the colon significantly ($p < 0.05$). In rats, a reverse trend was seen, with highest buffer capacity measured in gastric fluid, which was decreased down the small intestine and increased again in caecum and in colon (Figure 1a). It was interesting to note that the highest measured buffer capacity were not too different in rat and pig, but found in two opposite locations in the gut, i.e., stomach (rat) and distal small intestine (pig).

In the case of rabbit, the buffer capacity was shown to have a high inter-individual variability as seen in Figure 1b. Both 0.5 and 1 pH units trends were similar, differing only on their absolute values. As expected, buffer capacity reached a significant maximum ($p < 0.05$) at the jejunum probably due to food intake; and a second average peak at the caecum probably due to the increase levels of short chain fatty acids resulting from fermentation (though not significantly different from its adjacent intestinal parts).

Buffer capacity measured at a change of 0.5 and 1.0 pH units were significantly different ($p < 0.05$), in the small intestine of pigs, the stomach and large intestine of rats and the jejunum and caecum of rabbits (Figure 1, Table 1).

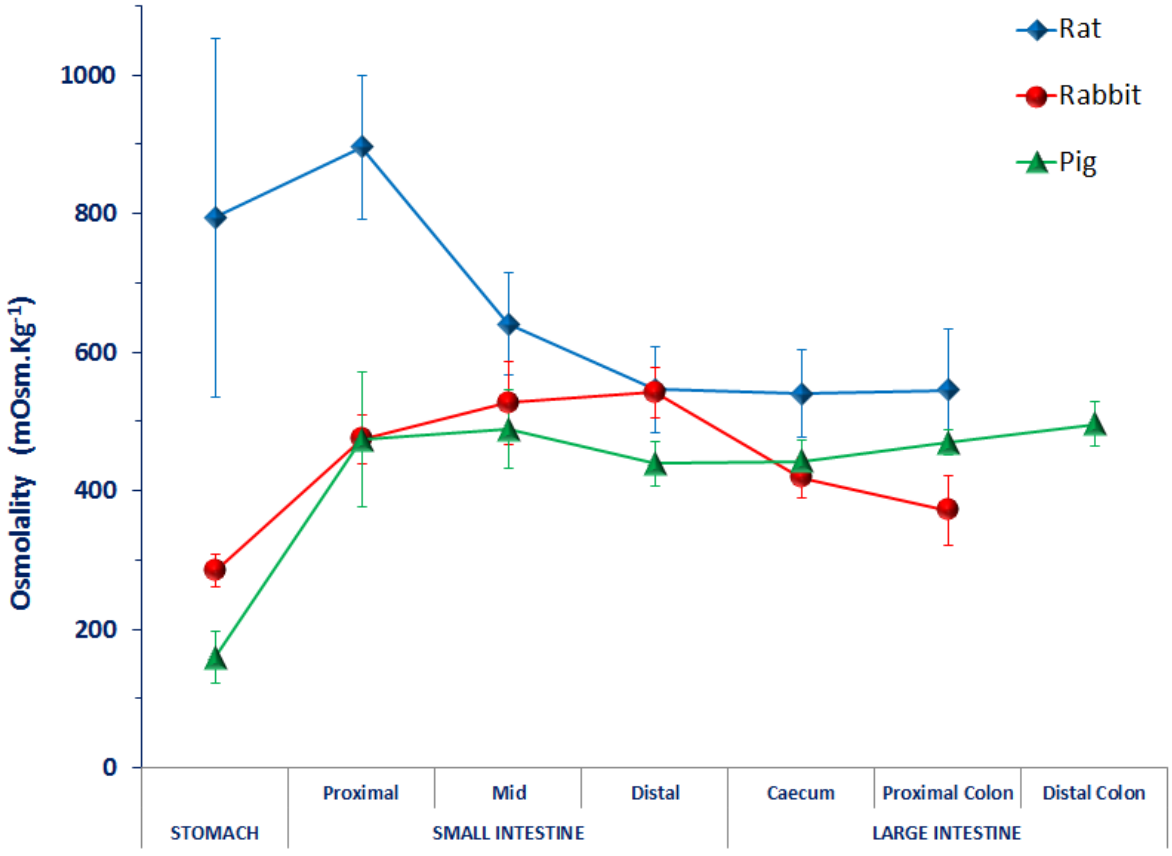
On comparison of the buffer capacity trend from these animal species to those reported in human, some interesting observations can be made. The buffer capacity of human gastric aspirates was reported to be 14 mmol/L/△pH thirty minutes post ingestion of liquid meal (Ensure® Plus) which increased to 28 mmol/L/△pH 210 minutes post-meal ingestion (Kalantzi et al., 2006). This was also related to the buffer capacity of the meal itself (Ensure Plus, 24 mmol/L/△pH). The buffer capacity measured thirty minutes post-ingestion of liquid meal in human was not dissimilar to those measured in pig gastric fluids, but surprisingly rat had remarkably higher buffer capacity in the stomach (Table 1). The buffer capacity of the duodenal aspirates varied between 18 and 30 mmol/L/△pH during 30 to 210 minutes post meal ingestion and were not too dissimilar to the buffer capacity in pig, rat and rabbit proximal small intestine (20.1, 28.2 and 25.4 mmol/L/△pH respectively). However it should be kept in mind that Kalantzi and colleagues measured buffer capacity in whole gastric and

duodenal aspirates whereas our measurements were performed in supernatants. The buffer capacity from human jejunal and ileal fluids were appreciably lower than those in rat, pig and rabbit (Table 1).

The buffer capacity of the supernatants from human ascending colon fluids was reported to be approximately 18.9 mmol/L/△pH (Diakidou et al., 2009) which is significantly lower than that in the proximal colon of pig and rat (33 and 42.7 mmol/L/△pH respectively) but not so different form the one found in rabbits (22.0 mmol/L/△pH). However a much higher buffer capacity (almost double) was estimated (37 mmol/L/△pH) when whole ascending colon fluids from human were tested instead of the supernatants. The authors suggested that this is due to the consumption of titrated acid by the bacteria-mediated reactions prevalent in the whole colonic fluids (Diakidou et al., 2009). This could also be attributed to the adsorption of acid on the colonic material, dominant in the whole fluid than in supernatants. The buffer capacity measured from a sample from human distal colon (Fadda et al., 2010) was almost double than what has been measured in pigs (Table 1), whereas the buffer capacity in rat and rabbit distal colon could not be measured due to lack of fluids in their respective distal gut.

3.2 Osmolality of the gastrointestinal fluids

Figure 2. Osmolality (mean ± standard deviation (SD)) of gastrointestinal fluids (supernatants) from rats (n = 3), rabbits (n = 6) and pigs (n = 3).



The osmolality of the gastrointestinal contents of the three animal models generally follows a trend, being low in the stomach, increasing in the proximal small intestine and decreasing distally. Pig seems to follow this pattern up the caecum, increasing slightly at the colon (Figure 2). This pattern of osmolality in general is in agreement with the physiology of the digestive system, where most of the food is digested and broken down into nutritional building blocks such as glucose, amino acids and fatty acids in the small intestine, hence there is a higher osmolality of the fluids. As nutrition gets absorbed further down the gut, osmolality of the contents decreases.

In rats, osmolality of the gastric contents was not statistically different from that in proximal and mid small intestine due to high variability in gastric osmolality. Osmolality in the distal small intestine was also statistically similar to that in caecum and in the proximal colon, in spite of the mean values decreasing considerably. Characteristic osmolality profile was noticed for rabbit gastrointestinal contents down the gut. Osmolality was lower in gastric fluids and increased in the small intestine ($p < 0.05$). The differences observed throughout the rabbit small intestine were not statistically significant. The osmolality then decreased in the caecum and colon to levels close of those of the stomach but still significantly different from the upper GI tract. In pigs, the osmolality was also lower in gastric fluids, which then increased in the small intestine ($p < 0.05$) but was then maintained at the same level ($p > 0.05$) in the caecum and the colon; unlike rabbits, where it dropped again in the distal gut.

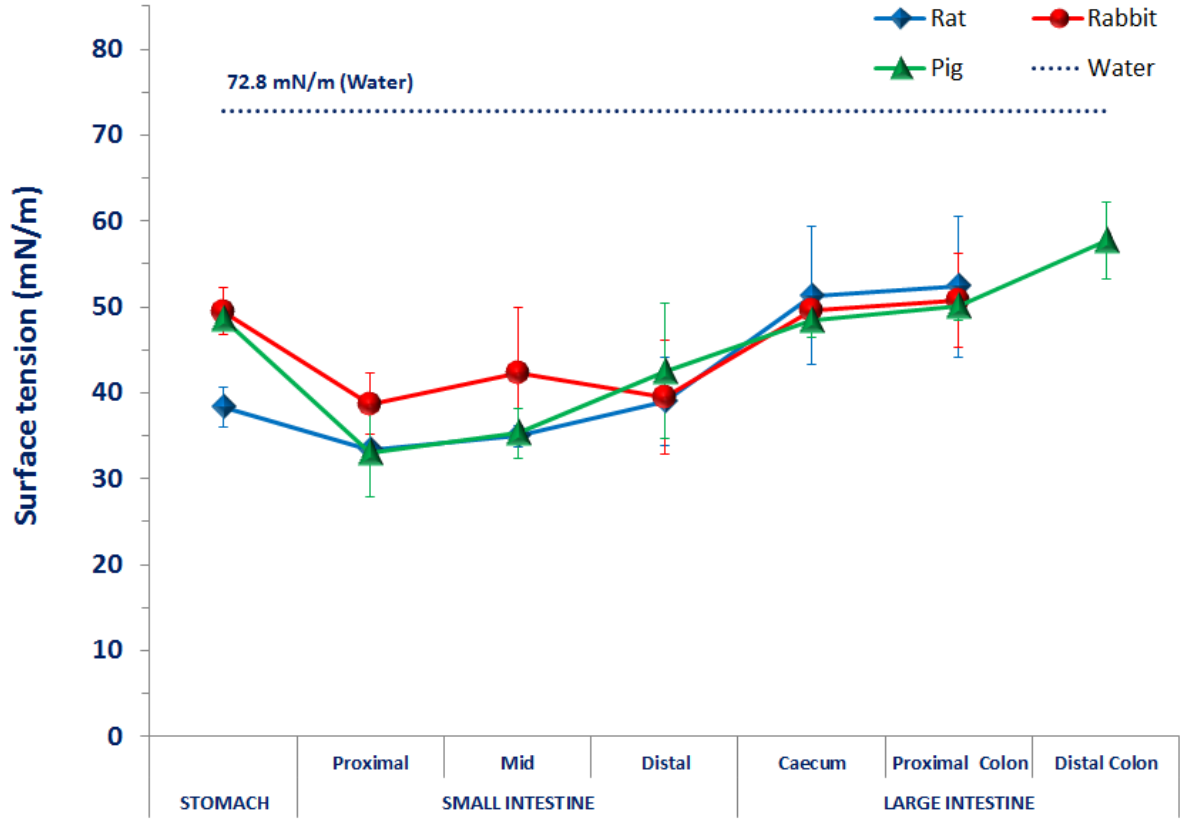
Osmolality of rat gastrointestinal contents were significantly ($p > 0.05$) higher than that in rabbit and pig in the stomach and proximal small intestine (Figure 2, Table 1). When comparing rat to pig fluids, statistical significance was only found in the proximal gut (stomach, proximal and mid small intestine), where osmolality was higher in rats than in pigs. Rabbits had significantly higher osmolality in the gastric and distal small intestine (ileum) than that in pig ($p < 0.05$), whereas no significant differences were noticed in fluids from the rest of the gastrointestinal tract.

Interesting trends were observed when osmolality data was compared with human data from published literature. Kalantzi and colleagues reported osmolality of the human gastric fluids to be 559 $mOsm.Kg^{-1}$, 30 minutes after ingestion of a liquid meal (Ensure® Plus), which is higher than that in rabbit and the pig gastric fluids in fed conditions. However, it not very dissimilar to fed gastric osmolality in rats (Table 1). It was also found that osmolality of the human gastric fluids were greatly influenced by the osmolality of the liquid meal (Ensure® Plus, 610 $mOsm.Kg^{-1}$) ingested before the study, hence a closely related osmolality of gastric fluids (559 $mOsm.Kg^{-1}$) post 30-mintues ingestion. This was later reduced to 217 $mOsm.Kg^{-1}$ post 210 min of meal ingestion, probably after the gastric emptying. The human duodenal aspirates showed an osmolality of ~400 $mOsm.Kg^{-1}$ which was relatively lower than that in rat, rabbit and pig, nevertheless it was not too dissimilar than in pig (492 ± 102 $mOsm.Kg^{-1}$) and specially in rabbit (475 ± 36 $mOsm.Kg^{-1}$). It should also be noted that our measurements in animals were performed on supernatants whereas Kalantzi et al (2006) used whole gastric

and duodenal aspirates. The osmolality of human jejunal aspirates under fasted conditions was significantly lower (200 mOsm.Kg^{-1}) than in fed rat, rabbit and pig (Perez de la Cruz Moreno, 2006). The supernatants from human ascending colon fluids (Diakidou et al., 2009) also showed considerably lower osmolality (224 mOsm.Kg^{-1}) than our measurements in the rat, rabbit and pig.

3.3 Surface tension of gastrointestinal fluids

Figure 3. Surface tension (mean ± standard deviation (SD)) of gastrointestinal fluids (supernatants) from rats (n = 3), rabbits (n = 6) and pigs (n = 3). Surface tension of water was used as reference and measured to be 72.8 mN/m.



Surface tension of the gastrointestinal fluids from rat, rabbit and pig was significantly lower than that of water throughout the gut. Surface tension in these animal species follows a general pattern, being higher in stomach and lower in the small intestine, and increasing again in the distal gut (Figure 3, Table 1). This behaviour of surface tension is also in agreement with the digestion processes in the gut. The digested nutrients (such as glucose, amino acids, fatty acids) and digestive secretions, such as bile, present in the small intestinal milieu decreases the surface tension of the fluids in this region, which gets absorbed in the distal gut resulting in a higher surface tension.

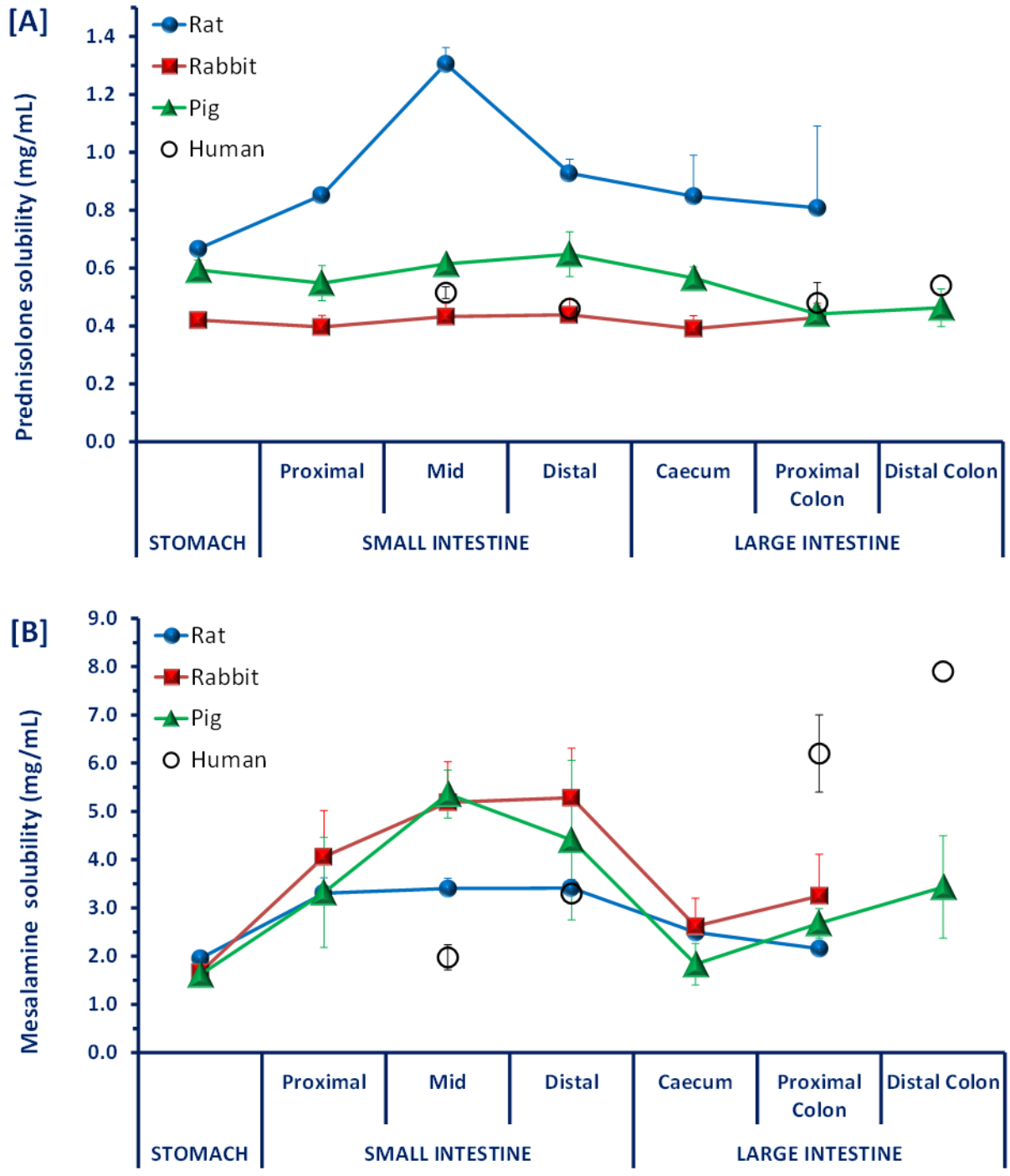
Surface tension of the rat gastric contents was not statistically different from that in the small intestine but was significantly lower ($p<0.05$) than caecum and colon. Characteristic surface tension curve was found for rabbit gastrointestinal contents. The surface tension was higher in gastric fluids, lower in the proximal small intestine ($p < 0.05$), which significantly ($p < 0.05$) rose again in the caecum and remained unchanged in the colon. In pigs, the surface tension was higher in gastric fluids, like in rabbits; it then decreased in the small intestine ($p < 0.05$) and increased in the caecum and proximal colon to match gastric levels, where it further increased, to some extent, in the distal colon ($p < 0.05$).

Inter-species differences in surface tension was apparent in gastric fluids, where surface tension of the rat gastric fluid was significantly lower than in the rabbit and pig ($p < 0.05$). In the proximal and mid small intestine, the surface tension in rabbit was significantly higher than in rat ($p < 0.05$). There was no significant difference in the surface tension of the fluids from the ileum, caecum and the colon ($p > 0.05$) from rats, rabbits and pigs.

The surface tension of human gastrointestinal aspirates reported in the literature was lower than our measurements in rat, rabbit and pig. Surface tension of the human gastric aspirates was reported to be ~30 mN/m in the fed state, which was relatively constant over a period of time (Kalantzi et al., 2006) and was lower than the surface tension of rat, rabbit and pig gastric fluids (Table 1) and can be rank ordered as follows: human < rat < pig ≅ rabbit. A similar observation was noticed with human duodenal aspirates, where surface tension was lowest (~28 mN/m) than in animal species (human < rat ≅ pig < rabbit). As stated in earlier sections, it should be noted that Kalantzi and co-worker tested whole gastric and duodenal aspirates whereas our measurements were performed in supernatants of the animal gastrointestinal fluids. Surface tension of the supernatants from human ascending colon fluids was also lowest (32.9 mN/m) (Diakidou et al., 2009) when compared to the surface tension of the proximal colon fluids from rat, rabbit and pig.

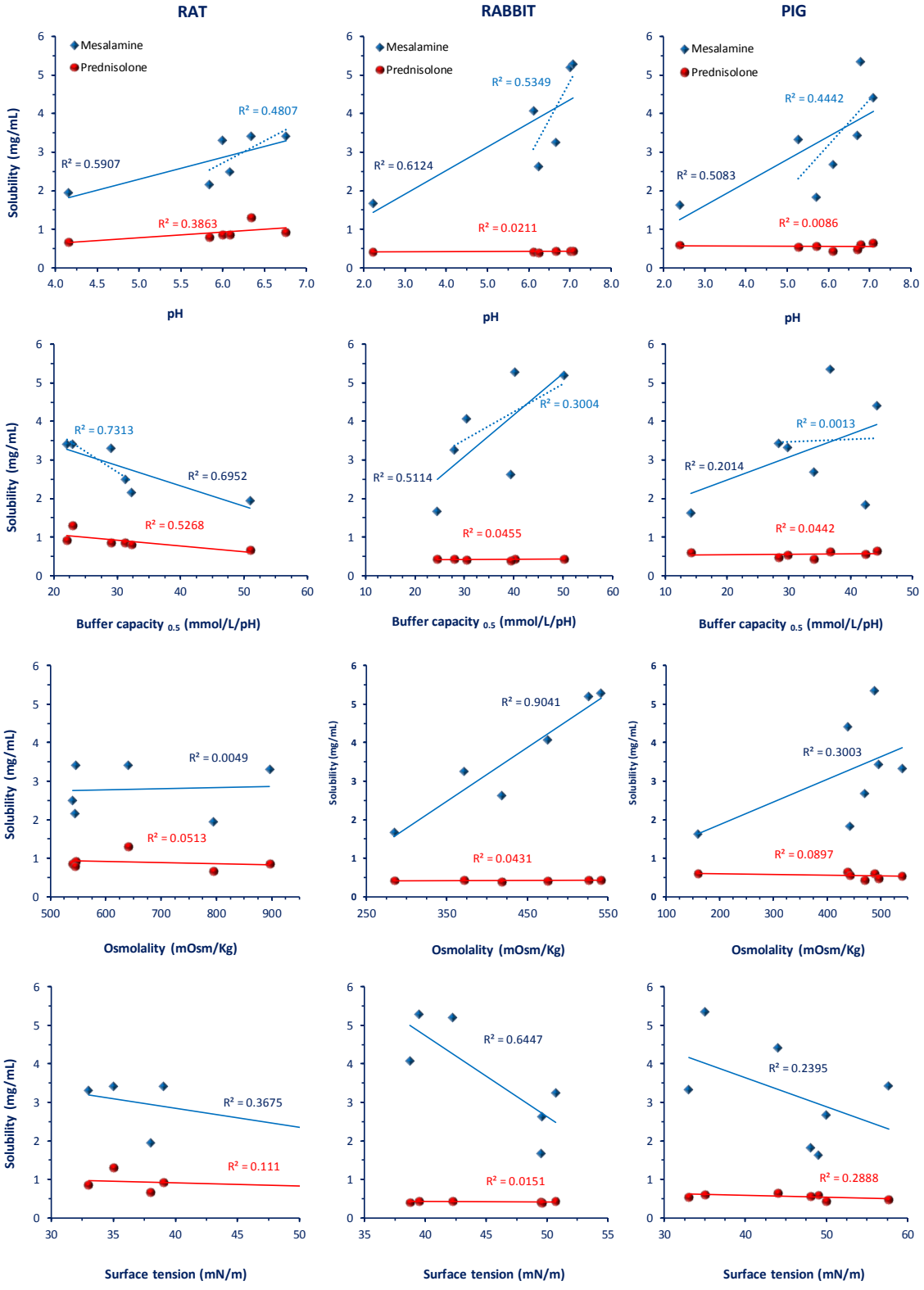
3.4 Drug solubility in gastrointestinal fluids

Figure 4. Solubility of (a) prednisolone and (b) mesalazine in gastrointestinal fluids (supernatants) from rat (pooled from eight rats), rabbits (n = 6) and pig (n = 3), human data from Fadda et al.(Fadda et al., 2010)



The solubility of prednisolone was fairly constant irrespective of the location of the sample from the pig and rabbit gut and was closer to the solubility measured earlier (Fadda et al., 2010) in human jejunal, ileal and colonic fluids (Figure 4, Table 1). Whereas an increasing trend was observed in the solubility of prednisolone in rats, measured from stomach until mid-small intestine, thereafter the solubility was decreased in distal small intestine but did not change significantly later further down the gut. Solubility of prednisolone in fluids from rat's gut was generally higher than those of man, except in proximal colon, where the differences do not attain statistical significance due to much higher variability. The peculiar solubility behaviour in rat proximal gut may be due to a relatively lower surface tension in the proximal and mid small intestine compared to gastric fluids in rats (Table 1). However, when prednisolone solubility was studied as a function of gastrointestinal pH, buffer capacity, osmolality and surface tension, no significant relationship was seen between solubility and any of these parameters in any of the animal species analysed (Figure 5).

Figure 5. Solubility of mesalazine (blue diamonds) and prednisolone (red circles) in rat, rabbit and pig gastrointestinal fluids (supernatants) as a function of its pH, buffer capacity, osmolality and surface tension, where dotted blue lines indicate mesalazine correlation excluding gastric fluids.



Solubility of mesalazine was comparable between rabbit and pig fluids from stomach, small intestine, caecum and proximal colon. Rat had comparable solubility in the stomach to rabbit and pig, but had considerably lower solubility in mid small intestine, which may be due to relatively higher pH and buffer capacity in mid small intestine of pigs and rabbits compared to rats. The reported mesalazine solubility from human fluids (Fadda et al., 2010) was only comparable to rat and pig at distal small intestine, whereas solubility in human jejunal aspirates was much less and solubility from human colonic fluids was much higher than in all three animals species (Table 1, Figure 5).

Solubility of mesalazine, when studied as a function of pH, buffer capacity, osmolality and surface tension, was found to be positively correlated to the gastrointestinal pH of rat and pig, which is in agreement with similar reports in humans (Fadda et al., 2010). However, the correlation with buffer capacity was very weak for pig fluids (for a ΔpH=0.5: standardized coefficient β=0.349, p=0.442; r^2=0.201). But a very strong correlation, although negative, was found with fluids from rats (for a ΔpH=0.5: standardized coefficient β=-0.835, p=0.039; r^2=0.695 / for a ΔpH=1.0 the: standardized coefficient β=-0.997, p=0.001; r^2=0.955) Figure 5), contrary to human fluids, where a positive correlation was reported for the both, pH and buffer capacity (Figure 5). This can be explained by notably higher buffer capacity of the gastric fluids from rat and typical decreasing trend of buffer capacity in rat fluids (Figure 5), suggesting that pH is relatively more important driver for the solubility of an ionisable drug – mesalazine.

In gastrointestinal fluids from rabbit, mesalazine solubility was found to correlate stronger with osmolality (standardized coefficient β=0.951, p=0.004, r^2=0.904) and pH (standardized coefficient β=0.783, p=0.066, r^2=0.612), rather than buffer capacity (for a ΔpH= 0.5: standardized coefficient β=0.716, p=0.109, r^2=0.511 / for a ΔpH= 1.0: standardized coefficient β=0.571, p=0.236, r^2=0.325). This is contrary to finding of Fadda *et al* 2010 where a stronger correlation with buffer capacity was reported.

It is to be noted that the correlation using human fluids reported in Fadda et al 2010 did not account for solubility in gastric fluids (Figure 5), which was accounted for the correlation in animal fluids presented here. It was interesting to note that correlation in human and animal fluids became similar on excluding gastric solubility from the animal fluids (dotted line in Figure 5). It was also interesting to note that the mean solubility was not higher in the distal small intestine of the pig compared to mid small intestine (Table 1) despite a higher regional pH and buffer capacity, which may be explained by a higher surface tension in this region (44 ± 6 vs. 35 ± 3). There is some affinity between surface tension and the solubility of mesalazine in rabbits (standardized coefficient β=-0.802, p=0.055; r^2=0.643).

This is to be noted that the pH values used for producing these correlations corresponds to the fluids measured after thawing and does not necessarily relate to the *in situ* pH values in the gut.

4. CONCLUSION

The inter-species differences in GI pH and buffer capacity are important considerations, particularly for pH-responsive formulations and ionisable drugs administered to the GI tract. Therefore, a relatively higher buffer capacity in jejunum, ileum and proximal colon of the rabbit and pig and a much lower buffer capacity in pig distal colon are very important considerations. A higher osmolality and surface tension were also found in fluids from the proximal small intestine and ascending colon of rat, rabbit and pig compared with those in human. These differences in GI features led to higher solubility of prednisolone in rats (except in proximal colon), whereas prednisolone solubility in pig and rabbit was comparable to human. Therefore, the solubility of prednisolone, a neutral compound, could be overestimated if measured in fluids from rat. On the other hand, the solubility of an ionisable drug, mesalazine, in rabbit and pig was higher in mid-small intestine and lower in colon than in human, and was comparable to human only at the distal small intestine. The differences in GI milieu, such as pH, buffer capacity, osmolality and surface tension, lead to differences in drug solubility. In rabbit and pig, the solubility of mesalazine changed significantly on moving down the GI tract, which was highly influenced by the pH and osmolality of the luminal fluids.

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6. REFERENCES

CALABRESE, E. J. 1984. GASTROINTESTINAL AND DERMAL ABSORPTION - INTERSPECIES DIFFERENCES. *Drug Metabolism Reviews,* 15**,** 1013-1032.

CLARYSSE, S., TACK, J., LAMMERT, F., DUCHATEAU, G., REPPAS, C. & AUGUSTIJNS, P. 2009. Postprandial evolution in composition and characteristics of human duodenal fluids in different nutritional states. *J Pharm Sci,* 98**,** 1177-92.

DIAKIDOU, A., VERTZONI, M., GOUMAS, K., SODERLIND, E., ABRAHAMSSON, B., DRESSMAN, J. & REPPAS, C. 2009. Characterization of the contents of ascending colon to which drugs are exposed after oral administration to healthy adults. *Pharm Res,* 26**,** 2141-51.

DRESSER, M. J., GRAY, A. T. & GIACOMINI, K. M. 2000. Kinetic and selectivity differences between rodent, rabbit, and human organic cation transporters (OCT1). *Journal of Pharmacology and Experimental Therapeutics,* 292**,** 1146-1152.

FADDA, H. M., SOUSA, T., CARLSSON, A. S., ABRAHAMSSON, B., WILLIAMS, J. G., KUMAR, D. & BASIT, A. W. 2010. Drug Solubility in Luminal Fluids from Different Regions of the Small and Large Intestine of Humans. *Mol Pharm*.

FINHOLT, P. & SOLVANG, S. 1968. Dissolution kinetics of drugs in human gastric juice--the role of surface tension. *Journal of pharmaceutical sciences,* 57**,** 1322-6.

FLAISHER-GRINBERG, S., KRONFELD-SCHOR, N. & EINAT, H. 2010. Models of mania: from facets to domains and from animal models to model animals. *Journal of Psychopharmacology,* 24**,** 437-438.

FORDTRAN, J. S. & LOCKLEAR, T. W. 1966. Ionic constituents and osmolality of gastric and small-intestinal fluids after eating. *The American journal of digestive diseases,* 11**,** 503-21.

FREIRE, A. C., BASIT, A. W., CHOUDHARY, R., PIONG, C. W. & MERCHANT, H. A. 2011. Does sex matter? The influence of gender on gastrointestinal physiology and drug delivery. *International Journal of Pharmaceutics,* 415**,** 15-28.

HANNAH-POQUETTE, C., ANDERSON, G. W., FLAISHER-GRINBERG, S., WANG, J., MEINERDING, T. M. & EINAT, H. 2011. Modeling mania: Further validation for Black Swiss mice as model animals. *Behavioural Brain Research,* 223**,** 222-226.

HÖRTER, D. & DRESSMAN, J. B. 2001. Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract. *Advanced Drug Delivery Reviews,* 46**,** 75-87.

INSEL, T. R. 2007. From animal models to model animals. *Biological Psychiatry,* 62**,** 1337-1339.

KALANTZI, L., GOUMAS, K., KALIORAS, V., ABRAHAMSSON, B., DRESSMAN, J. B. & REPPAS, C. 2006. Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. *Pharm Res,* 23**,** 165-76.

KARARLI, T. T. 1995. COMPARISON OF THE GASTROINTESTINAL ANATOMY, PHYSIOLOGY, AND BIOCHEMISTRY OF HUMANS AND COMMONLY USED LABORATORY-ANIMALS. *Biopharmaceutics & Drug Disposition,* 16**,** 351-380.

LAI, Y. 2009. Identification of interspecies difference in hepatobiliary transporters to improve extrapolation of human biliary secretion. *Expert Opinion on Drug Metabolism & Toxicology,* 5**,** 1175-1187.

LI, M., YUAN, H. D., LI, N., SONG, G. T., ZHENG, Y., BARATTA, M., HUA, F. M., THURSTON, A., WANG, J. & LAI, Y. R. 2008. Identification of interspecies difference in efflux transporters of hepatocytes from dog, rat, monkey and human. *European Journal of Pharmaceutical Sciences,* 35**,** 114-126.

MCCONNELL, E. L., BASIT, A. W. & MURDAN, S. 2008a. Measurements of rat and mouse gastrointestinal pH fluid and lymphoid tissue, and implications for in-vivo experiments. *Journal of Pharmacy and Pharmacology,* 60**,** 63-70.

MCCONNELL, E. L., FADDA, H. M. & BASIT, A. W. 2008b. Gut instincts: explorations in intestinal physiology and drug delivery. *Int J Pharm,* 364**,** 213-26.

MERCHANT, H. A., MCCONNELL, E. L., LIU, F., RAMASWAMY, C., KULKARNI, R. P., BASIT, A. W. & MURDAN, S. 2011. Assessment of gastrointestinal pH, fluid and lymphoid tissue in the guinea pig, rabbit and pig, and implications for their use in drug development. *Eur J Pharm Sci,* 42**,** 3-10.

MUDIE, D. M., AMIDON, G. L. & AMIDON, G. E. 2010. Physiological Parameters for Oral Delivery and in Vitro Testing. *Mol Pharm*.

PEREZ DE LA CRUZ MORENO, M., OTH, M., DEFERME, S., LAMMERT, F., TACK, J., DRESSMAN, J. AUGUSTIJNS, P. 2006. Characterization of fasted-state human intestinal fluids collected from duodenum and jejunum. *J Pharm Pharmacol,* 58**,** 1079-1089.

SCHILLER, C., FROHLICH, C. P., GIESSMANN, T., SIEGMUND, W., MONNIKES, H., HOSTEN, N. & WEITSCHIES, W. 2005. Intestinal fluid volumes and transit of dosage forms as assessed by magnetic resonance imaging. *Alimentary Pharmacology & Therapeutics,* 22**,** 971-979.

SMITH, H. W. 1965. OBSERVATIONS ON THE FLORA OF THE ALIMENTARY TRACT OF ANIMALS AND FACTORS AFFECTING ITS COMPOSITION. *The Journal of pathology and bacteriology,* 89**,** 95-122.

WARD, F. W. & COATES, M. E. 1987. GASTROINTESTINAL PH MEASUREMENT IN RATS - INFLUENCE OF THE MICROBIAL-FLORA, DIET AND FASTING. *Laboratory Animals,* 21**,** 216-222.

Chapter 2 - Sex differences in the gastrointestinal tract of adult Wistar rats

ABSTRACT

The aim of this study is to characterise and identify potential sex related differences in the gastrointestinal luminal environment of male and female rats.

Male and female rats were sacrificed, their gastrointestinal tracts were excised, segmented into stomach, duodenum, jejunum, ileum, caecum and colon, the respective contents collected and analysed for pH, buffer capacity, surface tension and osmolality. The solubility of two model drugs, prednisolone and 5-ASA was also quantified in the gastrointestinal fluids of male and female rats.

The pH in the stomach of females is lower than in males. The other intestinal sections did not show a sex difference. Buffer capacity was different in the sexes in the caecum and colon; females had higher values. Buffer capacity was also found to be higher at lower pHs. Males had higher osmolality than females in the duodenum, ileum and colon. Significant sex differences in surface tension were observed in the ileum where females had a higher surface tension. Solubility of 5-ASA was found to be significantly affected by the pH environment of the gastrointestinal tract. The female rat pH of the pooled fluids of the caecum and colon was lower than in the males, as a result, solubility of 5-ASA was lower in females in these segments.

The trend of each parameter is similar in the sexes, however differences in the values were observed. These were mainly identified in the stomach and in the distal portion of the gastrointestinal tract: ileum, caecum and colon. Understanding how the gastrointestinal environment is affected by sex is important, especially for pH responsive formulations and ionisable drugs intended for oral administration.

1. INTRODUCTION

Sex! It defines us. Animals frequently exhibit sexual dimorphism albeit in some circumstances the differences lie mainly on their gonads and gametes. Regardless of the apparent physical differences, animals tend to adapt to their sexual role with underlying genetic, hormonal, morphological and physiological differences: the sexes think and act differently (Tobet et al., 2009), have different nutritional needs (Li et al., 2012) and react differently to disease (manifestation, epidemiology and pathophysiology). A *"one size fits all"* approach for medical treatment, far from ideal, means that patients will not get the most suitable treatment (Regitz-Zagrosek, 2012).

Traditionally, medical and pharmaceutical research fails to consider differences between the sexes (CancerDiscovery, 2014). Biomedical research focuses more on males in animal studies and human clinical trials. The unacceptable consequence is a short-come to female healthcare which follows the fact that medicine is less evidence-based in women than in men (Nature, 2010). According to a study conducted in the USA between 2000 and 2002, sex dependent differences/phenomena where observed for 36% of the new drugs. It has even been suggested that women are possibly at a greater risk of suffering drug adverse effects than men (Rademaker, 2001).

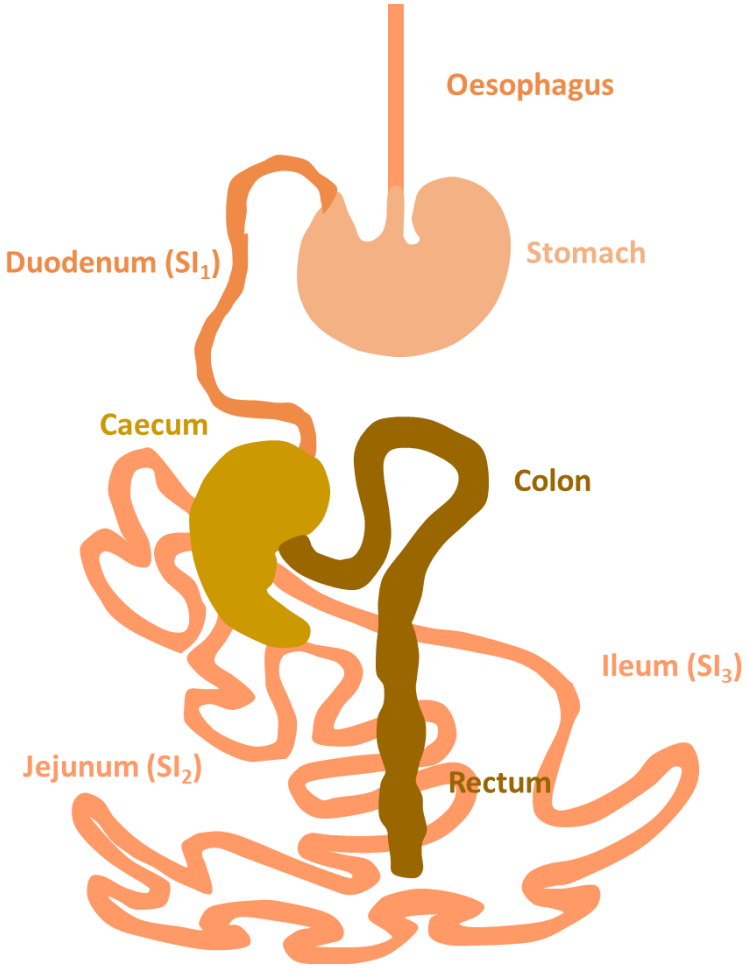
In an attempt to reduce the male oriented bias in research, in December 2013, the European Commission took a step demanding applicants to the research program, Horizons 2020, to include gender analysis in their projects. In the USA, women must be part of phase III clinical trials since 1993 (Schiebinger, 2014).

The US National Institute of Health requires applicants to include a sex-balance in the preclinical research funded by the organisation (Clayton and Collins, 2014, CancerDiscovery, 2014). Understanding how animal physiology varies according to sex is important to set the basis for preclinical research in drug development.

Laboratory animals are useful in preclinical research, not as surrogates of humans but to better guide drug development. For oral drug delivery, understanding the physiology of the gastrointestinal tract is essential. Because rats are a commonly used laboratory species, relatively easy to handle and inexpensive, they were chosen for this study where the pH, buffer capacity, surface tension and osmolality of gastro-intestinal (GI) luminal fluids of males and females will be investigated.

The rat GI tract is divided into oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon and rectum (Figure 1).

Figure 1. Rat gastrointestinal tract schematic view. Adapted (DeSesso and Jacobson, 2001).



The values of pH, buffer capacity, surface tension and osmolality are not constant through the gut, but change with the luminal environment. As digestion progresses and the ingested products travel down the gastro-intestinal tract (GIT) they are subjected to different secretions, enzymes and bacteria. To further complicate matters, sex also changes the activities of mucosal hydrolytic enzymes in the small intestine of the rat. Some enzymes are more affected than others and there are also differences in the adaptation of intestinal mucosa to environmental and hormonal factors. There are enzymes whose expression is increased quickly (within a day) in response to hormonal changes (folate-metabolizing and glycolytic enzymes), whereas others may take up to 9 weeks (lactose in rats) (Hietanen, 1975).

It is not surprising that sex is an important factor in the drug and disease susceptibility of the rat. For example female rats are more susceptible to mycophenolate mofetil toxicity due to a threefold lower glucuronidation capability in the upper jejunum compared with male rats (Stern et al., 2007)) and females are more resistant to colon cancer (Balish et al., 1977)). Even excipients can affect the sexes differently as was observed by our research group. In human

and rat males, polyethylene glycol 400 (PEG 400), a commonly used excipient induces a marked increase in the oral bioavailability of ranitidine. This effect was not seen in females (Afonso-Pereira et al., 2016, Ashiru et al., 2008).

Above are examples of sex differences in the rat's gut. The question on whether these biological differences are also mirrored by the physiochemical characteristics of the GI luminal fluids should also be addressed. Our research group characterised the gastrointestinal (GI) tracts of male pig, rat and rabbit in 2015 (Merchant et al., 2015). A separate study evaluated how ageing affects the male Wistar rat GI tract (Merchant et al., 2014). These studies were all conducted in male rats. A study on the GI tract physiology was conducted on female rats, however the aim of the study was not to identify any sex-related differences. In this female study pH, water content, lymphoid tissue distribution along the GIT and stomach volume were quantified (McConnell et al., 2008a). These parameters are not sufficient to evaluate an active substance solubility and overall disposition when orally administered.

The oral bioavailability of any given drug for systemic use will depend on the intrinsic properties of the drug (Pang, 2003), on the formulation approach used, and on the GI tract physiology (Chillistone, 2008, DeSesso and Jacobson, 2001). pH, buffer capacity, surface tension and osmolality were judged to be the most significant physicochemical factors affecting drug delivery through the gut.

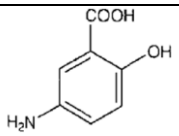
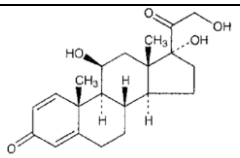
Gastrointestinal pH and buffer capacity are very important for ionisable drugs. The degree of ionisation of an active substance will influence its solubility, stability, absorption and ultimately, bioavailability. In addition, the change in gastrointestinal pH along the gastrointestinal tract affects the behaviour of modified release dosage forms, such as pH responsive enteric coated formulations (Merchant et al., 2014).

Surface tension contributes to the degree of solvation of drug particles and respective wettability. When added to a solution, surfactants increase solvation around dissolved molecules, increasing the energy of activation for the onset of precipitation, further contributing for an increased solubility of a given drug (Johnston et al., 2008). Bile salts, well known by their solubilisation capabilities, contribute to important reductions of media surface tension and to the possible creation of micelles (Rangel-Yagui et al., 2005). Surface tension has been reported to be inversely related to the dissolution rate of some active substances (Finholt and Solvang, 1968).

Osmolality can also contribute to solubility of a certain drug. It has been reported that the "salting-out" and "salting-in" effect can change solubility (Pegram and Record, 2008). Alterations to the fluids ionic content may furthermore influence drug ionisation, and hence limit absorption. The overall concentration of osmotic active species in GI fluids can be changed by food ingestion as well as the secretion of gastric acid, bile and pancreatic juices (Hörter and Dressman, 2001, Fordtran and Locklear, 1966).

The aim of this investigational study is to highlight sex differences, or their absence, in the GI tract fluids of fed rats. Due to the limited amount of gastrointestinal fluids available per rat, four fundamental parameters were chosen: pH, buffer capacity, surface tension and osmolality. In addition the solubility of two model drugs, 5-aminosalicylic acid (5-ASA) and prednisolone, was quantified. These drugs are models for ionisable and non-ionisable drugs, selected as they are predominantly administered as enteric, delayed/modified release formulations, see Table 1.

Table 1. Aqueous solubility, lipophilicity and ionization constants of mesalazine (5-ASA) and prednisolone, reproduced from(Fadda et al., 2010).

	5-ASA	*Prednisolone*
Structure		
Water solubility (mg/mL)[a]	1.32	0.223
pKa	2.3; 5.69	N/A
Log P	0.98	1.59

[a] solubility in water at 37°C (*in-house* measurement)

2. MATERIALS AND METHODS

2.1. Materials

5-ASA was obtained from Sigma Aldrich and prednisolone was obtained from Severn Biotech Ltd (Worcestershire, UK). HPLC-grade water, methanol, peroxide-free tetrahydrofuran and trifluoroacetic acid were purchased from Fisher Scientific (Loughborough, UK NaOH and HCl (0.1 M standards) were used for buffer capacity determinations and were procured from Sigma Aldrich (Dorset, UK).

2.2. Gastrointestinal tissues

Wistar rats (8 males; 6 females), 180 g and 235 g, 8 weeks old, were purchased from Harlan UK Ltd (Oxfordshire, UK). All animals were fed *ad libitum* with Teklad Global 18% Protein Rodent Diet (Harlan Ltd., Oxfordshire, UK) and given free access to tap water. All procedures were approved by the Home Office (PPL No.70/6421) and were conducted in accordance with the Animals (Scientific Procedures) Act 1986, UK.

2.3. Gastrointestinal fluids

All measurements were performed on supernatant obtained from the gastrointestinal fluids from the laboratory animals. The animals were sacrificed by CO_2 asphyxiation, the GI tract was promptly extracted and divided into stomach, duodenum, jejunum, ileum, caecum and colon (within 10 minutes). The gastrointestinal sections were emptied into 1.5 mL Eppendorf tubes and centrifuged (Centrifuge 5415D, Eppendorf AG, Hamburg, Germany) at 13000 rpm for 20 minutes. The supernatant obtained was kept at -80°C until analysed as follows:

Osmolality was measured with a Digital Micro-Osmometer (Type 5R), Hermann Roebling MESSTECHNIK, Berlin, Germany.

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

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

Buffer capacity, was measured at pH changes of 0.5 and 1.0 units by adding aliquots (10 µL) of 0.1 M HCl (for intestinal fluids) or 0.1 M NaOH (for gastric fluids) to a 300 µL supernatant pooled sample from GI fluid to achieve the desired pH change. Buffer capacity was then calculated using following equation:

$$\beta\ (mmol/L/\Delta\text{pH}) = \frac{M_a \times V_a}{\Delta pH} \times \frac{1000}{V_b}$$

Equation 1.

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

Due to the small amount of fluids available from some of the intestinal segments, some tests were run in pooled samples, in which fluids from the same segment of different animals were mixed to increase the available volume to perform the tests. As a result, in Table 2 values for pH, buffer capacity and solubility were determined with pooled fluids from the individual animals. Human values are mentioned to give an indication of typical human values (Merchant et al., 2014, Mudie et al., 2010, McConnell et al., 2008b).

2.4. Solubility Studies

Two model drugs were selected: an ionisable drug, 5-ASA, and a neutral drug, prednisolone. Solubility was measured in pooled supernatants from gastrointestinal fluids and the results were compared with published data from human fluids, and correlated with investigated gastrointestinal fluid parameters (pH, surface tension, osmolality and buffer capacity). To measure solubility, an excess of drug was added to microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) containing 200µL of the supernatants from the GI fluids. The samples were placed in a shaking bath (Gallenkamp, Loughborough, UK) at 37 °C at 170 rpm for 24 hours. After incubation, the samples were centrifuged at 5000 rpm for 10 minutes (Centrifuge 5804R, Eppendorf AG, Hamburg, Germany) at 37 °C. The supernatant was transferred to a centrifuge filter tube (Corning® Spin-X®, 0.22 µm cellulose acetate membrane, obtained from Fisher Scientific, Leicestershire, UK) and centrifuged at 5000 rpm for 20 minutes (Centrifuge 5804R, Eppendorf AG, Hamburg, Germany) at 37°C. 50 µL of the filtrate was removed and diluted with 1950 µL of mobile phase before analysis. The amount of prednisolone or 5-ASA dissolved in the sample was assayed by reversed-phase HPLC equipped with a UV detector (Agilent Technologies 1200 Series) (Fadda et al., 2010). The chromatographic conditions for 5-ASA were a mobile phase of 95% water, 5% methanol, 0.05% trifluoroacetic with a reverse phase column LiChrospher 100, C18, 5µm (Merck, Darmstadt, Germany). For prednisolone the mobile phase consisted of 68.8% water, 25% tetrahydrofuran, 6.2% methanol and a reverse phase column Water Symmetry C8 5µm (Waters, Massachusetts, USA) was used. Drugs were detected at 228 nm and 254 nm and retention times were 7.0 and 9.9 minutes for 5-ASA and prednisolone respectively. The other assay conditions were the same for both drugs (i.e., 20 µL injection volume, 1 mL/min flow rate at 124 bar, and 40□C column temperature), described in (Merchant et al., 2014).

2.5. Statistical analysis

The data was analysed by one-way ANOVA, followed by post-hoc Tukey with a 95 % confidence interval using IBM SPSS Statistics 19 (SPSS Inc., Illinois, USA). A Univariate General Linear Model tool was used with Tukey post-hoc analysis taking species and locations as fixed factors.

3. RESULTS

Table 2 features the pH, buffer capacity, osmolality, surface tension and solubility of the model drugs in the gastrointestinal fluids.

Table 2. pH, buffer capacity, osmolality, surface tension of the gastrointestinal fluids from male and female wistar rats and humans; solubility of 5-ASA and prednisolone in these fluids.

	Stomach	Small Intestine			Caecum	Colon
		Duodenum	Jejunum	Ileum		
*pH**						
Male rat	4.16 ± 0.01	5.99 ± 0.01	6.34 ± 0.01	6.76 ±0.04	6.08 ± 0.01	5.84 ± 0.01
Female rat	3.74 ± 0.01	6.12 ± 0.01	6.52 ± 0.01	7.01 ±0.02	5.50 ± 0.01	5.30 ± 0.01
Osmolality (mOsm.Kg^{-1})						
Male rat	802 ± 276	853 ± 90	627 ± 85	539 ± 70	512 ± 46	507 ± 87
Female rat	737 ± 247	665 ± 55	596 ± 70	451 ± 23	489 ± 26	418 ± 28
Surface tension (mN.m^{-1})						
Male rat	38 ± 2	33 ± 1	35 ± 1	39 ± 5	51 ± 8	52 ± 8
Female rat	39 ± 3	33 ± 1	34 ± 2	46 ± 4	45 ± 10	47 ± 10
*Buffer capacity (mmol.L^{-1}.△pH^{-1})**						
Male rat (0.5 unit)	51.0 ± 0.6	29.1 ± 2.6	23.0 ± 2.3	22.1 ± 1.7	31.3 ± 0.9	32.3 ± 0.4
(1.0 unit)	45.0 ± 1.9	28.2 ± 0.8	22.7 ± 2.4	20.1 ± 0.7	39.4 ± 0.6	42.7 ± 2.9
Female rat (0.5unit)	54.1 ± 2.5	34.0 ± 1.0	37.1 ± 1.2	38.0 ± 2.8	67.6 ± 4.0	80.4 ± 4.5
(1.0 unit)	50.2 ± 1.1	31.8 ± 0.5	34.2 ± 1.0	31.3 ± 0.2	83.9 ± 7.9	78.2 ± 1.6
*Prednisolone solubility (mg/mL)**						
Male rat	0.67 ± 0.02	0.85 ± 0.03	1.31 ± 0.06	0.93 ± 0.05	0.85 ± 0.14	0.81 ± 0.28
Female rat	0.76 ± 0.03	0.84 ± 0.02	1.22 ± 0.13	1.36 ± 0.1	0.73 ± 0.01	0.72 ± 0.03
*Mesalazine solubility (mg/mL)***						
Male rat	1.68 ± 0.15	2.75 ± 0.43	3.21 ± 0.31	2.94 ± 0.53	2.37 ± 0.30	1.77 ± 0.40
Female rat	1.02 ± 0.02	2.71 ± 0.03	3.21 ± 0.03	3.39 ± 0.01	1.36 ± 0.01	1.12 ± 0.01

* Parameter determined with pooled fluids.
'-' means insufficient supernatant.

3.1. pH

Figure 2 and Table 3 represent how the pH measured *in situ* changes along the GI tract. For the sexes, the global trend is similar with differences in the extremities, *i.e.* fundus and distal colon.

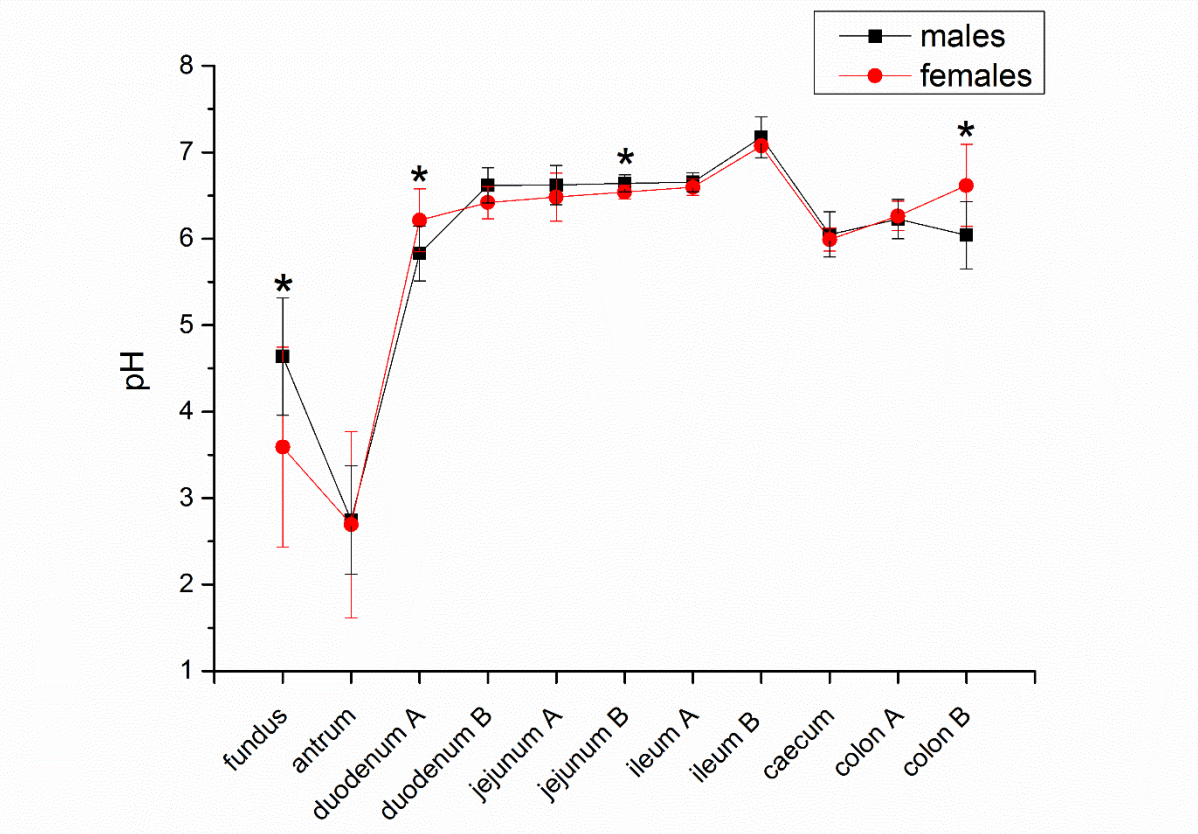
Table 3. Sex differences on gastrointestinal *in situ* pH of Wistar rats. A – Denotes proximal portion of the segment; B – denotes distal portion of the segment.

Gastrointestinal site	*In-situ* pH, Mean ± SD (Range)	
	Male rats	Female rats
Stomach		
Fundus	4.6 ± 0.7 (2.9-5.6)	3.6 ± 1.2 (1.6-5.1)
Antrum	2.7 ± 0.6 (2.1-4.4)	2.7 ± 1.1 (1.5-3.9)
Duodenum		
A	5.8 ± 0.3 (5.4-6.5)	6.2 ± 0.4 (5.7-6.7)
B	6.6 ± 0.2 (6.3-6.9)	6.4 ± 0.2 (6.2-6.6)
Jejunum		
A	6.6 ± 0.2 (6.2-6.9)	6.5 ± 0.3 (6.2-6.9)
B	6.6 ± 0.1 (6.5-6.8)	6.5 ± 0.1 (6.5-6.6)
Ileum		
A	6.7 ± 0.1 (6.5-6.9)	6.6 ± 0.1 (6.5-6.7)
B	7.2 ± 0.2 (6.8-7.5)	7.1 ± 0.0 (7.0-7.1)
Caecum		
	6.1 ± 0.3 (5.6-6.7)	6.0 ± 0.1 (5.7-6.1)
Colon		
A	6.2 ± 0.2 (5.8-6.6)	6.3 ± 0.1 (6.0-6.4)
B	6.0 ± 0.4 (5.4-6.9)	6.6 ± 0.5 (6.6-7.0)

The pH is lowest in the stomach. The antrum has a lower pH than the fundus. Females have a significantly ($p<0.05$) lower pH in the fundus than males. From the antrum to the duodenum there is a sharp rise in the pH that remains stable up to the distal ileum, where a small increase is observed. The pH then decreases in the caecum and colon. While pH values are approximately the same in the sexes, between the antrum and the proximal colon, there are statistically significant ($p<0.05$) differences in proximal duodenum, distal jejunum and distal colon, please refer to Figure 2. These differences are due to the small standard deviation and are not considered relevant.

The standard deviation pattern is similar in the sexes. Standard deviation is high in the stomach and tends to reduce along the GI tract with an increase in the distal colon. A high standard deviation reflects a high intersubject variability.

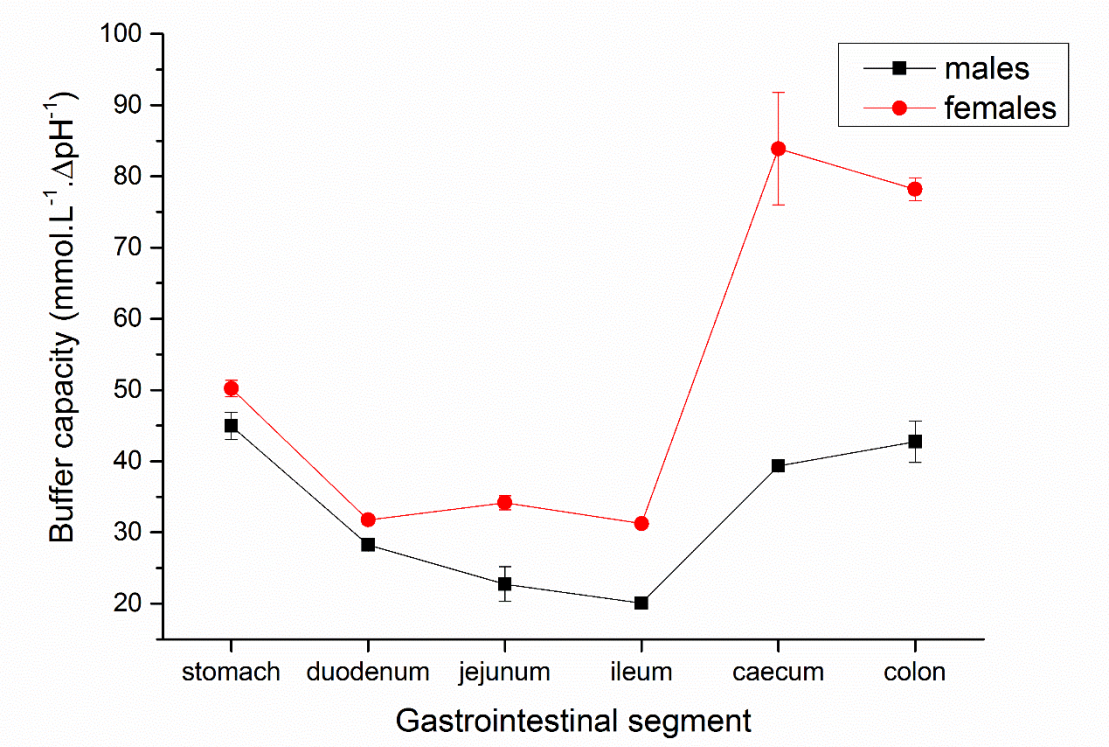
Figure 2. pH of the luminal environment of sections of the GI tract of male and female wistar rats. The results are measured *in situ*. *denotes a statistically significant ($p<0.05$) between males and females.



3.2. Buffer capacity

The buffer capacity was determined in pooled fluids from the rats, as a result no statistical tests were performed due to the small sample size; mean values will be presented in this section.

Figure 3. Buffer capacity (ΔpH=1.0) of pooled fluids of sections of the GI tract of male and female wistar rats. The values are the mean of the repeated measurements.

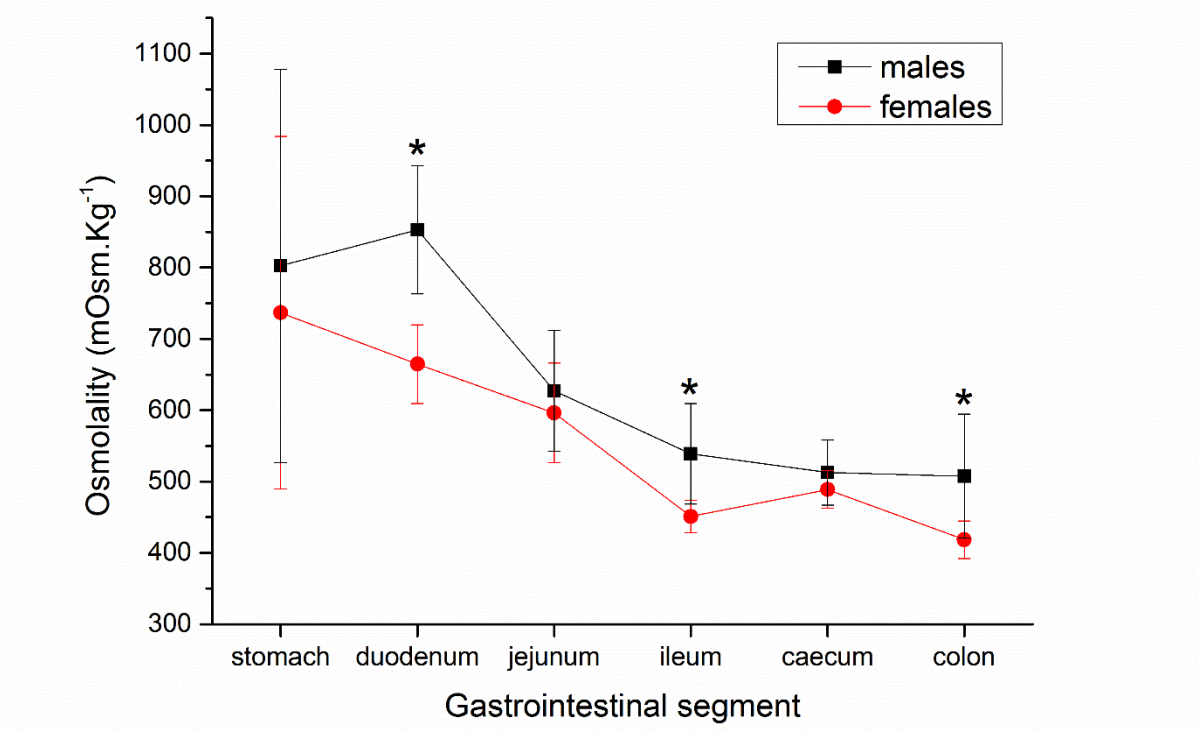


As seen in Figure 3, females have a higher buffer capacity than males in all the GI tract, especially in the caecum, where the female buffer capacity is double that of males and in the colon. The overall trend is similar in males and females. The buffer capacity is relatively high in the stomach and steadily reduces upon reaching the ileum; it increases (twofold in the females) in the caecum , and it remains stable in the colon. In Figure 3 it can be seen that the buffer capacity in the caecum is higher than in the colon, whilst it is the opposite for males. This is not considered to be significant, due to the higher standard deviation present in the colon in males and caecum in females.

3.3. Osmolality

Osmolality (Figure 4) is higher in the stomach and reduces distally. Males have a higher ($p<0.05$) osmolality than females, in the duodenum, ileum and colon. The osmolality, in males, increases slightly from the stomach to the duodenum whereas the females exhibit a reduction along the investigated GI tract sections. Standard deviation is much higher in the stomach than anywhere else. It is important to remember that the animals were fed and had free access to food and water prior to culling.

Figure 4. Osmolality of the fluids of sections of the GI tract of male and female wistar rats. *denotes a statistically significant ($p<0.05$) between males and females.

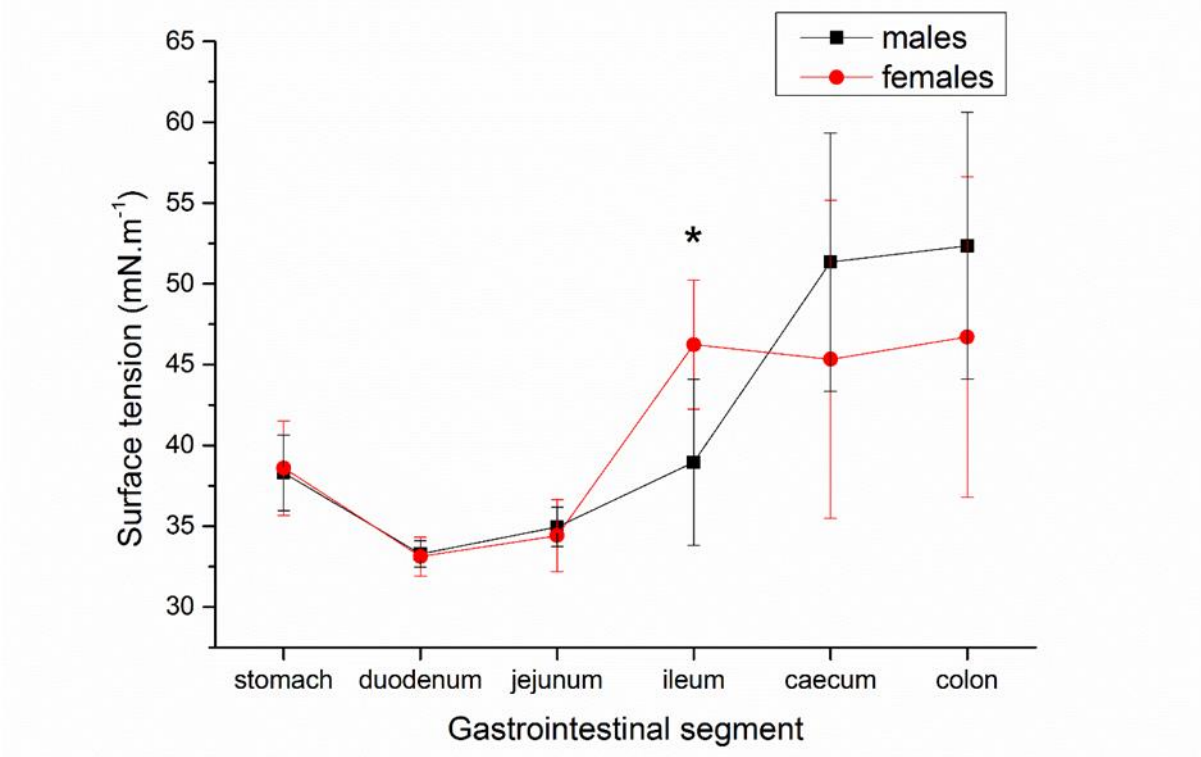


3.4. Surface tension

As seen in Figure 5, surface tension is minimal in the duodenum and then increases distally. No sex differences were observed in the surface tension of the stomach, duodenum and jejunum. Significant sex differences were observed in the ileum where females had a higher surface tension. Female surface tension is the same in the ileum, caecum and colon. The surface tension of the male fluids was higher in the caecum and colon when compared to the females; however this difference was not statistically significant.

The standard deviation is very small for the first three gastrointestinal segments and increases distally.

Figure 5. Surface tension of the fluids of sections of the GI tract of male and female wistar rats. *denotes a statistically significant ($p<0.05$) between males and females.



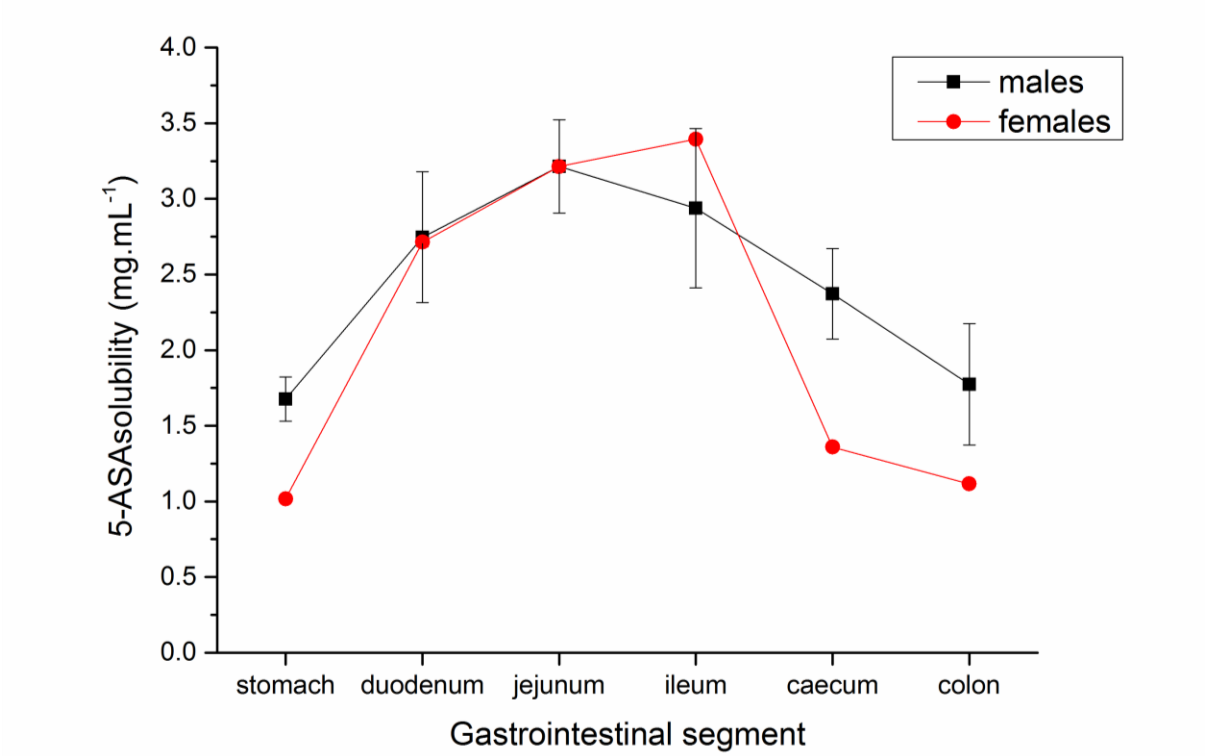
3.5. Solubility of the active substances: 5-ASA and prednisolone

The overall solubility trends for both active substances is similar in the sexes but varies at the ileum caecum and colon.

5-ASA

The solubility of mesalazine per gastrointestinal tract section follows a bell shape curve (Figure 6).

Figure 6. Solubility of 5-ASA in the fluids of different gastrointestinal segments of male and female rats.

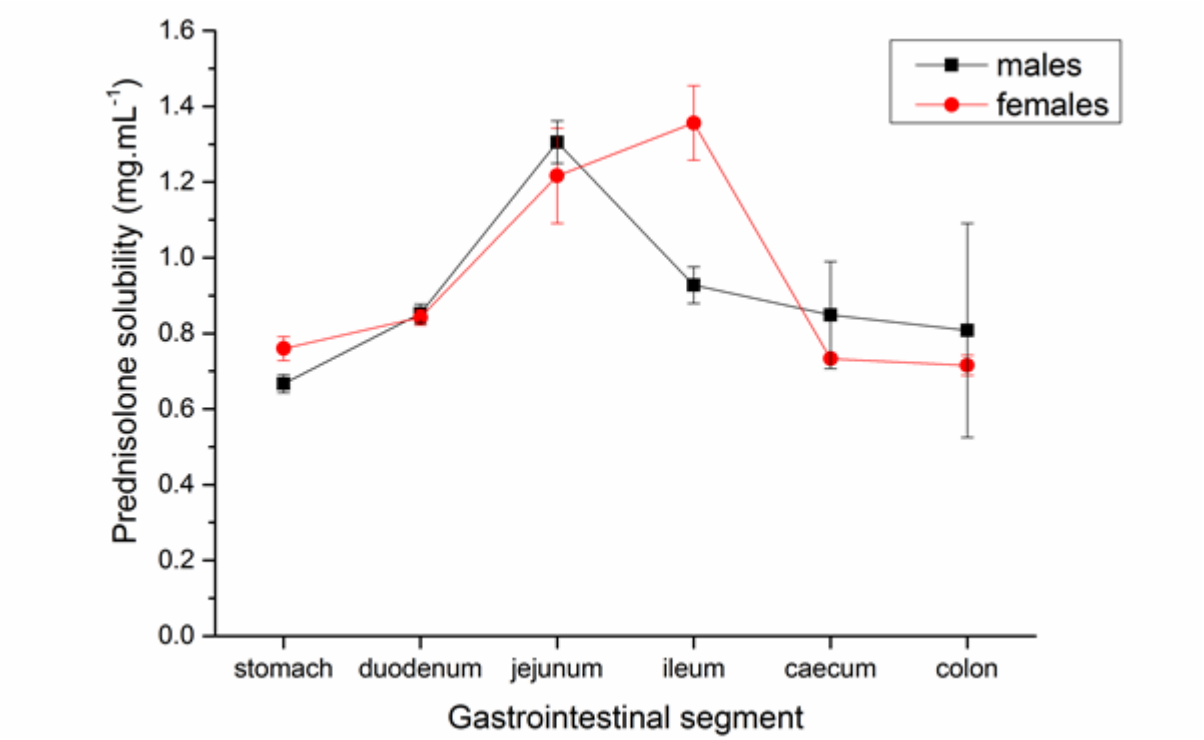


The solubility in the stomach and in the caecum/colon is lower and the maxima is reached in the small intestine, following a threefold increase in solubility. Mesalazine is less soluble in the female stomach than in the males, but is more soluble in the female ileum. Interestingly, the reduction in the solubility from the ileum to the caecum is more pronounced in females than in males.

Prednisolone

Prednisolone solubility in the stomach, duodenum, caecum and colon is the same and does not change with the sex of the animal (Figure 7).

Figure 7. Solubility of prednisolone in the fluids of different gastrointestinal segments of male and female rats.



The peak in solubility (a twofold increase compared to the stomach) is observed in the jejunum (males) and ileum (females). The solubility of prednisolone in the female's ileum is approximately 50% higher than the males.

4. DISCUSSION

4.1. pH

There were some differences between the pH measured *in situ* (Figure 2 and Table 3) and the pH from the pooled fluids (Table 2). The pooled and *in situ* pH is similar in males but there are differences in the females: the pH from the pooled fluids was higher in the ileum (+0.4) and lower in the caecum (-0.5) and colon (-1.0). The fluids, after being extracted and frozen will inevitably be altered. Pooling of the samples also tends to make population differences less evident to measure/observe as the biological parameters tend to be averaged. These differences between pooled fluids and individual *in situ* measurements are expected.

The graphical representation between pH and buffer capacity is better related if the pH of the pooled fluids is considered (and not that of *in situ* measurements). This is expected, given that buffer capacity was measured in the same pooled fluids and not individually for each rat.

The observed pH profile in males is similar to the one reported in (Merchant et al., 2014). Also, the female pH data of the pooled fluids follows the same pattern as identified in (McConnell et al., 2008a). However, in the latter publication the pH of the female pooled fluids was on average 1.0 unit lower than in our study. This could be explained by the difference in experimental conditions and equipment used Hence the importance of the current work. We have determined the pH of the different segments in the sexes with the same equipment, the same operators under the same conditions. As a result, variability due to these factors has been considerably reduced.

The results for the i*n situ* pH values were generally expected. The pH is lower in the stomach due to the active secretion of hydrochloric acid; increases in the small intestine where the bicarbonate ions, bile and other species neutralise the stomach acid; peaks at the ileum and then reduces markedly in the caecum, where fermentation occurs and acid species are produced (Barnes, 1962).

Females, however, have a lower pH ($p < 0.05$) in the stomach than males (Figure 2). There are conflicting literature views on this subject in rats. Some research has shown that oestrogens are inhibitors of the gastric acid secretion while testosterone is an inducer (Amure and Omole, 1970) and (Maitrya et al., 1979); hence, a higher pH in females due to the lower acid secretion would be expected however, this is not supported by the current data. In another study, oestrogen and progesterone have demonstrated a protective activity on the stomach (Aguwa, 1984). However, these did not affect acid secretion, which would not explain why females have indeed a lower gastric pH. However, this paper also states that oestrogens may have an enhanced mucus activity, which is further supported by the following study from 2017. In a recent study, it was found that males have a higher gastric blood flow than females. Oestrogen administration reduced the mean blood flow in the gastric mucosa by 31% in males whilst it remained largely unchanged in females. The mucus layer also thickened more quickly in females than males (Shore et al., 2017). This suggests females are more “resistant” to feminine

hormones and are more effective in repairing damage to the gastric wall. If the mucus producing rate of the females is higher it may suggest an evolutionary biological adaptation to higher stomach acidity. It is important to note that none of these studies investigated the pH of the contents of the stomach.

Another interesting fact is that standard deviation is higher in the stomach, both fundus and antrum, and in the distal portion of the colon, remaining quite low throughout the small intestine. This suggests interindividual variability is higher in these portions. The animals had free access to water and food, and upon culling it was observed they had different amounts of gastric contents, which contributed to the variability observed. As for the colon, the higher variability may have been due to the different microbial populations (total amount and relative proportions) on each animal.

4.2. Buffer capacity

The buffer capacity follows an expected trend in the sexes (Merchant et al., 2015, Merchant et al., 2014): higher in the stomach, lower in the small intestine, where it remains constant, and higher again in the caecum and colon. A higher buffer capacity in the stomach may be explained by the large amount of chyme that is gradually released into the duodenum. As the chyme is converted into chyle the buffer capacity is reduced by a factor of approximately 20 $mmol.L^{-1} \triangle pH^{-1}$. The subsequent increase of the buffer capacity in the caecum can be attributed to the gut microflora. The caecum and colon are the fermenting powerhouses of the gut: its by-products include phenols, indoles, amines, sulfides, ammonia, short-chain fatty acids (SCFA), produced from indigestible carbohydrates and branched-chain fatty acids (BCFA) produced by the fermentation of aminoacids (Liu et al., 2014). These compounds have different pKas, positively contributing to the buffer capacity of the fluids, and have been reported in high levels in both cases.

Sex differences in the buffer capacity were observed in the caecum and colon where females doubled that of males. This suggests a higher production of buffering species in females. As stated above, SCFAs can contribute to the overall buffer capacity of the luminal fluids. There are mainly three different types of SCFAs: acetate, propionate and butyrate; and each type is mainly produced by certain bacteria taxa (Ríos-Covián et al., 2016). For example, the total faecal propionate concentration is linked to the abundance of Bacteroidetes (Salonen et al., 2014), whereas butyrate is mainly produced by certain Coprococcus (Flint et al., 2015). Both taxa have been reported to be more abundant in female rats (Shastri et al., 2015) (Org et al., 2016). The higher amounts of SCFA producing bacteria in the female distal gut may explain why the buffer capacity was higher in the female rats. Sex plays a role in the microflora distribution in rats which affects the buffer capacity of the luminal environment.

4.3. Osmolality

As seen in Figure4, the osmolality of the gastrointestinal contents of both sexes decreases distally. Osmolality and fluid volume have been investigated in relation to buffer capacity and pH, given that the alteration of salt concentration and ingested fluids can alter both parameters through stimulating the secretion of gastric acid, bile and pancreatic juices (Hörter and

Dressman, 2001, Fordtran and Locklear, 1966). The continuous digestion and absorption of osmotically active species may contribute to a distal reduction of the osmolality of the luminal environment. This finding is important because the alteration of ionic content may furthermore influence drug ionisation, and hence limit absorption.

Males had a higher osmolality ($p<0.05$) than females in the duodenum, ileum and colon. No literature reference was found that might explain the reason behind this difference.

In male rats, osmolality of the gastric contents was not statistically different from that in proximal and mid small intestine due to high variability in gastric osmolality. Osmolality in the distal small intestine was also statistically similar to that in caecum and in the proximal colon, in spite of the mean values decreasing considerably.

Interestingly, as seen with pH, the standard deviation is considerably higher in the stomach and tends to reduce distally. The higher gastric variability may be justified by the different gastric contest found in the animals. This was also observed in humans (Kalantzi et al., 2006).

4.4. Surface tension

Surface tension of the gastrointestinal fluids from rat is significantly lower than that of water, approximately 72 $mN.m^{-1}$ throughout the gut ref. This can be easily understood by the presence of a myriad of different compounds that act as surfactants in the GI tract of which bile salts are the most widely known. Bile salts are released in the upper duodenum and, as a result, the surface tension of the chyme is reduced. Rats lack a gall bladder, meaning the bile is not concentrated and stored but released continuously in the small intestine (DeSesso and Jacobson, 2001). As a result rat bile tends to be more diluted but secreted in relative higher amounts than in humans (DeSesso and Jacobson, 2001, Kararli, 1995). This might lead to more constantly high levels of bile salts in the rat gut during fasted state, allowing for a lower surface tension.

Female surface tension at the ileum is higher ($p < 0.05$), but in the caecum and colon the surface tension in females stabilizes (equals that of the ileum) whereas the males increases.

The higher surface tension in the ileum of females suggests that the females are swift in removing bile salts from the luminal environment (leading to an increase of the surface tension). The reason behind this is unclear, however it may be related to a combined effect of higher reabsorption rates and a more efficient metabolism of bile salts. It could also be considered that females secrete less bile salts than males, however, this does not appear to be the case because the surface tension in males and females is the same in the duodenum and in the jejunum. Also, the standard deviation of these mean values is very low in the duodenum and jejunum, suggesting the luminal environments in the upper GI tract are homogeneous in regards to surface tension and there is little intersubject variability. However, in the ileum, caecum and colon, the standard deviation is very high, suggesting that the mechanism behind the distal increase of the surface tension is more variable. This supports the hypothesis of a variable, albeit more efficient, degradation/scavenging mechanism of retrieving bile salts in females rats if compared to males.

4.5. Solubility of the active substances: 5-ASA and prednisolone

Overall, male and females have similar trends for each drug throughout the length of the GI tract (Figure 6 and Figure 7). Nevertheless there are some interesting points, mainly distally, where the sexes diverge.

Prednisolone

The solubility of prednisolone is relatively constant throughout the GI tract with the exception of the jejunum and the ileum where prednisolone solubility nearly doubles relatively to the stomach. It is interesting to note that males only have a higher prednisolone solubility at the jejunum, dropping in the ileum to similar values of the other gastrointestinal segments. On the other hand females have a higher solubility in both jejunum and ileum. both maximums in the sexes are very similar, just in different, yet contiguous, intestinal segments. This increase in the solubility of prednisolone in the jejunum and ileum was possibly due to a lower surface tension that would enhance wettability of the active substance, thus increase its solubility (Merchant et al., 2015). pH is not considered to influence the solubility of prednisolone significantly as this is an unionisable active substance.

Multiple linear regressions were carried out between the prednisolone solubility values and pH, buffer capacity, surface tension and osmolality. Only a weak correlation with surface tension in females (standardized coefficient β=-0.765, p=0.076; R^2=0.585) was observed. This is not sufficient to account for the increased active substance solubility in the female ileum. The gastrointestinal environment is complex and there is a large plethora of factors that may influence the solubility of active substances other than the four that were studied in this work.

5-ASA

Mesalazine is an ionisable drug with two pKa values: 2.3 and 5.69. This is an acidic compound and as such it that at lower pHs the solubility is lower than at higher pHs. If the pH of the pooled fluids in Table 2 is plotted, the shape of the solubility graph and the pH graph is inversely similar. The same is observed with buffer capacity.

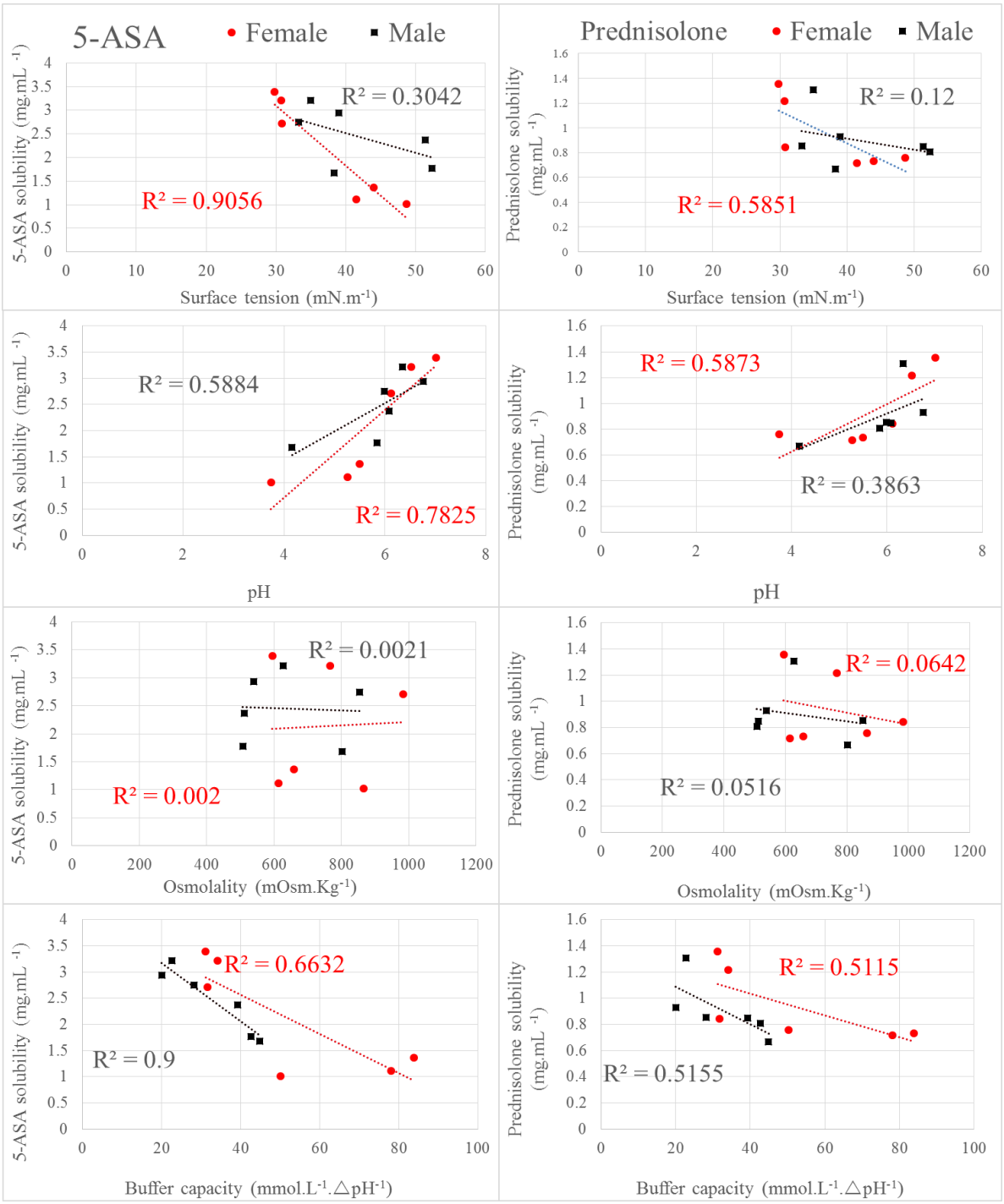
The sex differences observed in the solubility of 5-ASA are mainly related to the pH differences observed in the pooled fluids (Table 2) from the different segments.

The female rat has a lower gastric pH and as such also has an expected lower 5-ASA solubility. The higher pH in the duodenum and the jejunum allows for more 5-ASA to go in solution but no sex differences are observed because the pHs of the pooled fluids is very similar. The maximum solubility was achieved in the female ileum, where the pH of the pooled fluid was also the highest.

In the caecum and colon the pH is reduced and so is the solubility of 5-ASA. However, there is an interesting finding. The solubility of 5-ASA in the female caecum and colon is much lower than in males. The explanation is related to the second pKa of 5-ASA, 5.69. The pH of the male caecum and colon pooled fluids is above the pKa (6.08 and 5.84 respectively), whereas

the female pH is below (5.50 and 5.3 respectively). These pH values are considered to be close to the equivalence point of the titration curve (pKa) where a small change in the pH will produce a significant change in the ionisation degree of the active substance and as a result, impact solubility. Solubility was found to be positively correlated to the gastrointestinal pH of male (standardized coefficient β=0.767, p=0.075; R^2=0.588) and female (standardized coefficient β=0.885, p=0.019; R^2=0.728) rats.

Figure 8. Solubility of 5-ASA and prednisolone as a function of surface tension, pH, osmolality and buffer capacity. The dotted lines are the correlation between solubility and the physiologic parameter (the r^2 is represented on the graph).



Buffer capacity on the other hand was found to be negatively correlated with solubility (males: for a ΔpH=1.0, standardized coefficient β=-0.949, p=0.001; R^2=0.900 / females: standardized coefficient β=-0.814, p=0.048; R^2=0.663). This is also expected because the higher buffer capacities coincided with the lower pHs. And as discussed above the active substance is a weak acid, and finds difficulty in dissolving in an acidic media that is maintained so by a higher buffer capacity. 5-ASA is intended to be delivered to the colon and as such, a lower pH in female rats may have reduced the efficacy of the active substance when compared to male rats.

5. CONCLUSION

With the aim to identify whether rats sex played a role in their gastrointestinal luminal physicochemical characteristics male and female rats were sacrificed, their gastrointestinal tracts excised and the respective contents collected and analysed for pH, buffer capacity, surface tension and osmolality.

The trend of each parameter is similar in the sexes, however differences in the values were observed. These were mainly identified in the stomach and in the distal portion of the gastrointestinal tract: ileum, caecum and colon.

The pH in the stomach of females is lower than in males. The remainder of the sections did not show a sex difference. Buffer capacity was different in the sexes in the caecum and colon; females had higher values. Buffer capacity was also found to be higher at lower pHs. Males had higher osmolality than females in the duodenum, ileum and colon. Significant sex differences in surface tension were observed in the ileum where females had a higher surface tension. Solubility of 5-ASA was found to be significantly affected by the pH environment of the gastrointestinal tract. The female rat pH of the pooled fluids of the caecum and colon was lower than in the males, as a result, solubility of 5-ASA was lower in females in these segments.

Understanding how the gastrointestinal environment is affected by sex is important, especially for pH responsive formulations and ionisable drugs intended for oral administration.

6. ACKNOWLEDGEMENTS

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7. REFERENCES

AFONSO-PEREIRA, F., MURDAN, S., SOUSA, J., VEIGA, F. & BASIT, A. W. 2016. Sex differences in excipient effects: Enhancement in ranitidine bioavailability in the presence of polyethylene glycol in male, but not female, rats. *Int J Pharm,* 506**,** 237-41.

AGUWA, C. N. 1984. Effects of exogenous administration of female sex hormones on gastric secretion and ulcer formation in the rat. *European Journal of Pharmacology,* 104**,** 79-84.

AMURE, B. O. & OMOLE, A. A. 1970. Sex hormones, and acid gastric secretion induced with carbachol, histamine, and gastrin. *Gut,* 11**,** 641-5.

ASHIRU, D. A., PATEL, R. & BASIT, A. W. 2008. Polyethylene glycol 400 enhances the bioavailability of a BCS class III drug (ranitidine) in male subjects but not females. *Pharm Res,* 25**,** 2327-33.

BALISH, E., SHIH, C. N., CROFT, W. A., PAMUKCU, A. M., LOWER, G., BRYAN, G. T. & YALE, C. E. 1977. Effect of age, sex, and intestinal flora on the induction of colon tumors in rats. *J Natl Cancer Inst,* 58**,** 1103-6.

BARNES, R. H. 1962. NUTRITIONAL IMPLICATIONS OF COPROPHAGY. *Nutrition Reviews,* 20**,** 289-291.

CANCERDISCOVERY 2014. NIH to require both sexes in preclinical studies. *Cancer Discov,* 4**,** 860.

CHILLISTONE, S. H., J. 2008. Factors affecting drug absorption and distribution. *Anaesthesia and Intensive Care Medicine,* 5.

CLAYTON, J. A. & COLLINS, F. S. 2014. Policy: NIH to balance sex in cell and animal studies. *Nature,* 509**,** 282-3.

DESESSO, J. M. & JACOBSON, C. F. 2001. Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. *Food Chem Toxicol,* 39**,** 209-28.

FADDA, H. M., SOUSA, T., CARLSSON, A. S., ABRAHAMSSON, B., WILLIAMS, J. G., KUMAR, D. & BASIT, A. W. 2010. Drug Solubility in Luminal Fluids from Different Regions of the Small and Large Intestine of Humans. *Mol Pharm*.

FINHOLT, P. & SOLVANG, S. 1968. Dissolution kinetics of drugs in human gastric juice--the role of surface tension. *Journal of pharmaceutical sciences,* 57**,** 1322-6.

FLINT, H. J., DUNCAN, S. H., SCOTT, K. P. & LOUIS, P. 2015. Links between diet, gut microbiota composition and gut metabolism. *Proc Nutr Soc,* 74**,** 13-22.

FORDTRAN, J. S. & LOCKLEAR, T. W. 1966. Ionic constituents and osmolality of gastric and small-intestinal fluids after eating. *The American journal of digestive diseases,* 11**,** 503-21.

HIETANEN, E. 1975. Sex variation in the activities of mucosal hydrolytic enzymes in the small intestine of the rat. *Digestion,* 13**,** 33-41.

HÖRTER, D. & DRESSMAN, J. B. 2001. Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract. *Advanced Drug Delivery Reviews,* 46**,** 75-87.

JOHNSTON, K. P., OVERHOFF, K. A., MCCONVILLE, J. T., YANG, W., PETERS, J. I. & WILLIAMS, R. O. 2008. Effect of stabilizer on the maximum degree and extent of supersaturation and oral absorption of tacrolimus made by ultra-rapid freezing. *Pharmaceutical Research,* 25**,** 167-175.

KALANTZI, L., GOUMAS, K., KALIORAS, V., ABRAHAMSSON, B., DRESSMAN, J. B. & REPPAS, C. 2006. Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. *Pharm Res,* 23**,** 165-76.

KARARLI, T. T. 1995. COMPARISON OF THE GASTROINTESTINAL ANATOMY, PHYSIOLOGY, AND BIOCHEMISTRY OF HUMANS AND COMMONLY USED LABORATORY-ANIMALS. *Biopharmaceutics & Drug Disposition,* 16**,** 351-380.

LI, K. K., CONCEPCION, R. Y., LEE, H., CARDINAL, B. J., EBBECK, V., WOEKEL, E. & READDY, R. T. 2012. An examination of sex differences in relation to the eating habits and nutrient intakes of university students. *J Nutr Educ Behav,* 44**,** 246-50.

LIU, X., BLOUIN, J.-M., SANTACRUZ, A., LAN, A., ANDRIAMIHAJA, M., WILKANOWICZ, S., BENETTI, P.-H., TOMÉ, D., SANZ, Y., BLACHIER, F. & DAVILA, A.-M. 2014. *High protein diet modifies colonic microbiota and luminal environment but not colonocyte metabolism in the rat model: The increased luminal bulk connection*.

MAITRYA, B. B., GAHLOT, S. & MAITRYA, B. 1979. Effect of estrogen and testosterone on the gastric secretion of rats and conscious rabbits. *Indian J Physiol Pharmacol,* 23**,** 105-9.

MCCONNELL, E. L., BASIT, A. W. & MURDAN, S. 2008a. Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments. *J Pharm Pharmacol,* 60**,** 63-70.

MCCONNELL, E. L., SHORT, M. D. & BASIT, A. W. 2008b. An in vivo comparison of intestinal pH and bacteria as physiological trigger mechanisms for colonic targeting in man. *J Control Release,* 130**,** 154-60.

MERCHANT, H. A., AFONSO-PEREIRA, F., RABBIE, S. C., YOUSSEF, S. A. & BASIT, A. W. 2015. Gastrointestinal characterisation and drug solubility determination in animals. *J Pharm Pharmacol*.

MERCHANT, H. A., RABBIE, S. C., VARUM, F. J., AFONSO-PEREIRA, F. & BASIT, A. W. 2014. Influence of ageing on the gastrointestinal environment of the rat and its implications for drug delivery. *Eur J Pharm Sci*.

MUDIE, D. M., AMIDON, G. L. & AMIDON, G. E. 2010. Physiological Parameters for Oral Delivery and in Vitro Testing. *Mol Pharm*.

NATURE 2010. Putting gender on the agenda. *Nature,* 465**,** 665.

ORG, E., MEHRABIAN, M., PARKS, B. W., SHIPKOVA, P., LIU, X., DRAKE, T. A. & LUSIS, A. J. 2016. Sex differences and hormonal effects on gut microbiota composition in mice. *Gut Microbes,* 7**,** 313-322.

PANG, K. S. 2003. Modeling of intestinal drug absorption: roles of transporters and metabolic enzymes (for the Gillette Review Series). *Drug Metab Dispos,* 31**,** 1507-19.

PEGRAM, L. M. & RECORD, M. T., JR. 2008. Thermodynamic origin of hofmeister ion effects. *J Phys Chem B,* 112**,** 9428-36.

RADEMAKER, M. 2001. Do women have more adverse drug reactions? *Am J Clin Dermatol,* 2**,** 349-51.

RANGEL-YAGUI, C. O., PESSOA, A., JR. & TAVARES, L. C. 2005. Micellar solubilization of drugs. *J Pharm Pharm Sci,* 8**,** 147-65.

REGITZ-ZAGROSEK, V. 2012. Sex and gender differences in health: Science & Society Series on Sex and Science. *EMBO Reports,* 13**,** 596-603.

RÍOS-COVIÁN, D., RUAS-MADIEDO, P., MARGOLLES, A., GUEIMONDE, M., DE LOS REYES-GAVILÁN, C. G. & SALAZAR, N. 2016. Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. *Frontiers in Microbiology,* 7**,** 185.

SALONEN, A., LAHTI, L., SALOJARVI, J., HOLTROP, G., KORPELA, K., DUNCAN, S. H., DATE, P., FARQUHARSON, F., JOHNSTONE, A. M., LOBLEY, G. E., LOUIS, P., FLINT, H. J. & DE VOS, W. M. 2014. Impact of diet and individual variation on intestinal microbiota composition and fermentation products in obese men. *Isme j,* 8**,** 2218-30.

SCHIEBINGER, L. 2014. Scientific research must take gender into account. *Nature,* 507**,** 9.

SHASTRI, P., MCCARVILLE, J., KALMOKOFF, M., BROOKS, S. P. & GREEN-JOHNSON, J. M. 2015. Sex differences in gut fermentation and immune parameters in rats fed an oligofructose-supplemented diet. *Biol Sex Differ,* 6**,** 13.

SHORE, R., BJÖRNE, H., OMOTO, Y., SIEMIATKOWSKA, A., GUSTAFSSON, J.-Å., LINDBLAD, M. & HOLM, L. 2017. Sex differences and effects of oestrogen in rat gastric mucosal defence. *World Journal of Gastroenterology,* 23**,** 426-436.

STERN, S. T., TALLMAN, M. N., MILES, K. K., RITTER, J. K., DUPUIS, R. E. & SMITH, P. C. 2007. Gender-related differences in mycophenolate mofetil-induced gastrointestinal toxicity in rats. *Drug Metab Dispos,* 35**,** 449-54.

TOBET, S., KNOLL, J. G., HARTSHORN, C., AURAND, E., STRATTON, M., KUMAR, P., SEARCY, B. & MCCLELLAN, K. 2009. Brain sex differences and hormone influences: A moving experience? *Journal of neuroendocrinology,* 21**,** 387-392.

Chapter 3 - Sex differences in excipient effects: Enhancement in ranitidine bioavailability in the presence of polyethylene glycol in male, but not female, rats*

* This chapter has been published, please refer to the List of Publications.

ABSTRACT

Males and females respond differently to drugs: indeed, sex plays a crucial role in determining drug pharmacokinetics and pharmacodynamics. Excipients have also been shown to enhance the biovailability of drugs differently in men and women. The aim of this work was to investigate whether rodents are a good model in which to study sex-specific effects of polyethylene glycol 400 (PEG 400) on the bioavailability of ranitidine. Ranitidine (50 mg/Kg) was dissolved in water with different amounts of PEG 400 - 0 (control), 13, 26, 51, 77, 103, and 154 mg/Kg; these solutions were dosed orally by gavage to male and female Wistar rats. Blood samples were withdrawn over 480 minutes and assayed via HPLC-UV. Individual ranitidine plasma profiles were constructed for each rat, and standard pharmacokinetic parameters were determined. In the male rats, the change in the area under the plasma ranitidine curve (AUC_{0-480}) compared to the control group, was +18%; +49% ($p<0.05$); +37% ($p<0.05$); +31% ($p<0.05$); +8% and -22% ($p<0.05$) for PEG 400 doses of 13; 26; 51; 77; 103; and 154 mg/Kg respectively. On the other hand, females showed no statistically significant difference between the groups. In conclusion, low doses of PEG 400 enhanced the bioavailability of ranitidine in male, but not female, rats. These findings are in agreement with previously published human data, therefore confirming he validity of the rodent model, and highlight the unusual and clinically significant phenomenon that an excipient can influence drug bioavailability in one gender and not the other.

1. INTRODUCTION

Variability in drug effects is governed by a multitude of factors. Age, race, genetics, hormones, diet and disease can potentially alter the activity of a drug through either or both its pharmacokinetic and pharmacodynamic parameters (Jamei et al., 2009, Nicolas et al., 2009). Yet sex also acts as an important marker of inter-individual variability: sex-related differences have been evidenced in areas ranging from selective serotonin reuptake inhibitors' efficacy (Berlanga and Flores-Ramos, 2006) to cardiovascular pharmacology (Oertelt-Prigione and Regitz-Zagrosek, 2009, Ueno and Sato, 2012), pain management (Fillingim et al., 2009, Pleym et al., 2003), incidence of adverse drug effect, and drug toxicity (Bigos et al., 2009, Nicolas et al., 2009, Makkar et al., 1993, Aichhorn et al., 2005).

Though there remain wide gaps in our knowledge insofar as gender-specific drug effects are concerned, this should not undermine their wider importance - evidently, sex does matter. Indeed, such sex-related differences in gastrointestinal physiology have been increasingly well documented, including luminal pH (Feldman and Barnett, 1991), acid secretion (Prewett et al., 1991), fluid volume and composition (Lindahl et al., 1997, Bouras et al., 2002), transit times (Sadik et al., 2003, Metcalf et al., 1987), and even the extent to which they have been seen to influence oral drug bioavailability (Woodhead et al., 1991). The implications of such differences relate not only to the achievement of therapeutic benefit in sex groups and circumvention of adverse effects but, likewise, the potential for the personalization of medicines (Freire et al., 2011, Florence and Lee, 2011).

This also has special importance for the understanding of drug and dosage form behaviour in the context of sex specificity; though we might anticipate some differences in drug behaviour owing to such inter-individual variability between the sexes, this would not necessarily be the case for excipients included in a given formulation. Excipients are small or large molecules, such as mixtures of polymers (Raymond, 2009), incorporated into the final drug product to fulfil a particular role in drug delivery such as lubrication, dilution of the active ingredient or bulking, and are formally defined by the US Food and Drug Administration (FDA) as "inactive" substances. Strikingly, however, we have previously demonstrated the influence of the widely available and utilised solubility enhancer, polyethylene glycol (PEG) 400, on the enhancement of ranitidine bioavailability in men when given at low doses (Ashiru et al., 2008). The same effect was, surprisingly, absent in women – highlighting the complexity of sex influences in the context of oral drug delivery, as well as bringing into question the inert status of PEG 400 and excipients.

The aim of the current work was to investigate the potential of a rat model to identify sex-specific differences in the effects of excipients on drug bioavailability. As such, we aimed to determine the effects of PEG 400 on orally-delivered ranitidine in male and female rats.

2. MATERIALS AND METHODS

2.1 Materials and animals

Ranitidine, polyethylene Glycol 400, glacial acetic acid and sodium acetate trihydrate were obtained from Sigma Aldrich (Dorset UK). Water and acetonitrile were purchased from Fisher Scientific (Loughborough, UK), and were of HPLC grade.

Male and female Wistar rats (8 weeks old) were purchased from Harlan UK Ltd (Oxfordshire, UK).

2.2 Animal procedures

The rats were fasted for twelve hours in metabolic cages prior to the start of the experiment; food was made available four hours after dosing of the animals. A dose of 50mg/Kg of ranitidine was administered via oral gavage in an aqueous solution to all animals. The dose of ranitidine was chosen based on literature (Eddershaw et al., 1996), and on the quantification capabilities of the method used. The groups differed only according to sex and dose of PEG 400 administered (0mg/Kg; 13 mg/Kg; 26 mg/Kg; 51mg/Kg; 77mg/Kg; 103 mg/Kg; 154mg/Kg). The doses of PEG 400 were extrapolated to rats based on the human doses previously used (Ashiru et al., 2008), which produced an increase in the oral bioavailability of ranitidine in human volunteers. The dose extrapolation from human to rat was based on species body surface area (BSA). In order to extrapolate the animal dose from a human equivalent dose (HED), Reagen-Shaw et al. (Reagan-Shaw et al., 2008) explained that BSA would enable the most appropriate conversion as it provides acceptable correlations across different biological parameters, including "oxygen utilization, caloric expenditure, basal metabolism, blood volume, circulating plasma proteins, and renal function". The following equation was applied for the conversion:

$$HED(mg/Kg) = AD(mg/Kg)\frac{Animal\ Km}{Human\ Km}$$

Where HED is the human equivalent dose, AD the animal dose, Km is the BSA conversion factor which is 37 for humans and 6 for Wistar rats (Reagan-Shaw et al., 2008).

After dosing, the rats were placed in individual metabolic cages and were allowed to move freely. Approximately 200µL of blood was collected from the tail vein into anticoagulant centrifuge tubes (BD Microtainer® K2E Becton, Dickinson and Company, USA) at the following time points: 30min, 1.25h, 2h, 3h, 4h, and 6h. In between sampling, the rats were placed back in the metabolic cages. Blood volumes were taken in accordance with the project license and were stored on ice until the last collection point. After 8h, the animals were sacrificed in a CO_2 euthanasia chamber (Schedule 1 method), and approximately 2mL of blood were obtained via cardiac puncture.

Blood samples were centrifuged at 10000rpm (930g) for 10 min on a Centrifuge 5804R (Eppendorf AG, 22331 Hamburg, Germany) within 8h of sampling. 50µL of the supernatant

(plasma) was collected and placed into a 1.5mL Eppendorf tube, and immediately frozen at -20°C prior to analysis.

2.3 Sample analysis

Plasma samples were thawed and assayed for the amount of ranitidine: for each sample, 50µL of plasma was mixed with the same volume of acetonitrile in order to precipitate the plasma proteins. After 1 min of vortex-mixing, a further 100µL of HPLC grade water was added to the mixture, and after subsequent vortex-mixing, the samples were centrifuged at 4ºC for 10 min at 10000rpm. The resulting supernatant was subjected to HPLC-UV analysis using a previously validated method (Ashiru et al., 2007). The column used was a 5µm Luna SCX (Phenomenex, UK); the mobile phase was a mixture of 20:80 acetonitrile:0.1M sodium acetate pH=5.0 with a flow rate of 2ml/min and 40µL of injection volume. Calibration standards were prepared with blank rat plasma samples spiked with drug subjected to the above-mentioned treatment.

Plasma ranitidine concentration versus time profiles were produced for each animal. C_{max} and t_{max} were taken from these profiles. The cumulative area under the plasma concentration versus time curve ($AUC_{0\text{-}480}$) was calculated using the integration method with OriginPro 9.0 (OriginLab, Northampton, MA, USA).

2.4 Statistical analysis

The overall data was analysed by two-way ANOVA, followed by a Tukey post-hoc analysis and by individual t-student comparisons between individual groups and the appropriate controls, with IBM SPSS Statistics 19 (SPSS Inc., Illinois, USA).

3. RESULTS

The mean variations in the AUC in the presence and absence of PEG 400 are shown in Figures 1, 2 and Table 1.

Table 1. The effect of different doses of PEG400 on the bioavailability of ranitidine in male and female rats; mean ± standard deviation are shown; n=5-6

Dose of PEG 400 (mg/Kg)	*Male*		*Female*	
	AUC_{0-480} (µg.min/mL)	*Variation against the control*	*AUC_{0-480} (µg.min/mL)*	*Variation against the control*
0 (control)	347.6±42.5	NA	409.8±175.4	NA
13	410.2±182.0	+18%	403.3±53.2	-2%
26	517.6±140.0	+49%*	437.6±90.3	+7%
51	476.3±130.0	+37%*	416.1±117.0	+2%
77	456.5±80.4	+31%*	425.3±71.9	+4%
103	375.0±58.5	+8%	368.8±104.0	-10%
154	271.9±17.6	-22%*	394.4±45.4	-4%

***** Indicates statistical difference ($p<0.05$) from the control group.

Two-way ANOVA showed a statistically significant influence of sex ($p<0.05$) and different doses of PEG 400 ($p<0.05$) on variation of ranitidine AUC_{0-480} compared with the control.

More specifically, from Figure 1 and Table 1 it can be seen that: PEG 400 influenced ranitidine bioavailability in male but not females rats. The influence of PEG 400 in male rats was dose-dependent, with three of the doses of PEG 400 leading to significant increases in ranitidine bioavailability, while the highest PEG 400 dose led to a significant decrease in ranitidine bioavailability.

Figure 1. Mean AUC_{0-480} of the animal groups.***** Denotes statistically significant difference compared to the control group ($p<0.05$).

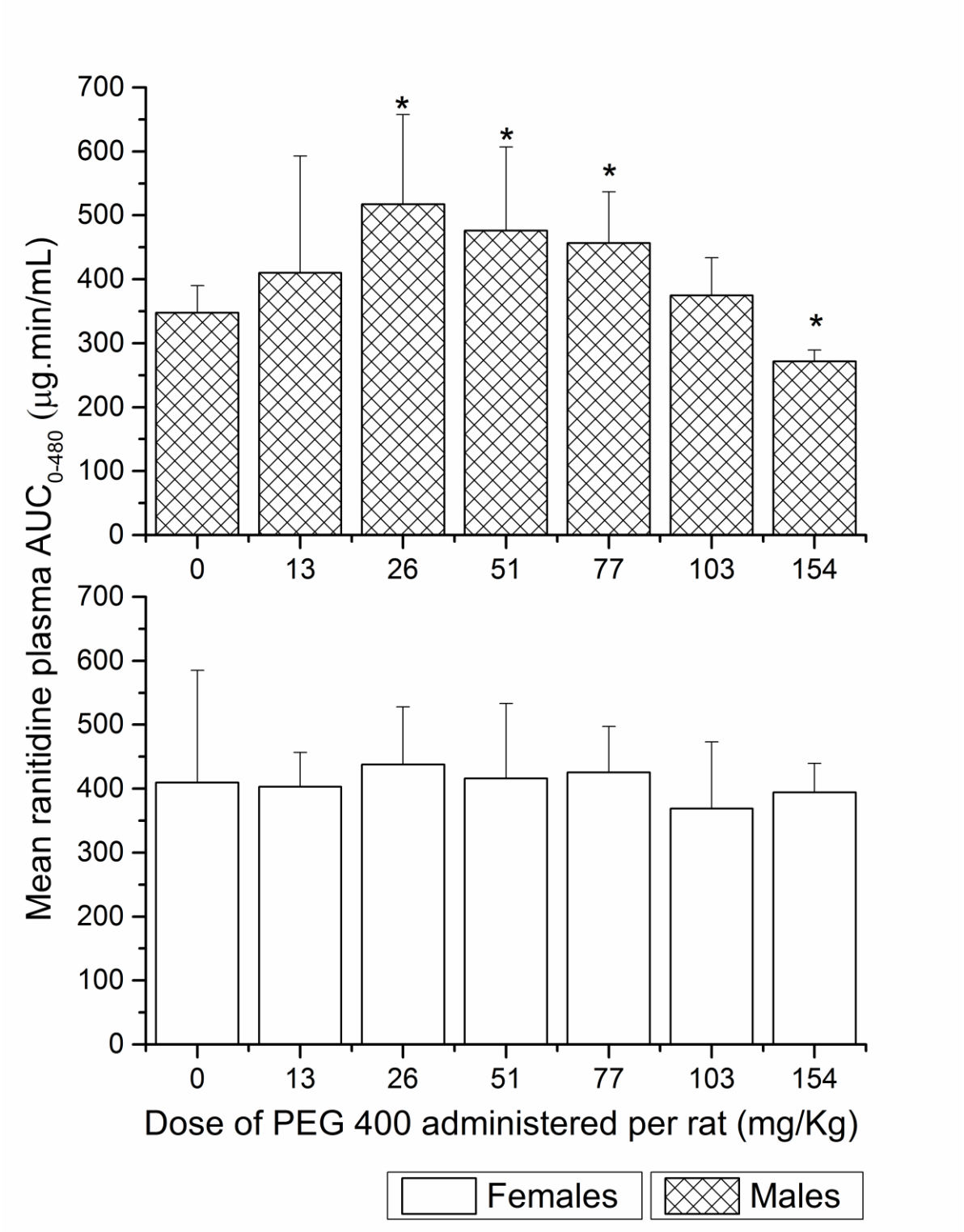
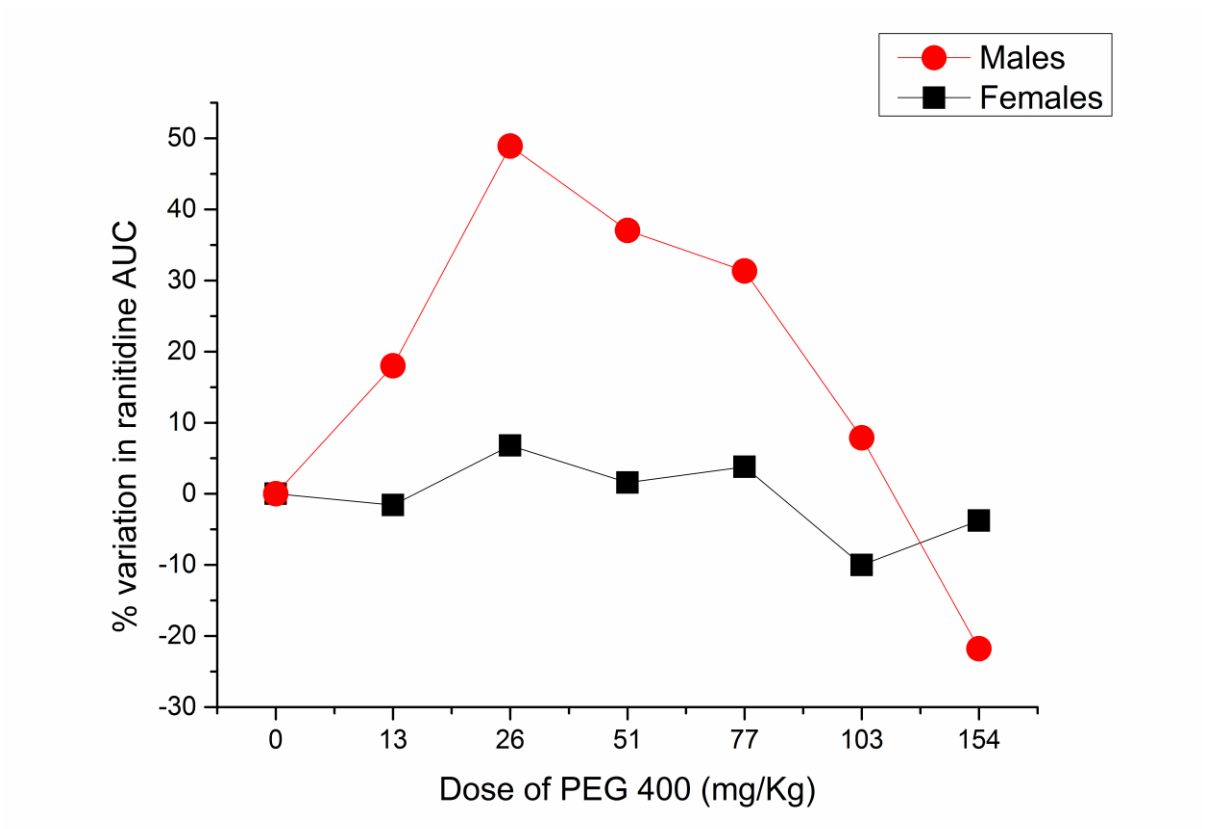


Figure 2. Percentage variation of the mean AUC_{0-480} of ranitidine of the different PEG 400 dose groups against the control group.



4. DISCUSSION

Our results in the rat model demonstrating the enhancement of ranitidine bioavailability by low doses of PEG 400 in male, but not female, rats is in agreement with results reported for human volunteers (Ashiru et al., 2008). In the latter study, an increase in ranitidine bioavailability of up to 63% ($p<0.05$) was achieved in men, but not in women, when PEG 400 was used at a dose of 0.75 g. High doses of PEG 400 led to reductions in the bioavailability of ranitidine. High doses of PEG 400, by retaining water in the intestinal lumen, reduces the gastrointestinal transit time, thus the time ranitidine has to be absorbed (Basit et al., 2002, Schulze et al., 2003, Basit et al., 2001). Based on the profiles of bioavailability changes in humans (Ashiru et al., 2008) and rats (Figures 1 and 2), this animal model may be considered appropriate for carrying out further studies investigating excipient effects on ranitidine bioavailability.

Ranitidine is a BCS class III drug that possesses a high aqueous solubility but low permeability through gastrointestinal membranes (Kortejarvi et al., 2005). As such, 40% of the overall bioavailability of ranitidine in humans is mediated by membrane transporters, with the paracellular route via tight junctions accounting for the remaining 60% (Bourdet et al., 2006). Ranitidine has also been reported to act as a substrate for efflux and influx membrane transporters and a considerable amount of membrane secretion of ranitidine into the luminal environment, mainly in the small intestine has been observed (Collett et al., 1999). Taking these into consideration, two possible reasons arise for the bioavailability enhancing effect of ranitidine by PEG 400: intercellular space disorganisation (opening of tight junctions) by PEG 400 with a subsequent increase in the absorption of ranitidine via the paracellular route; or the interactions of PEG 400 with membrane transporters on the intestinal wall. The fact that PEG 400 does not affect the permeation of drugs that permeate exclusively via the paracellular route (Johnson et al., 2002, Rege et al., 2001) without necessitating membrane transporters, indicates that the PEG 400 enhancing effect on ranitidine bioavailability in males occurs by interaction with membrane transporters. Furthermore, studies which have identified regional differences in the absorption of ranitidine from the small intestine (Mummaneni and Dressman, 1994) suggest that the effects of PEG 400 are not necessarily homogeneous throughout the gastrointestinal tract. Regionally differing permeation can be associated with the combination of different transporters (influx and efflux) with regional differences in their expression levels in the small intestine (Kagan et al., 2010, Lindell et al., 2003).

The most extensively described interaction of ranitidine with a membrane transporter is not with uptake transporters, but instead with a transporter responsible for its luminal secretion (efflux transporters). Examples of commonly occurring efflux transporters in biological membranes are P-glycoprotein (P-gp), MDR-associated protein (MRP1 and MRP2), and breast cancer resistance proteins (BRCP) (Collett et al., 1999). It is therefore unsurprising that drugs which function as substrates for these efflux transporters may be subject to variations in their bioavailability when the efflux is somehow affected. While ranitidine is not a substrate for MDR-associated proteins or BCRP (following inhibition of these transporters, no difference was observed in terms of ranitidine secretion) it is a substrate for P-gp (Collett et al., 1999).

Excipients have been reported to elicit effects on efflux transporters (Li et al., 2011, Rege et al., 2001, Johnson et al., 2002, Shen et al., 2006), and specifically in the case of PEG 400, its influence on P-gp transporters has been investigated in excised male rat intestine (Johnson et al., 2002). The latter study revealed an important dose-dependent inhibition of P-gp without affecting the integrity of tight junctions between cells. This data was later corroborated by Ashiru et al (Ashiru-Oredope et al., 2011), who reached similar conclusions, namely that the efflux of ranitidine by Caco-2 cells was effectively inhibited by PEG 400 (Zhang et al., 2008). The reasons as to why Caco-2 permeation do not allow for a full explanation of the PEG 400 effect, including the marked sex difference observed *in vivo*, is that Caco-2 is a male-derived cell line from a colon adenocarcinoma (Sambuy et al., 2005) - there is no corresponding female parental cell line to mimic permeation in females. PEG 400 was also shown to affect the permeation of substances other than ranitidine. Shen (Hugger et al., 2002) found that PEG 400, 2000 and 20000, as well as PEG monooleate and PEG monostearate, inhibited the transport of rhodamine by P-gp across isolated rat intestinal membranes in a dose dependent way.

The bioavailability enhancement of ranitidine in the presence of low doses of PEG 400 in male rats may, therefore, be partially due to the inhibition of P-gp. The mechanism by which P-gp might be inhibited by excipients is still unclear. Nevertheless there have been some studies trying to elucidate this. Surfactants and solvents, for example, by fluidising the lipid bilayer have been shown to modulate P-gp activity (Lo, 2003); Pluronics on the other hand lead to an ATP depletion by reducing the ATPase activity of P-gp (Batrakova and Kabanov, 2008); other interactions between excipients and P-gp have been suggested as down regulation of the MDR1 gene (the gene responsible for P-gp expression) (Sachs-Barrable et al., 2007) and steric impediment or inhibition of the protein kinase C activity (Cornaire et al., 2004). As far as PEG 400 is concerned, no study on the nature of the inhibition mechanism was found, but the idea of modification of the fluidity of the polar head regions of the cell membranes with PEG 300 has been put forward (Werle, 2008), and most likely will be an analogous situation with PEG 400.

From the above information it may be possible to find an explanation for the bioavailability-enhancing effect in the male rats. If intestinal membrane P-gp is inhibited by PEG 400, ranitidine absorbed into the enterocytes will not be secreted back into the intestinal lumen. This will raise the concentration of ranitidine inside the enterocyte, in turn leading to enhanced absorption into the blood stream, raising the overall exposure of the male rat to ranitidine. The fact that such enhancement of bioavailability does not occur in females indicates sex-based differences in: the level of expression of epithelial transporters and/or on the location(s) of the small intestine where these transporters are expressed in higher amounts. It is hypothesised that females will be less susceptible to P-gp inhibition than males, likely resulting from either an overall lower amount of active P-gp transporters in the gut or in lower expression of P-gp at the preferred absorption sites of ranitidine in the small intestine.

Indeed, sex differences in gut transporter levels have been reported. For example, an important influx transporter is the OCT/OCAT (organic cation uptake transporter) whose substrates include antagonists of the histamine type 2 receptor (Bourdet et al., 2006, Bourdet et al., 2005).

Though no reports on the relative amount of the OCT/OCAT in different regions of the intestinal mucosa have been found, their expression in rat kidneys is known to be much higher in males than in females (Urakami et al., 2000). This may provide an alternative explanation for our results. Sex-related differences have also been identified in the renal and hepatic expressions of OAT (organic anion transporters) (OAT1-3 and SLc22a6-8) in mice (Buist and Klaassen, 2004), although whether these differences in the kidney translate to differences in the intestinal mucosa is unknown. It is also known that, some efflux transporters such as BCRP are generally more prevalent in females than males (Zamber et al., 2003), except for the liver, where the levels of BCRP and P-gp are higher in males (Schuetz et al., 1995, Merino et al., 2005). Cummins et al. (Cummins et al., 2002) and Chen (Chen, 2005) suggested that differences in the clearance of CYP3A4/P-gp substrates could be due to different expression of P-gp in males and females and not so much due to differences in the amount of the related enzymes.

It stands that lower levels of gut P-gp in females could therefore contribute to the differences in bioavailability, as noted in this rat study and in others which have shown healthy women to have significantly increased intestinal epithelial permeability compared to men in response to incoming stimuli in the jejunum (Alonso et al., 2012). Assuming these sex differences are similar to those in rats, the potential inhibitory effect of PEG 400 would not be as pronounced as in males, which could provide an explanation as to why, with the used doses of PEG 400, the bioavailability of ranitidine in female rats only varied slightly from -10% to +7%.

5. CONCLUSION

We have shown that the widely-used so-called “inert” excipient PEG 400 influences the bioavailability of ranitidine in rats in both a gender-specific and dose-dependent manner. Here, high doses of PEG 400 were seen to reduce ranitidine absorption, by decreasing intestinal transit time. Low doses of PEG 400 had the contrary effect of increasing absorption of ranitidine in male rats but not in females. The exact mechanism for these effects remains unknown, but may be related to the role of intestinal P-gp drug efflux transporters present in different regions and at different concentrations in male and female gastrointestinal tracts.

Other factors such as influx transporters, GIT luminal fluid volumes, and GIT transit time may additionally play a role in these gender differences. Furthermore, these PEG 400 effects may be extendable to other BCS III drugs and those drugs which also function as substrates for intestinal membrane transporters. Consequently, the potential for an “active” role of excipients in pharmaceutical formulations should not be underestimated, and particularly in the case of formulations for poorly permeable and/or soluble compounds belonging to BCS classes III and IV. This also applies to the potential for previously unrecognized gender-specific effects, which may prove to be dangerous in the case of some drug compounds should these excipient effects be replicable, predisposing to significant decreases and/or increases in drug bioavailability in one sex but not the other.

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7. REFERENCES

AICHHORN, W., GASSER, M., WEISS, E. M., ADLASSNIG, C. & MARKSTEINER, J. 2005. Gender differences in pharmacokinetics and side effects of second generation antipsychotic drugs. *Current Neuropharmacology,* 3**,** 73-85.

ALONSO, C., GUILARTE, M., VICARIO, M., RAMOS, L., REZZI, S., MARTINEZ, C., LOBO, B., MARTIN, F. P., PIGRAU, M., GONZALEZ-CASTRO, A. M., GALLART, M., MALAGELADA, J. R., AZPIROZ, F., KOCHHAR, S. & SANTOS, J. 2012. Acute experimental stress evokes a differential gender-determined increase in human intestinal macromolecular permeability. *Neurogastroenterol Motil,* 24**,** 740-6, e348-9.

ASHIRU-OREDOPE, D. A., PATEL, N., FORBES, B., PATEL, R. & BASIT, A. W. 2011. The effect of polyoxyethylene polymers on the transport of ranitidine in Caco-2 cell monolayers. *International Journal of Pharmaceutics,* 409**,** 164-8.

ASHIRU, D. A., PATEL, R. & BASIT, A. W. 2007. Simple and universal HPLC-UV method to determine cimetidine, ranitidine, famotidine and nizatidine in urine: application to the analysis of ranitidine and its metabolites in human volunteers. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences,* 860**,** 235-40.

ASHIRU, D. A., PATEL, R. & BASIT, A. W. 2008. Polyethylene glycol 400 enhances the bioavailability of a BCS class III drug (ranitidine) in male subjects but not females. *Pharm Res,* 25**,** 2327-33.

BASIT, A. W., NEWTON, J. M., SHORT, M. D., WADDINGTON, W. A., ELL, P. J. & LACEY, L. F. 2001. The effect of polyethylene glycol 400 on gastrointestinal transit: implications for the formulation of poorly-water soluble drugs. *Pharmaceutical Research,* 18**,** 1146-50.

BASIT, A. W., PODCZECK, F., NEWTON, J. M., WADDINGTON, W. A., ELL, P. J. & LACEY, L. F. 2002. Influence of polyethylene glycol 400 on the gastrointestinal absorption of ranitidine. *Pharmaceutical Research,* 19**,** 1368-74.

BATRAKOVA, E. V. & KABANOV, A. V. 2008. Pluronic block copolymers: evolution of drug delivery concept from inert nanocarriers to biological response modifiers. *J Control Release,* 130**,** 98-106.

BERLANGA, C. & FLORES-RAMOS, M. 2006. Different gender response to serotonergic and noradrenergic antidepressants. A comparative study of the efficacy of citalopram and reboxetine. *Journal of Affective Disorders,* 95**,** 119-123.

BIGOS, K. L., POLLOCK, B. G., STANKEVICH, B. A. & BIES, R. R. 2009. Sex Differences in the Pharmacokinetics and Pharmacodynamics of Antidepressants: An Updated Review. *Gender Medicine,* 6**,** 522-543.

BOURAS, E. P., DELGADO-AROS, S., CAMILLERI, M., CASTILLO, E. J., BURTON, D. D., THOMFORDE, G. M. & CHIAL, H. J. 2002. SPECT imaging of the stomach: comparison with barostat, and effects of sex, age, body mass index, and fundoplication. *Gut,* 51**,** 781-786.

BOURDET, D. L., POLLACK, G. M. & THAKKER, D. R. 2006. Intestinal absorptive transport of the hydrophilic cation ranitidine: a kinetic modeling approach to elucidate

the role of uptake and efflux transporters and paracellular vs. transcellular transport in Caco-2 cells. *Pharmaceutical Research,* 23**,** 1178-87.

BOURDET, D. L., PRITCHARD, J. B. & THAKKER, D. R. 2005. Differential substrate and inhibitory activities of ranitidine and famotidine toward human organic cation transporter 1 (hOCT1; SLC22A1), hOCT2 (SLC22A2), and hOCT3 (SLC22A3). *J Pharmacol Exp Ther,* 315**,** 1288-97.

BUIST, S. C. & KLAASSEN, C. D. 2004. Rat and mouse differences in gender-predominant expression of organic anion transporter (Oat1-3; Slc22a6-8) mRNA levels. *Drug Metab Dispos,* 32**,** 620-5.

CHEN, M. L. 2005. Confounding factors for sex differences in pharmacokinetics and pharmacodynamics: focus on dosing regimen, dosage form, and formulation. *Clin Pharmacol Ther,* 78**,** 322-9.

COLLETT, A., HIGGS, N. B., SIMS, E., ROWLAND, M. & WARHURST, G. 1999. Modulation of the permeability of H-2 receptor antagonists cimetidine and ranitidine by P-glycoprotein in rat intestine and the human colonic cell line Caco-2. *J Pharmacol Exp Ther,* 288**,** 171-178.

CORNAIRE, G., WOODLEY, J., HERMANN, P., CLOAREC, A., ARELLANO, C. & HOUIN, G. 2004. Impact of excipients on the absorption of P-glycoprotein substrates in vitro and in vivo. *International Journal of Pharmaceutics,* 278**,** 119-31.

CUMMINS, C. L., WU, C. Y. & BENET, L. Z. 2002. Sex-related differences in the clearance of cytochrome P450 3A4 substrates may be caused by P-glycoprotein. *Clin Pharmacol Ther,* 72**,** 474-89.

EDDERSHAW, P. J., CHADWICK, A. P., HIGTON, D. M., FENWICK, S. H., LINACRE, P., JENNER, W. N., BELL, J. A. & MANCHEE, G. R. 1996. Absorption and disposition of ranitidine hydrochloride in rat and dog. *Xenobiotica,* 26**,** 947-56.

FELDMAN, M. & BARNETT, C. 1991. FASTING GASTRIC PH AND ITS RELATIONSHIP TO TRUE HYPOCHLORHYDRIA IN HUMANS. *Digestive Diseases and Sciences,* 36**,** 866-869.

FILLINGIM, R. B., KING, C. D., RIBEIRO-DASILVA, M. C., RAHIM-WILLIAMS, B. & RILEY, J. L., III 2009. Sex, Gender, and Pain: A Review of Recent Clinical and Experimental Findings. *Journal of Pain,* 10**,** 447-485.

FLORENCE, A. T. & LEE, V. H. L. 2011. Personalised medicines: More tailored drugs, more tailored delivery. *International Journal of Pharmaceutics,* 415**,** 29-33.

FREIRE, A. C., BASIT, A. W., CHOUDHARY, R., PIONG, C. W. & MERCHANT, H. A. 2011. Does sex matter? The influence of gender on gastrointestinal physiology and drug delivery. *International Journal of Pharmaceutics,* 415**,** 15-28.

HUGGER, E. D., NOVAK, B. L., BURTON, P. S., AUDUS, K. L. & BORCHARDT, R. T. 2002. A comparison of commonly used polyethoxylated pharmaceutical excipients on their ability to inhibit P-glycoprotein activity in vitro. *J Pharm Sci,* 91**,** 1991-2002.

JAMEI, M., DICKINSON, G. L. & ROSTAMI-HODJEGAN, A. 2009. A Framework for Assessing Inter-individual Variability in Pharmacokinetics Using Virtual Human Populations and Integrating General Knowledge of Physical Chemistry, Biology, Anatomy, Physiology and Genetics: A Tale of 'Bottom-Up' vs 'Top-Down' Recognition of Covariates. *Drug Metabolism and Pharmacokinetics,* 24**,** 53-75.

JOHNSON, B. M., CHARMAN, W. N. & PORTER, C. J. 2002. An in vitro examination of the impact of polyethylene glycol 400, Pluronic P85, and vitamin E d-alpha-tocopheryl polyethylene glycol 1000 succinate on P-glycoprotein efflux and enterocyte-based metabolism in excised rat intestine. *AAPS PharmSci,* 4**,** E40.

KAGAN, L., DREIFINGER, T., MAGER, D. E. & HOFFMAN, A. 2010. Role of p-glycoprotein in region-specific gastrointestinal absorption of talinolol in rats. *Drug Metab Dispos,* 38**,** 1560-6.

KORTEJARVI, H., YLIPERTTULA, M., DRESSMAN, J. B., JUNGINGER, H. E., MIDHA, K. K., SHAH, V. P. & BARENDS, D. M. 2005. Biowaiver monographs for immediate release solid oral dosage forms: ranitidine hydrochloride. *Journal of Pharmaceutical Sciences,* 94**,** 1617-25.

LI, M., SI, L., PAN, H., RABBA, A. K., YAN, F., QIU, J. & LI, G. 2011. Excipients enhance intestinal absorption of ganciclovir by P-gp inhibition: assessed in vitro by everted gut sac and in situ by improved intestinal perfusion. *Int J Pharm,* 403**,** 37-45.

LINDAHL, A., UNGELL, A. L., KNUTSON, L. & LENNERNAS, H. 1997. Characterization of fluids from the stomach and proximal jejunum in men and women. *Pharmaceutical Research,* 14**,** 497-502.

LINDELL, M., KARLSSON, M. O., LENNERNAS, H., PAHLMAN, L. & LANG, M. A. 2003. Variable expression of CYP and Pgp genes in the human small intestine. *Eur J Clin Invest,* 33**,** 493-9.

LO, Y. L. 2003. Relationships between the hydrophilic-lipophilic balance values of pharmaceutical excipients and their multidrug resistance modulating effect in Caco-2 cells and rat intestines. *J Control Release,* 90**,** 37-48.

MAKKAR, R. R., FROMM, B. S., STEINMAN, R. T., MEISSNER, M. D. & LEHMANN, M. H. 1993. FEMALE GENDER AS A RISK FACTOR FOR TORSADES-DE-POINTES ASSOCIATED WITH CARDIOVASCULAR DRUGS. *Jama-Journal of the American Medical Association,* 270**,** 2590-2597.

MERINO, G., VAN HERWAARDEN, A. E., WAGENAAR, E., JONKER, J. W. & SCHINKEL, A. H. 2005. Sex-dependent expression and activity of the ATP-binding cassette transporter breast cancer resistance protein (BCRP/ABCG2) in liver. *Mol Pharmacol,* 67**,** 1765-71.

METCALF, A. M., PHILLIPS, S. F., ZINSMEISTER, A. R., MACCARTY, R. L., BEART, R. W. & WOLFF, B. G. 1987. Simplified Assessment of Segmental Colonic Transit. *Gastroenterology,* 92**,** 40-47.

MUMMANENI, V. & DRESSMAN, J. B. 1994. Intestinal uptake of cimetidine and ranitidine in rats. *Pharmaceutical Research,* 11**,** 1599-604.

NICOLAS, J.-M., ESPIE, P. & MOLIMARD, M. 2009. Gender and interindividual variability in pharmacokinetics. *Drug Metabolism Reviews,* 41**,** 408-421.

OERTELT-PRIGIONE, S. & REGITZ-ZAGROSEK, V. 2009. Gender Aspects in Cardiovascular Pharmacology. *Journal of Cardiovascular Translational Research,* 2**,** 258-266.

PLEYM, H., SPIGSET, O., KHARASCH, E. D. & DALE, O. 2003. Gender differences in drug effects: implications for anesthesiologists. *Acta Anaesthesiologica Scandinavica,* 47**,** 241-259.

PREWETT, E. J., SMITH, J. T. L., NWOKOLO, C. U., SAWYERR, A. M. & POUNDER, R. E. 1991. 24 HOUR INTRAGASTRIC ACIDITY AND PLASMA GASTRIN-CONCENTRATION PROFILES IN FEMALE AND MALE-SUBJECTS. *Clinical Science,* 80**,** 619-624.

RAYMOND, C. R. P. J. S. M. E. Q. 2009. *Handbook of Pharmaceutical Excipients*, Pharmaceutical Press.

REAGAN-SHAW, S., NIHAL, M. & AHMAD, N. 2008. Dose translation from animal to human studies revisited. *Faseb j,* 22**,** 659-61.

REGE, B. D., YU, L. X., HUSSAIN, A. S. & POLLI, J. E. 2001. Effect of common excipients on Caco-2 transport of low-permeability drugs. *J Pharm Sci,* 90**,** 1776-86.

SACHS-BARRABLE, K., THAMBOO, A., LEE, S. D. & WASAN, K. M. 2007. Lipid excipients Peceol and Gelucire 44/14 decrease P-glycoprotein mediated efflux of rhodamine 123 partially due to modifying P-glycoprotein protein expression within Caco-2 cells. *J Pharm Pharm Sci,* 10**,** 319-31.

SADIK, R., ABRAHAMSSON, H. & STOTZER, P. O. 2003. Gender differences in gut transit shown with a newly developed radiological procedure. *Scandinavian Journal of Gastroenterology,* 38**,** 36-42.

SAMBUY, Y., DE ANGELIS, I., RANALDI, G., SCARINO, M. L., STAMMATI, A. & ZUCCO, F. 2005. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol,* 21**,** 1-26.

SCHUETZ, E. G., FURUYA, K. N. & SCHUETZ, J. D. 1995. Interindividual variation in expression of P-glycoprotein in normal human liver and secondary hepatic neoplasms. *J Pharmacol Exp Ther,* 275**,** 1011-8.

SCHULZE, J. D., WADDINGTON, W. A., ELI, P. J., PARSONS, G. E., COFFIN, M. D. & BASIT, A. W. 2003. Concentration-dependent effects of polyethylene glycol 400 on gastrointestinal transit and drug absorption. *Pharmaceutical Research,* 20**,** 1984-8.

SHEN, Q., LIN, Y., HANDA, T., DOI, M., SUGIE, M., WAKAYAMA, K., OKADA, N., FUJITA, T. & YAMAMOTO, A. 2006. Modulation of intestinal P-glycoprotein function by polyethylene glycols and their derivatives by in vitro transport and in situ absorption studies. *International Journal of Pharmaceutics,* 313**,** 49-56.

UENO, K. & SATO, H. 2012. Sex-related differences in pharmacokinetics and pharmacodynamics of anti-hypertensive drugs. *Hypertension Research,* 35**,** 245-250.

URAKAMI, Y., OKUDA, M., SAITO, H. & INUI, K. 2000. Hormonal regulation of organic cation transporter OCT2 expression in rat kidney. *FEBS Lett,* 473**,** 173-6.

WERLE, M. 2008. Natural and Synthetic Polymers as Inhibitors of Drug Efflux Pumps. *Pharmaceutical Research,* 25**,** 500-511.

WOODHEAD, J. C., DRULIS, J. M., NELSON, S. E., JANGHORBANI, M. & FOMON, S. J. 1991. GENDER-RELATED DIFFERENCES IN IRON-ABSORPTION BY PREADOLESCENT CHILDREN. *Pediatric Research,* 29**,** 435-439.

ZAMBER, C. P., LAMBA, J. K., YASUDA, K., FARNUM, J., THUMMEL, K., SCHUETZ, J. D. & SCHUETZ, E. G. 2003. Natural allelic variants of breast cancer resistance protein (BCRP) and their relationship to BCRP expression in human intestine. *Pharmacogenetics,* 13**,** 19-28.

ZHANG, X. X., PAN, W. S., GAN, L., ZHU, C. L. & GAN, Y. 2008. Effects of breast cancer resistance protein inhibitors and pharmaceutical excipients on decreasing gastrointestinal toxicity of camptothecin analogs. *Acta Pharmacol Sin,* 29**,** 1391-8.

Chapter 4 - Sex differences in the gut: influence of an excipient on cimetidine bioavailability in humans and rats

ABSTRACT

Males and females respond differently to drugs: sex plays a crucial role in determining drug pharmacokinetics and pharmacodynamics. It has been found, in two separate studies, that polyethylene glycol 400 (PEG 400) enhances the oral bioavailability of the BCS III drug ranitidine in males but not females in both humans (Ashiru et al., 2008) and Wistar rats (Afonso-Pereira et al., 2016).

The aim of this study was to understand if such a sex-related influence of PEG 400 is more universal (i.e. applies to other drugs) and if the rat is a robust predictor of bioavailability trends in humans. Cimetidine was co-administered with PEG 400 (0 -control-; 0.5; 0.75; 1; 1.5 and 5g) to fasted men (n=6) and women (n=6). The cimetidine amount excreted in urine over 24 hours was quantified as an indication of oral bioavailability. Fasted male and female Wistar rats were dosed with 50mg/Kg of cimetidine and PEG 400 (0; 13; 26; 51; 77 mg/Kg).

Low doses of PEG 400 increase the bioavailability of cimetidine in men and male rats. The degree at which PEG 400 modifies the oral bioavailability of cimetidine is dose dependent. In men, PEG 400 in a dose of 0.5, 0.75 and 1.0 g significantly ($p<0.05$) increased the bioavailability of cimetidine by 29%, 53% and 41% respectively. In male rats, doses of PEG 400 of 12.9 and 25.7 mg/kg significantly ($p<0.05$) increased the bioavailability of cimetidine by 36% and 37% respectively, Women and female rats are not affected by low doses of PEG 400. The effect of PEG 400 is sex dependent in both species.

The changes in the bioavailability of cimetidine in humans and rats by PEG 400 are common to ranitidine and this is likely due to sex differences in the intestinal transporters.

1. INTRODUCTION

Variability in drug effects is governed by a multitude of factors. Diet, genetics, hormones and disease can potentially alter the activity of drugs in any individual through either or both its pharmacokinetics and pharmacodynamics (Jamei et al., 2009), with race, age and even sex playing fundamental roles (Perrie et al., 2012, Freire et al., 2011, Gandhi et al., 2004, Yood et al., 1998). Specifically in the context of sex, differences in responses to drug therapy between males and females have been noted in several cases, including antidepressants (Bigos et al., 2009); anti-hypertensives (Ueno and Sato, 2012); iron supplements (Woodhead et al., 1991) and pain relief (Fillingim et al., 2009). Indeed, 25 of the 67 new molecular entities (NMEs) approved by the U.S. Food and Drug Administration (FDA) between 2000-2002 were shown to be influenced by sex with respect to pharmacokinetics, efficacy and/or the incidence of adverse effects (Yang et al., 2009). Prior to this, eight out of ten drugs withdrawn from the U.S. market between 1997 and 2000 were shown to have a higher incidence of adverse effects in females than in males ((GAO), 2001). Sex-determined differences are arguably to be expected, given the broad variations in male and female gastrointestinal physiology (Gandhi et al., 2004, Freire et al., 2011). In addition, sex-differences in disease prevalence are also thought to strongly contribute to unequal participation in clinical trials as well as differences in clinical outcomes (Poon et al., 2011).

What might be considered unexpected, however, is the influence on drug bioavailability between the sexes, mediated by excipients in each formulation. Excipients have traditionally been classified as inactive or inert ingredients per drug monographs and regulatory bodies such as the US Food and Drug Administration (FDA), and are not considered to have a direct influence on the activity of an active pharmaceutical ingredient (API) with which they are co-formulated. However, there is recent evidence to suggest that excipients may perform other roles in the context of drug delivery, bringing their “inert” status into question (Basit et al., 2001, Basit et al., 2002, Schuetz et al., 1995, Schulze et al., 2003a, Ashiru et al., 2007, Garcia-Arieta, 2014).

The long-held assumption of excipient “inertness” was contradicted when PEG 400, at certain doses, was shown to stimulate intestinal motility, reducing intestinal transit time, thus limiting absorption of ranitidine, and reducing its bioavailability (Basit et al., 2001, Basit et al., 2002, Schulze et al., 2003b). Interestingly, lower doses of PEG 400 were found to have the opposite effect: that of increasing the bioavailability of ranitidine in both men (Ashiru et al., 2008) and male rats (Afonso-Pereira et al., 2016) but not in the females of either species, in a dose dependent manner. The fact that the same bioavailability enhancing effect of PEG 400 was observed for ranitidine in male humans and rats, in two separate studies, indicates that the mechanism responsible for this sex differentiation is common to both species. This assumes special relevance considering the possibility of other drugs being affected in the same way by PEG 400. If they are, the implications of this effect are extremely important not only for the achievement of a bioequivalent therapeutic benefit in males and females, but also for the circumvention of adverse effects and for the potentiation of the personalization of medicines.

Based on previous studies with ranitidine in rats and humans, and to allow for a more effective *in vivo* extrapolation from rat to man, a new study was conducted (and is reported here) where the effects of PEG 400 at different doses on the bioavailability of cimetidine in both healthy men and women, and in male and female Wistar rats was determined. The cumulative urine excretion in human volunteers was measured, while in rats, drug levels in plasma were quantified. Thus, the aim of this study was to determine whether a similar sex-based influence of PEG 400 occurs on the oral bioavailability of cimetidine. The latter was chosen due to the fact that it is functionally related to ranitidine as an H_2-antagonist that is primarily absorbed from the small intestine. As for ranitidine, the narrow absorption window of cimetidine means that its bioavailability is vulnerable to factors influencing intestinal transit or absorption.

In healthy volunteers, cimetidine has been reported to have an oral bioavailability of approximately 60% (Somogyi and Gugler, 1983). It has been reported in the same paper that between 50%-80% of an intravenous administered dose is eliminated unchanged in urine. It is safe to assume that 50%-80% of the amount of cimetidine in the plasma is eliminated unchanged in urine. If 60 % of the orally administered dose reaches the plasma, between 50 to 80% of this amount will be eliminated unchanged in urine. This corresponds to 30%-48% of the orally administered dose which would be excreted unchanged in urine. Metabolism does play a role in cimetidine elimination accounting for up to a maximum of 40% of the administered dose (Somogyi and Gugler, 1983). These excretion values allow us to indirectly infer on cimetidine oral bioavailability variation based on its cumulative urinary excretion in humans.

2. MATERIALS AND METHODS

2.1 Materials and animals

Cimetidine, Polyethylene glycol 400, glacial acetic acid and sodium acetate trihydrate were obtained from Sigma Aldrich (Dorset UK). Water and acetonitrile were purchased from Fisher Scientific (Loughborough, UK), and were of HPLC grade.

Male and female Wistar rats (8 weeks old) were purchased from Harlan UK Ltd (Oxfordshire, UK).

2.2 Human Study

The dosage forms comprised oral solutions consisting of 150 mL of water containing 150 mg of cimetidine (GlaxoSmithKline, Harlow, UK) with one of the following doses of PEG 400: 0g (control), 0.5g, 0.75g, 1.0g, 1.5g, and 5g; a washout period of one week was given in-between dosing. Twelve volunteers (6 males and 6 females) within the following ranges were included - age: males [24-40 years (median 26 years)], females [23-27 years (median 24 years)]; weight: males [55-90 Kg (median 62 Kg)] , females [50-76 Kg (median 60 Kg)]; height: males [1.66–1.84 m (median 1.73 m)], females 1.58-1.7 m (median 1.69 m)]. All 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 with the Helsinki guidelines for ethics in research (1965) and its subsequent revisions up to the revision of Edinburgh 2000.

The volunteers reported to the study centre after an overnight fast and each received, on six separate occasions, 150mL of a cimetidine solution containing the required dose of PEG 400. A standardised lunch consisting of a two-piece cheese or egg sandwich, a 32.5g packet of crisps and a 250 mL juice drink was provided 4 hours post-dose, and water was available *ad libitum* from this point onwards.

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 2h, 2 to 4h, 4 to 6h, 6 to 12h, and 12 to 24 h. For each time point, a 20 mL aliquot was taken and stored at -20°C.

Urine samples were assayed for the amount of unchanged cimetidine. Frozen aliquots were thawed at room temperature, and 0.65mL of each sample was added to 0.65mL of a 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 a validated HPLC-UV method (Ashiru et al, 2007). The mobile phase was 20:80 acetonitrile: 0.1M sodium acetate, with a flow rate of 2ml/min. Calibration standards were prepared with blank human

urine, spiked with drug, also diluted (50%) with 20:80, acetonitrile: water. The results obtained for the cumulative excretion of cimetidine in urine for male and female human volunteers were subjected to one-way ANOVA to assess the effects of the different concentrations of PEG 400 on the bioavailability of cimetidine in males and females separately, and then a *post hoc* Turkey's test, with IBM SPSS Statistics 19 (SPSS Inc., Illinois, USA).

2.3 Rat study

Male and female Wistar rats were fasted for 12h in metabolic cages prior to the start of the experiment; food was made available four hours after dosing. A dose of 50mg/Kg of cimetidine in water was administered via oral gavage. The dose of cimetidine was chosen based on previous literature doses for cimetidine (Franca et al., 2000) , and on the quantification capabilities of the method used. The groups differed only according to sex and dose of PEG400 administered (0mg/Kg; 13mg/Kg; 26mg/Kg; 51mg/Kg; 77mg/Kg; mg). The doses of PEG 400 were extrapolated to the rat model based on the human doses previously used (Ashiru et al., 2008), which had been shown to produce an increase in the oral bioavailability of ranitidine in human volunteers. The dose extrapolation from human to rat was based on the species body surface area as explained (Reagan-Shaw et al., 2008, Afonso-Pereira et al., 2016).

After dosing, the rats were placed individually in a metabolic cage and were allowed to move freely. Approximately 200µL of blood was collected from the tail vein into anticoagulant centrifuge tubes (BD Microtainer® K2E Becton, Dickinson and Company, USA) at the following time points: 30min, 1h, 1.5h, 2h, 3h, 4h, and 6h. In between sampling, rats were placed back in the metabolic cages. Blood volumes were taken in accordance with the project licence and were stored on ice until the last collection point. After 8h, the animals were sacrificed in a CO_2 euthanasia chamber (Schedule 1 method), and 2mL of blood was obtained via cardiac puncture. Within 8h of sampling, blood samples were centrifuged at 10000rpm (930g) for 10 min on a Centrifuge 5804R (Eppendorf AG, 22331 Hamburg, Germany). 50µL of the supernatant (plasma) was collected and placed into a 1.5mL Eppendorf tube, and immediately frozen at -20°C.

2.3.1 Plasma sample analysis

Plasma samples were thawed and assayed for the amount of unchanged cimetidine. For each sample, 50µL of plasma were mixed with the same volume of acetonitrile in order to precipitate the plasma proteins. After 1 min of vortex-mixing, a further 100µL of HPLC grade water was added to the mixture, and after subsequent vortex-mixing, the samples were centrifuged at 4ºC for 10 min at 10000rpm. The resulting supernatant was collected and subjected to HPLC-UV analysis using a previously validated method (Ashiru et al., 2007). The column used was a 5µm Luna SCX (Phenomenex, UK); the mobile phase was a mixture of 20:80 (acetonitrile):(0.1M sodium acetate pH=5.0) with a flow rate of 2ml/min and 40µL of injection volume. Calibration standards were prepared with blank rat plasma samples spiked with cimetidine and subjected to the same treatment.

Plasma cimetidine concentration *versus* time profiles were produced for each animal, and C_{max} and t_{max} were obtained. The cumulative area under curve of plasma concentration versus time (AUC_{0-480}) was calculated using the integration method with OriginPro 9.0 (OriginLab, Northampton, MA, USA). The overall data was analysed by one-way ANOVA followed by a Tukey post-hoc analysis with a 95 % confidence interval and by comparisons between individual groups and relevant controls, with IBM SPSS Statistics 19 (SPSS Inc., Illinois, USA).

3. RESULTS

In Table 1, the bioavailability of cimetidine in human subjects is indicated by the cumulative amounts of unchanged cimetidine excreted in urine over 24 h. Table 2 shows the bioavailability of cimetidine in rats as represented by the AUC_{0-480} of the plasma concentration of cimetidine over an 8h period.

Table 1. Cumulative amount of cimetidine excreted in urine by human volunteers in 24 hours (mg); the mean percentage difference between the control and the groups is also expressed.

Dose of PEG 400	**0g (control)**		**0.5g**		**0.75g**		**1.0g**		**1.5g**	
Volunteer	Men	Women	Men	Women	Men	Women	Men	Women	Men	Women
1	71	60	98	70	111	50	119	50	76	43
2	61	67	124	89	142	74	80	95	54	90
3	73	98	67	63	93	85	100	84	56	108
4	79	67	88	64	90	59	111	62	67	33
5	93	66	96	60	120	70	129	75	62	50
6	57	64	88	39	108	55	75	56	54	19
Mean	72	70	94	64	111	66	102	70	62	57
SD	13	14	24	12	24	13	21	18	6	32
% mean diff	-	-	+29%*	-9%	+53%*	-7%	+41%*	0%	-15%	-19%

***** Indicates statistical difference ($p<0.05$) from the control group.

Table 2. The effect of different doses of PEG 400 on the bioavailability of cimetidine in male and female rats; mean ± standard deviation are shown; n=5-6

Dose of PEG400 (mg/Kg)	**0 (Control)**	**12.9**	**25.7**	**51.4**	**77**
Male AUC_{0-480} (µg.min/mL)	879±68	1196±189*	1204±152*	1115±221	698±276
% difference between AUC_{0-480} at PEG 400 dose and the control against the AUC_{0-480} in males	NA	+36%*	+37%*	+27%	-21%
Female AUC_{0-480} (µg.min/mL)	1585±316	1403±189	1625±463	1321±180	735±90*
% difference between AUC_{0-480} at PEG 400 dose and the control against the AUC_{0-480} in females	NA	-11%	+3%	-17%	-54%*

***** Indicates statistical difference ($p<0.05$) from the control group. NA – not applicable

Statistical analysis shows that for both species there was a significant ($p<0.05$) influence of sex and different doses of PEG 400 on the bioavailability of cimetidine.

In humans, the dose-dependent influence of PEG 400 on cimetidine excretion in urine is shown in Figures 1 and 2. The different doses of PEG 400 not affect the amount of cimetidine excreted in women. In men, the excreted amount as a function of the PEG 400 dose is bell shaped with a maximum effect at 0.75 g of PEG 400. It is also observed that there is a reduction of the excreted amount of cimetidine at the highest dose, 1.5 g in men and women.

Figure 1. Mean cumulative urine excretion of cimetidine in healthy human volunteers.
* Indicates statistically significant difference to the control ($p<0.05$).

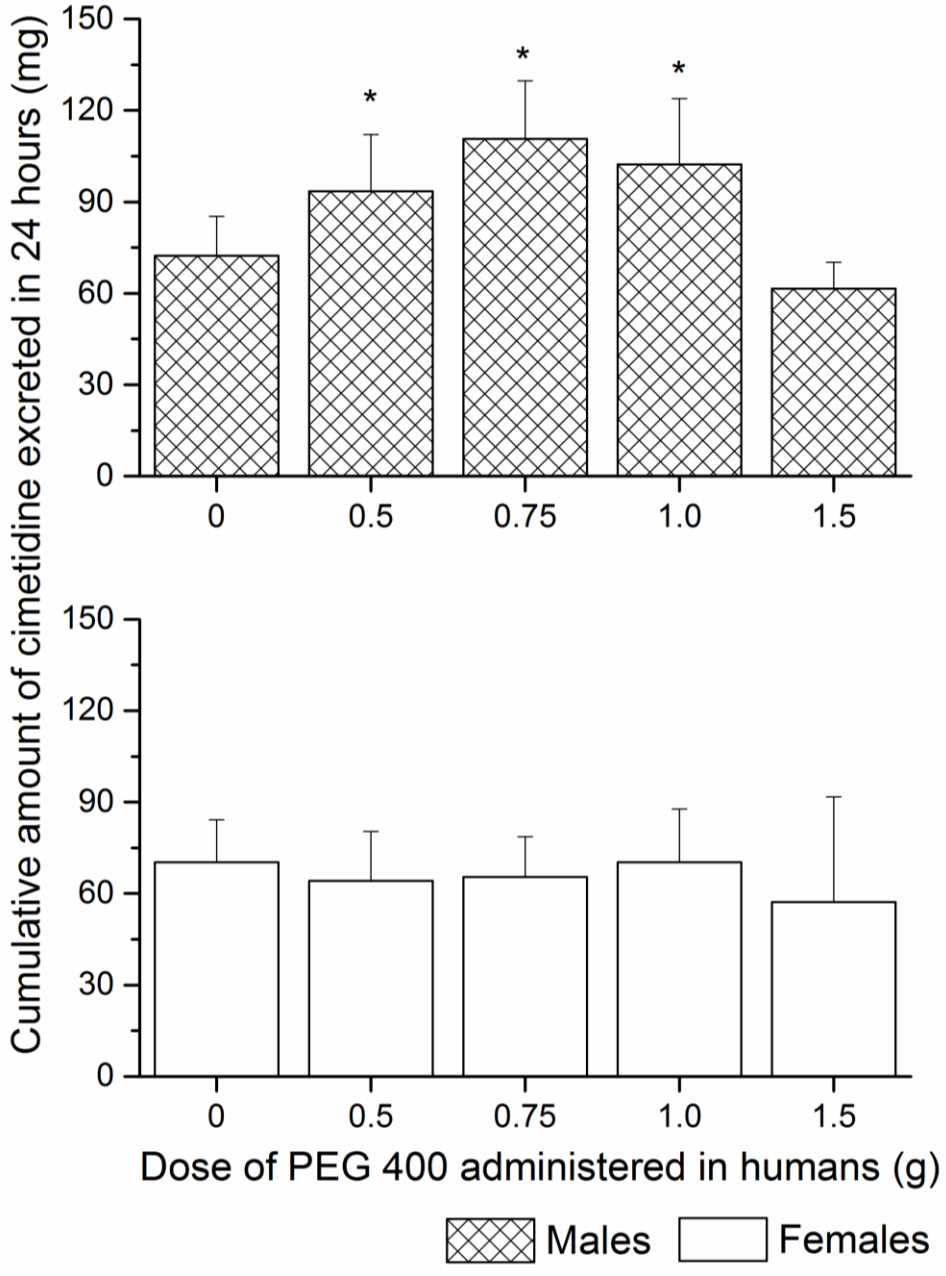
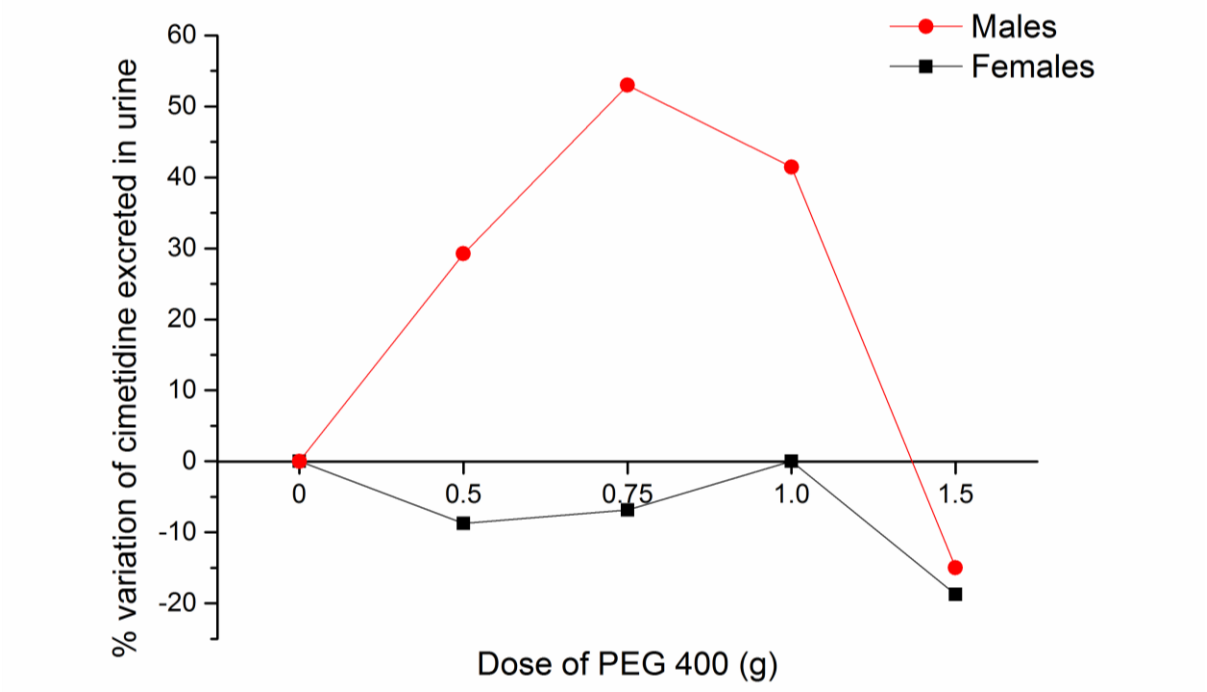


Figure 2. Percentage variation of cimetidine cumulative urinary excretion for the different doses of PEG 400 groups against the control (0g of PEG 400) in humans.



In rats, PEG 400 influenced cimetidine bioavailability in male but not females. The influence of PEG 400 in male rats was dose-dependent, with two of the doses of PEG 400 leading to significant increases in cimetidine bioavailability. The highest dose of PEG 400 led to a decrease in the bioavailability of cimetidine in males and females. The rat data is graphically represented in Figures 3 and 4.

Figure 3. Mean area under the cimetidine plasma concentration versus time curve (AUC_{0-480}) of the rats * Denotes statistically significant difference to the control ($p<0.05$).

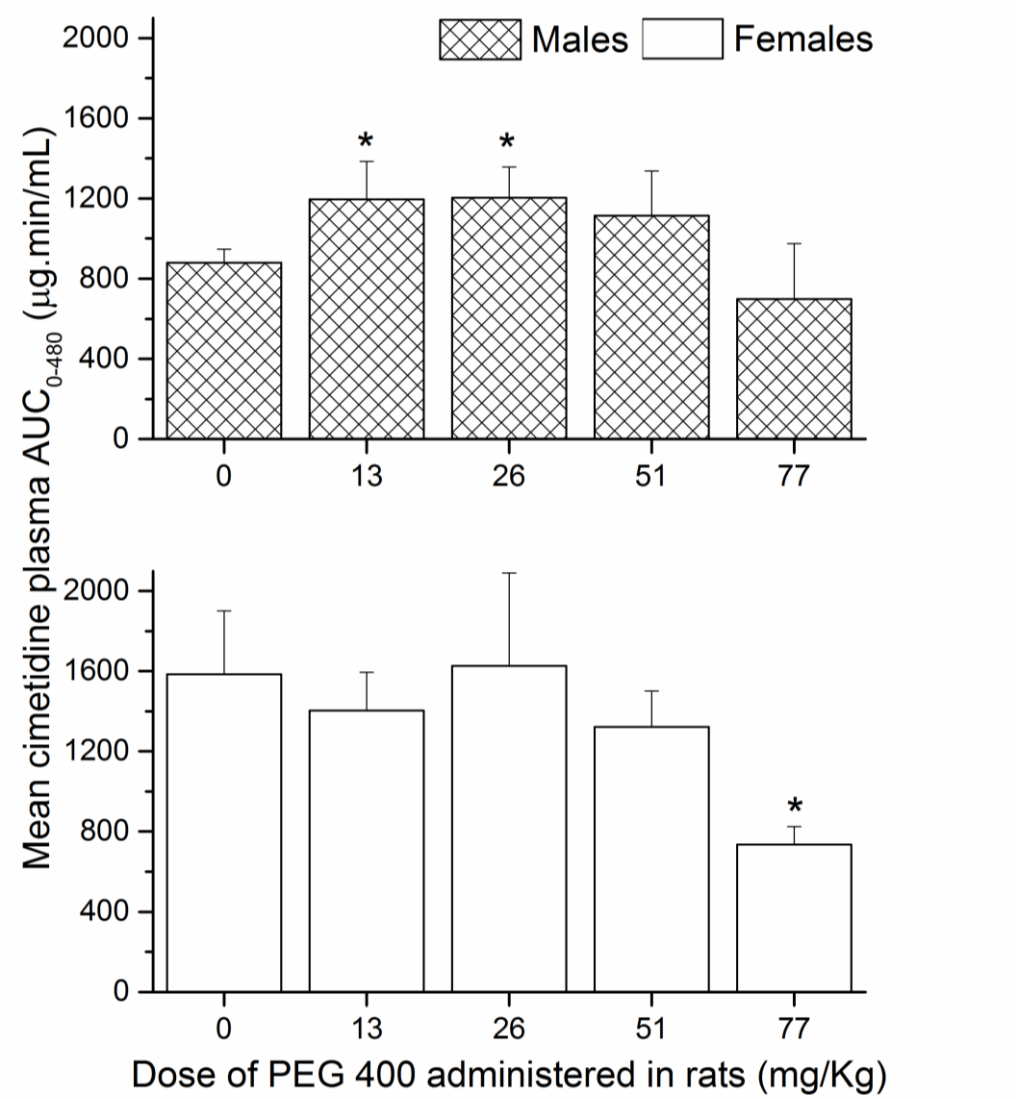
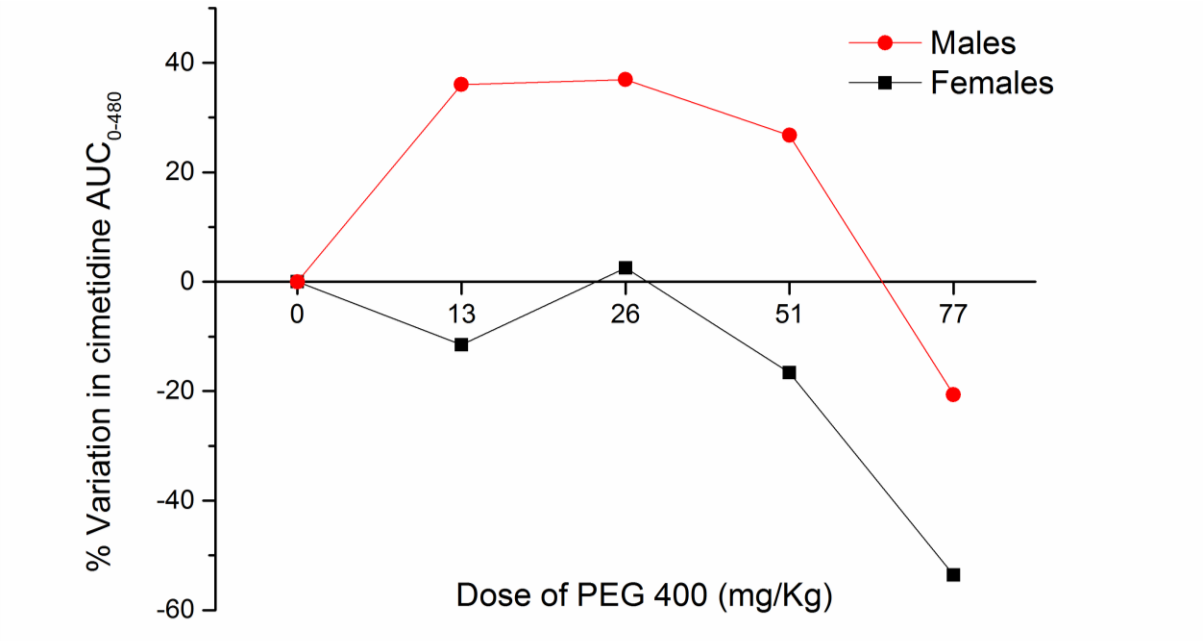


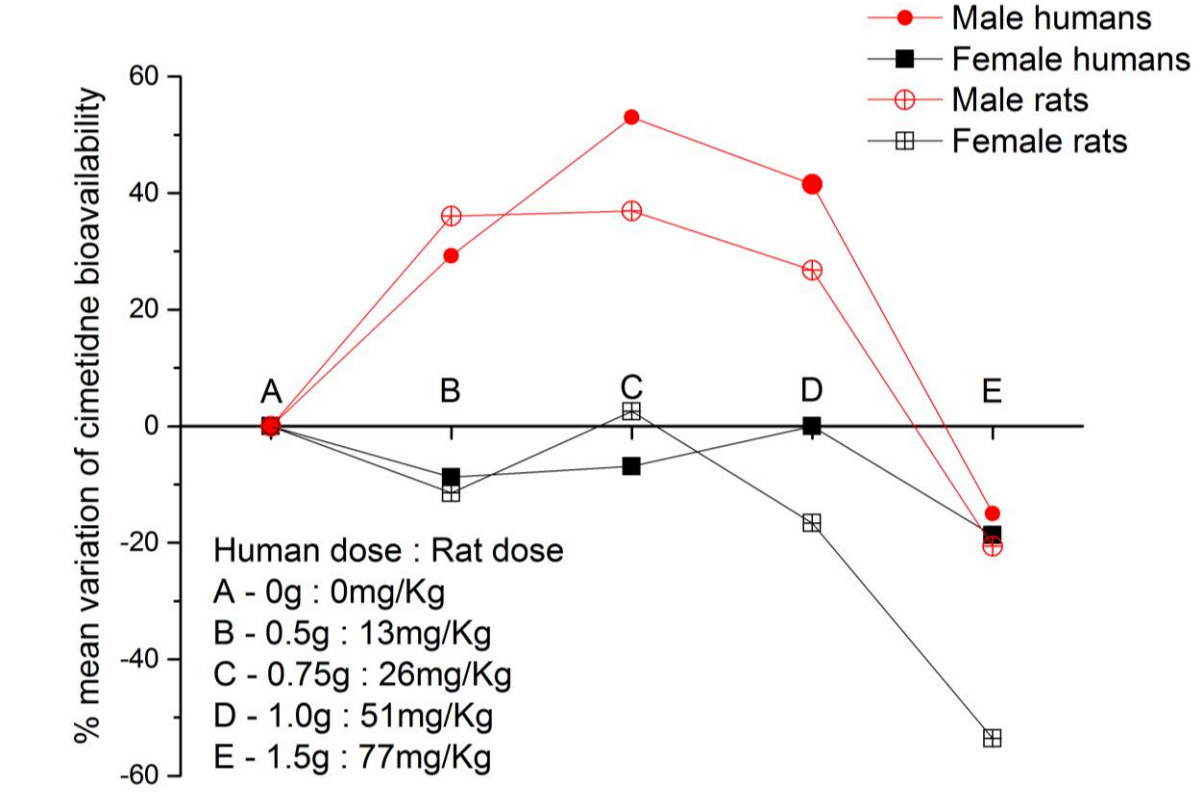
Figure 4. Percentage of variation of cimetidine AUC0-480 for the different PEG 400 dose groups against the control (0mg/Kg of PEG 400) in rats.



The effects of PEG 400 on the bioavailability of cimetidine in male and female rats is shown in Figure 4. As in humans, the effect is dependent on the dose of PEG 400 and on the sex. For male rats there is an increase of the mean $AUC_{0\text{-}480}$ followed by a decrease at the highest dose of PEG 400. Female rats, on the other hand, did not show a difference in their $AUC_{0\text{-}480}$, except for the highest dose of PEG 400 where a significant decrease in the mean $AUC_{0\text{-}480}$ was observed.

It is observed that the identified trend in humans and rats is similar. PEG 400 increases the bioavailability of Cimetidine in a dose dependent manner in males but not in females. The effect of PEG 400 depends on the dose and on the sex of the subject. This can be visually understood from Figure 5.

Figure 5. Percentage variation of cimetidine cumulative urinary excretion for the different doses of PEG 400 groups against the control (0g of PEG 400) in humans and of cimetidine $AUC_{0\text{-}480}$ for the different PEG 400 dose groups against the control (0mg/Kg of PEG 400) in rats.



4. DISCUSSION

This discussion will focus on explaining the following findings:

- Low doses of PEG 400 increase the bioavailability of cimetidine in men and male rats.
- The degree at which PEG 400 modifies the oral bioavailability of cimetidine is dose dependent.
- Women and female rats are not affected by low doses of PEG 400. The effect of PEG 400 is sex dependent in both species.
- The changes in the bioavailability of cimetidine in humans and rats by PEG 400 follow the same trend as for ranitidine (Ashiru et al., 2008, Afonso-Pereira et al., 2016).

The previously-reported sex-specific bioavailability-enhancing effect of low doses of PEG 400 on ranitidine in humans (Ashiru et al., 2008) and rats (Afonso-Pereira et al., 2016) is confirmed with cimetidine in both species.

The effect of PEG 400 is dose dependent. High doses of PEG 400 lead to a decrease in the bioavailability of cimetidine in both sexes due to the laxative osmotic effect of PEG 400. By retaining fluid in the intestinal lumen, PEG 400 reduces the transit time of the drug through the small intestine, reducing its absorption, thus its bioavailability (Schulze et al., 2003a). Whereas lower doses increase the overall bioavailability

PEG 400 can affect active substances other than ranitidine; as such this is not a specific ranitidine-PEG interaction. The question is, how many other drugs are affected by PEG 400? Naturally it becomes important to carefully analyze the results in the light of current knowledge to provide an explanation. What can be safely assumed is that the mechanisms behind the effect of PEG 400 on the bioavailability of ranitidine and cimetidine are are common to humand and rats.

Also, a sex-determined effect is undisputedly evident. There may be physical sex differences, related to both size and physiology between male and female humans and rats (McConnell et al., 2008, Merchant et al., 2014, Afonso-Pereira et al., 2017). Sex differences have been observed in the fluid volumes of the human small intestine, with higher absolute fluid volumes measured in men, compared to women (Gotch et al., 1957). This difference could also be responsible for differences in the concentration of drug and PEG 400 in the intestinal lumen in both humans and rats, which in turn could affect transit and permeability between genders. In rats, a study was performed comparing male and female gastrointestinal contents (Afonso-Pereira et al., 2017). The trend of each parameter is similar in the sexes, however differences in the values were observed. These were mainly identified in the stomach and in the distal portion of the gastrointestinal tract: ileum, caecum and colon. The pH in the stomach of females

is lower than in males. The remainder of the sections did not show a sex difference. Buffer capacity was higher in the female caecum and colon. Buffer capacity was also found to be higher at lower pHs. Males had higher osmolality than females in the duodenum, ileum and colon. Significant sex differences in surface tension were observed in the ileum where females had a higher surface tension. These differences identified in the rats would mainly affect the solubility of the active substances. It is important to note that cimetidine and ranitidine are very soluble active substances. The differences identified in the rat and human intestinal lumen environments would not modify the solubility of these drugs in a way that could significantly affect their availability to be absorbed. As such, physicochemical differences in the sexes in both species would not give a major contribute to the observed PEG 400 effect.

Another possible source of sex-differences observed in the behaviour of PEG 400 could be sex-differences in drug metabolism, in the liver or the enterocytes (Kagan et al., 2010, Lindell et al., 2003, Rademaker, 2001, Axiotis et al., 1991). However, PEG 400 does not affect the metabolism of ranitidine in males and female humans (Ashiru et al., 2008), thus making it unlikely that such a phenomenon would explain the present results with cimetidine in either humans or rats.

As previously explained for ranitidine, the main reason for the bioavailability enhancing effect of PEG 400 (Afonso-Pereira et al., 2016), and cimetidine in this study, may lie with membrane transporters in the small intestine. Some of these transporters might limit drug absorption (efflux transporters) or enhance it (influx transporters). For the influx transporters, the organic cation uptake transporter family (OCT/OCAT) and the organic anion transporters (OAT) will be considered. While for the efflux transporters special attention will be given to P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP). PEG 400 has been previously reported to be capable of interacting with some membrane transporters, mainly inhibiting them through an unknown mechanism.

Ranitidine and cimetidine, are known substrates for OCT/OCAT in both the kidney (OCT2) and the liver (OCT1) (Kimura et al., 2005, Bourdet et al., 2006). OCAT expression in rat kidneys was found to be much higher in males than in females (Urakami et al., 1999). If this was to translate to the intestine, a higher drug absorption in males than females could be expected in the control group. This is not observed in both species. Differences have been reported in the renal and hepatic expression of OAT (organic anion transporters) (OAT1-3 and SLc22a6-8) in male and female mice (Buist et al., 2002); again, how this translates to intestinal levels is unclear. If males did possess higher levels of OAT uptake transporters, a higher absorption of cimetidine would be expected in males than females of the control group. Again this was not seen. Furthermore, PEG 400 would have to have a stimulating effect in the transporter and not an inhibitory one, otherwise the levels of cimetidine in males would fall instead of rising, which does not seem to be the case. As a result, uptake transporters do not seem to be the primary cause bearing in mind that extrapolating transport-related sex differences between species might be difficult (Groves et al., 2006). More details on hepatic and renal sex differences in OATs are reviewed elsewhere (VanWert et al., 2010).

Cimetidine is a substrate for the efflux transporters P-glycoprotein (P-gp) (Collett et al., 1999) and breast cancer resistance protein (BCRP) (Pavek et al., 2005). BCRP has greater expression 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). If BCRP was to be inhibited, females would predictably be more sensitive to the effects of the inhibitor. However, it appears that males are more sensitive to the presence of low doses of PEG 400, as the females seem unaffected. Furthermore, cimetidine is a substrate for both BCRP and P-gp but ranitidine only acts as a substrate for P-gp (Collett et al., 1999, Pavek et al., 2005). This may allow us to exclude BCRP as the main reason for the bioavailability enhancing effect of PEG 400 on cimetidine in males and not in females.

P-gp assumes a particular relevance as a possible explanation, given that both cimetidine and ranitidine are known substrates and it is present in both rats (Estudante et al., 2013) and humans. The effect of sex on transporters can be contradictory, however, with one study reporting that women have a lower enterocyte P-gp content then men (Potter et al., 2004), and another study suggesting no difference (Paine et al., 2005). If men do possess a higher level of intestinal P-gp males would be rendered more sensitive to detect its inhibition. By inhibiting P-gp, after crossing the enterocyte apical membrane, less cimetidine would be secreted back to the intestinal lumen. Thus, the concentration of cimetidine would increase in the enterocyte, increasing the amount that would then be absorbed by the systemic circulation. In the case of women, if their P-gp levels are lower in the small intestine, they would not be as influenced by its inhibition, naturally rendering them more resistant to the effects of PEG 400, allowing for a justification as to why PEG 400 only seems to increase the bioavailability of cimetidine in males but not females. Also, it was found that oral female steroids (e.g. oral contraceptives and HRT) can modulate P-gp expression on the gut wall (Frohlich et al., 2004a). Some studies do suggest that P-gp itself can be inhibited by progestin/progesterone (Kim and Benet, 2004, Frohlich et al., 2004b), and that P-gp expression inducement occurs by oestrogens (Bebawy and Chetty, 2009). This seems contradictory but expression of the transporter and its activity are two different subjects. It may be that female hormones do play a role in increasing the expression of P-gp, but at the same time partially limit its activity after the transporter is functional in the membrane. Other studies suggested that testosterone is a potent inhibitor of P-gp, more so than female hormones (Hamilton et al., 2001, Suzuki et al., 2006). In our study the menstrual cycle of women volunteers and female rats was not controlled. The possibility of sex hormones affecting the expression of P-gp and as such modulating the absorption of ranitidine and cimetidine following P-gp inhibition should be considered.

In order organs sex differences in the expression of P-gp have been reported: in the kidney and lung, both Mdr1a and Mdr1b genes (genes that code for P-gp) have a female-dominant expression pattern; the expression of Mdr1b in the brain is male-dominant; there were no sex differences in Mdr1a in the duodenum (Cui et al., 2009).

It is clear that the scenario is very complex, and not one of these sex differences alone seems to explain the intriguing results of why PEG 400, in low doses, only affects males of both species by enhancing cimetidine and ranitidine bioavailability (Ashiru et al., 2008, Afonso-

Pereira et al., 2016). It is worth noting that the increase in cimetidine bioavailability in the presence of 0.75 g PEG in humans is less than that noted for ranitidine (53% compared to 63%). This can be explained by the fact that the baseline oral bioavailability of cimetidine is higher than ranitidine, hence there is less scope for an increase in bioavailability in the presence of excipients such as PEG 400, or simply because of different affinities to the intestinal transporters involved.

5. CONCLUSION

Low doses of PEG 400 were found to significantly increase the bioavailability of cimetidine in male, but not female, rats and humans in a dose dependent manner. As expected, the highest PEG 400 doses led to a decrease of the bioavailability of cimetidine in both sexes and both species.

Although the definitive mechanism for the enhancement of bioavailability of ranitidine and cimetidine in male subjects has not yet been elucidated, nor the reasons for the observed sex-determined differences in drug bioavailability explained, this study confirms that the effect of a low dose of PEG 400 is not limited to ranitidine in either rats or humans. Our results show that PEG 400 even at low concentrations should not be considered to be an "inert" excipient. The absorption enhancing effects observed with low PEG 400 doses are suggested to be due to its effects on drug permeability through the gastrointestinal tract via the PEG 400 effects on efflux transporters.

In order to gain a good understanding of sex differences in treatment response, information on basic biologic differences, participation of both women and men in clinical trials, and analysis of safety and efficacy data are important. As such, it is anticipated that our findings will influence the reconsideration of excipient classification in drug monographs and help determine the clinical consequences of these excipient effects on drug toxicity profiles, and especially in the case for those drugs with narrow therapeutic indices for which these excipients may otherwise have pronounced effects on drug safety and dosing.

6. REFERENCES

(GAO), U. S. G. A. O. 2001. Drug Safety: Most Drugs Withdrawn In Recent Years Had Greater Health Risks For Women. *General Accounting Report.* Washington, D. C.: United States General Accounting Office (GAO).

AFONSO-PEREIRA, F., MURDAN, S., SOUSA, J., VEIGA, F. & BASIT, A. W. 2016. Sex differences in excipient effects: Enhancement in ranitidine bioavailability in the presence of polyethylene glycol in male, but not female, rats. *Int J Pharm,* 506**,** 237-41.

AFONSO-PEREIRA, F., VEIGA, F., SOUSA, J., MURDAN, S. & BASIT, A. W. 2017. Sex differences in the gastrointestinal tract of adult Wistar rats. UCL School of Pharmacy.

ASHIRU, D. A., PATEL, R. & BASIT, A. W. 2007. Simple and universal HPLC-UV method to determine cimetidine, ranitidine, famotidine and nizatidine in urine: application to the analysis of ranitidine and its metabolites in human volunteers. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences,* 860**,** 235-40.

ASHIRU, D. A., PATEL, R. & BASIT, A. W. 2008. Polyethylene glycol 400 enhances the bioavailability of a BCS class III drug (ranitidine) in male subjects but not females. *Pharm Res,* 25**,** 2327-33.

AXIOTIS, C. A., GUARCH, R., MERINO, M. J., LAPORTE, N. & NEUMANN, R. D. 1991. P-Glycoprotein Expression Is Increased in Human Secretory and Gestational Endometrium. *Laboratory Investigation,* 65**,** 577-581.

BASIT, A. W., NEWTON, J. M., SHORT, M. D., WADDINGTON, W. A., ELL, P. J. & LACEY, L. F. 2001. The effect of polyethylene glycol 400 on gastrointestinal transit: implications for the formulation of poorly-water soluble drugs. *Pharmaceutical Research,* 18**,** 1146-50.

BASIT, A. W., PODCZECK, F., NEWTON, J. M., WADDINGTON, W. A., ELL, P. J. & LACEY, L. F. 2002. Influence of polyethylene glycol 400 on the gastrointestinal absorption of ranitidine. *Pharmaceutical Research,* 19**,** 1368-74.

BEBAWY, M. & CHETTY, M. 2009. Gender differences in p-glycoprotein expression and function: effects on drug disposition and outcome. *Current drug metabolism,* 10**,** 322-8.

BIGOS, K. L., POLLOCK, B. G., STANKEVICH, B. A. & BIES, R. R. 2009. Sex Differences in the Pharmacokinetics and Pharmacodynamics of Antidepressants: An Updated Review. *Gender Medicine,* 6**,** 522-543.

BOURDET, D. L., POLLACK, G. M. & THAKKER, D. R. 2006. Intestinal absorptive transport of the hydrophilic cation ranitidine: a kinetic modeling approach to elucidate the role of uptake and efflux transporters and paracellular vs. transcellular transport in Caco-2 cells. *Pharmaceutical Research,* 23**,** 1178-87.

BUIST, S. C. N., CHERRINGTON, N. J., CHOUDHURI, S., HARTLEY, D. P. & KLAASSEN, C. D. 2002. Gender-specific and developmental influences on the expression of rat organic anion transporters. *Journal of Pharmacology and Experimental Therapeutics,* 301**,** 145-151.

COLLETT, A., HIGGS, N. B., SIMS, E., ROWLAND, M. & WARHURST, G. 1999. Modulation of the permeability of H-2 receptor antagonists cimetidine and ranitidine by P-glycoprotein in rat intestine and the human colonic cell line Caco-2. *J Pharmacol Exp Ther,* 288**,** 171-178.

CUI, Y. J., CHENG, X., WEAVER, Y. M. & KLAASSEN, C. D. 2009. Tissue Distribution, Gender-Divergent Expression, Ontogeny, and Chemical Induction of Multidrug Resistance Transporter Genes (Mdr1a, Mdr1b, Mdr2) in Mice. *Drug Metabolism and Disposition,* 37**,** 203-210.

ESTUDANTE, M., MAYA, M., MORAIS, J. G., SOVERAL, G. & BENET, L. Z. 2013. Effect of P-Glycoprotein on the Rat Intestinal Permeability and Metabolism of the BDDCS Class 1 Drug Verapamil. *Mol Pharm,* 10**,** 4038-45.

FILLINGIM, R. B., KING, C. D., RIBEIRO-DASILVA, M. C., RAHIM-WILLIAMS, B. & RILEY, J. L., III 2009. Sex, Gender, and Pain: A Review of Recent Clinical and Experimental Findings. *Journal of Pain,* 10**,** 447-485.

FRANCA, L. R., LEAL, M. C., SASSO-CERRI, E., VASCONCELOS, A., DEBELJUK, L. & RUSSELL, L. D. 2000. Cimetidine (Tagamet) is a reproductive toxicant in male rats affecting peritubular cells. *Biol Reprod,* 63**,** 1403-12.

FREIRE, A. C., BASIT, A. W., CHOUDHARY, R., PIONG, C. W. & MERCHANT, H. A. 2011. Does sex matter? The influence of gender on gastrointestinal physiology and drug delivery. *International Journal of Pharmaceutics,* 415**,** 15-28.

FROHLICH, M., ALBERMANN, N., SAUER, A., WALTER-SACK, I., HAEFELI, W. E. & WEISS, J. 2004a. In vitro and ex vivo evidence for modulation of P-glycoprotein activity by progestins. *Biochemical pharmacology,* 68**,** 2409-16.

FROHLICH, M., BURHENNE, J., MARTIN-FACKLAM, M., WEISS, J., VON WOLFF, M., STROWITZKI, T., WALTER-SACK, I. & HAEFELI, W. E. 2004b. Oral contraception does not alter single dose saquinavir pharmacokinetics in women. *British Journal of Clinical Pharmacology,* 57**,** 244-252.

GANDHI, M., AWEEKA, F., GREENBLATT, R. M. & BLASCHKE, T. F. 2004. Sex differences in pharmacokinetics and pharmacodynamics. *Annual Review of Pharmacology and Toxicology,* 44**,** 499-523.

GARCIA-ARIETA, A. 2014. Interactions between active pharmaceutical ingredients and excipients affecting bioavailability: impact on bioequivalence. *Eur J Pharm Sci,* 65**,** 89-97.

GOTCH, F., NADELL, J. & EDELMAN, I. S. 1957. Gastrointestinal Water and Electrolytes .4. The Equilibration of Deuterium Oxide (D2o) in Gastrointestinal Contents and the Proportion of Total Body Water (Tbw) in the Gastrointestinal Tract. *Journal of Clinical Investigation,* 36**,** 289-296.

GROVES, C. E., SUHRE, W. B., CHERRINGTON, N. J. & WRIGHT, S. H. 2006. Sex differences in the mRNA, protein, and functional expression of organic anion transporter (Oat) 1, Oat3, and organic cation transporter (Oct) 2 in rabbit renal proximal tubules. *Journal of Pharmacology and Experimental Therapeutics,* 316**,** 743-752.

HAMILTON, K. O., YAZDANIAN, M. A. & AUDUS, K. L. 2001. Modulation of P-glycoprotein activity in Calu-3 cells using steroids and beta-ligands. *International Journal of Pharmaceutics,* 228**,** 171-179.

JAMEI, M., DICKINSON, G. L. & ROSTAMI-HODJEGAN, A. 2009. A Framework for Assessing Inter-individual Variability in Pharmacokinetics Using Virtual Human Populations and Integrating General Knowledge of Physical Chemistry, Biology, Anatomy, Physiology and Genetics: A Tale of 'Bottom-Up' vs 'Top-Down' Recognition of Covariates. *Drug Metabolism and Pharmacokinetics,* 24**,** 53-75.

KAGAN, L., DREIFINGER, T., MAGER, D. E. & HOFFMAN, A. 2010. Role of P-Glycoprotein in Region-Specific Gastrointestinal Absorption of Talinolol in Rats. *Drug Metabolism and Disposition,* 38**,** 1560-1566.

KIM, W. Y. & BENET, L. Z. 2004. P-glycoprotein (P-gp/MDR1)-mediated efflux of sex-steroid hormones and modulation of P-gp expression in vitro. *Pharmaceutical Research,* 21**,** 1284-1293.

KIMURA, N., MASUDA, S., TANIHARA, Y., UEO, H., OKUDA, M., KATSURA, T. & INUI, K. 2005. Metformin is a superior substrate for renal organic cation transporter OCT2 rather than hepatic OCT1. *Drug Metab Pharmacokinet,* 20**,** 379-86.

LINDELL, M., KARLSSON, M. O., LENNERNAS, H., PAHLMAN, L. & LANG, M. A. 2003. Variable expression of CYP and Pgp genes in the human small intestine. *European Journal of Clinical Investigation,* 33**,** 493-499.

MCCONNELL, E. L., FADDA, H. M. & BASIT, A. W. 2008. Gut instincts: Explorations in intestinal physiology and drug delivery. *International Journal of Pharmaceutics,* 364**,** 213-226.

MERCHANT, H. A., RABBIE, S. C., VARUM, F. J., AFONSO-PEREIRA, F. & BASIT, A. W. 2014. Influence of ageing on the gastrointestinal environment of the rat and its implications for drug delivery. *Eur J Pharm Sci*.

MERINO, G., VAN HERWAARDEN, A. E., WAGENAAR, E., JONKER, J. W. & SCHINKEL, A. H. 2005. Sex-dependent expression and activity of the ATP-binding cassette transporter breast cancer resistance protein (BCRP/ABCG2) in liver. *Mol Pharmacol,* 67**,** 1765-71.

PAINE, M. F., LUDINGTON, S. S., CHEN, M. L., STEWART, P. W., HUANG, S. M. & WATKINS, P. B. 2005. Do men and women differ in proximal small intestinal CYP3A or P-glycoprotein expression? *Drug Metabolism and Disposition,* 33**,** 426-433.

PAVEK, P., MERINO, G., WAGENAAR, E., BOLSCHER, E., NOVOTNA, M., JONKER, J. W. & SCHINKEL, A. H. 2005. Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine. *J Pharmacol Exp Ther,* 312**,** 144-52.

PERRIE, Y., BADHAN, R. K. S., KIRBY, D. J., LOWRY, D., MOHAMMED, A. R. & OUYANG, D. 2012. The impact of ageing on the barriers to drug delivery. *Journal of Controlled Release,* 161**,** 389-398.

POON, R., KHANIJOW, K., UMARJEE, S., FADRIAN, E., YU, M., ZHANG, L. & PAREKH, A. 2011. Participation of Women in Late Phase Clinical Trials of New

Molecular Entity (NME) Drugs and Biologics Approved by the Food and Drug Administration Between 2007 and 2009. *Journal of Womens Health,* 20**,** 495-496.

POTTER, J. M., MCWHINNEY, B. C., SAMPSON, L. & HICKMAN, P. E. 2004. Area-under-the-curve monitoring of prednisolone for dose optimization in a stable renal transplant population. *Therapeutic Drug Monitoring,* 26**,** 408-414.

RADEMAKER, M. 2001. Do women have more adverse drug reactions? *Am J Clin Dermatol,* 2**,** 349-51.

REAGAN-SHAW, S., NIHAL, M. & AHMAD, N. 2008. Dose translation from animal to human studies revisited. *Faseb j,* 22**,** 659-61.

SCHUETZ, E. G., FURUYA, K. N. & SCHUETZ, J. D. 1995. Interindividual variation in expression of P-glycoprotein in normal human liver and secondary hepatic neoplasms. *J Pharmacol Exp Ther,* 275**,** 1011-8.

SCHULZE, J. D., WADDINGTON, W. A., ELI, P. J., PARSONS, G. E., COFFIN, M. D. & BASIT, A. W. 2003a. Concentration-dependent effects of polyethylene glycol 400 on gastrointestinal transit and drug absorption. *Pharmaceutical Research,* 20**,** 1984-8.

SCHULZE, J. D. R., WADDINGTON, W. A., ELL, P. J., PARSONS, G. E., COFFIN, M. D. & BASIT, A. W. 2003b. Concentration-dependent effects of polyethylene glycol 400 on gastrointestinal transit and drug absorption. *Pharm Res,* 20**,** 1984-1988.

SOMOGYI, A. & GUGLER, R. 1983. Clinical pharmacokinetics of cimetidine. *Clin Pharmacokinet,* 8**,** 463-95.

SUZUKI, T., ZHAO, Y. L., NADAI, M., NARUHASHI, K., SHIMIZU, A., TAKAGI, K. & HASEGAWA, T. 2006. Gender-related differences in expression and function of hepatic P-glycoprotein and multidrug resistance-associated protein (Mrp2) in rats. *Life Sciences,* 79**,** 455-461.

UENO, K. & SATO, H. 2012. Sex-related differences in pharmacokinetics and pharmacodynamics of anti-hypertensive drugs. *Hypertension Research,* 35**,** 245-250.

URAKAMI, Y., NAKAMURA, N., TAKAHASHI, K., OKUDA, M., SAITO, H., HASHIMOTO, Y. & INUI, K. 1999. Gender differences in expression of organic cation transporter OCT2 rat kidney. *Febs Letters,* 461**,** 339-342.

VANWERT, A. L., GIONFRIDDO, M. R. & SWEET, D. H. 2010. Organic Anion Transporters: Discovery, Pharmacology, Regulation and Roles in Pathophysiology. *Biopharmaceutics & Drug Disposition,* 31**,** 1-71.

WOODHEAD, J. C., DRULIS, J. M., NELSON, S. E., JANGHORBANI, M. & FOMON, S. J. 1991. GENDER-RELATED DIFFERENCES IN IRON-ABSORPTION BY PREADOLESCENT CHILDREN. *Pediatric Research,* 29**,** 435-439.

YANG, Y., CARLIN, A. S., FAUSTINO, P. J., MOTTA, M. I. P., HAMAD, M. L., HE, R., WATANUKI, Y., PINNOW, E. E. & KHAN, M. A. 2009. Participation of Women in Clinical Trials for New Drugs Approved by the Food and Drug Administration in 2000-2002. *Journal of Womens Health,* 18**,** 303-310.

YOOD, M. U., ROTHMAN, K. J., JOHNSON, C. C., JICK, S. S., LANG, J., WELLS, K. E. & JICK, H. 1998. Race and gender differences in the effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on the risk for colorectal cancer. *American Journal of Epidemiology,* 147**,** S10-S10.

ZAMBER, C. P., LAMBA, J. K., YASUDA, K., FARNUM, J., THUMMEL, K., SCHUETZ, J. D. & SCHUETZ, E. G. 2003. Natural allelic variants of breast cancer resistance protein (BCRP) and their relationship to BCRP expression in human intestine. *Pharmacogenetics,* 13**,** 19-28.

Chapter 5 - Establishing Bioequivalence: Can sex play a role in the effect of excipients in the bioavailability of BCS III drugs?

ABSTRACT

The aims of this work are to understand if different BCS class III compounds are affected by low doses of polyethylene glycol (PEG) 400; if the effect of PEG 400 is only prevalent on males (not females); and, in doing so, to gain a better understanding of the mechanisms underlying the bioavailability enhancing effect of PEG 400.

Male and female wistar rats were administered 50 mg/kg of ranitidine, cimetidine and ampicillin or 20 mg/kg of metformin in the presence and absence (control) of 26 mg/kg of PEG 400. The pharmacokinetic profiles of the active substances were evaluated.

It was found that PEG 400 increased the AUC_{0-480} of ranitidine, cimetidine and ampicillin by 49%, 37% and 81% respectively when compared to the control groups. This effect was only observed in male rats and not in female rats, where PEG 400 did not significantly change the AUC_{0-480} of the drugs. Metformin was not affected by dosing with PEG 400 in either sex.

PEG 400, in small amounts, is capable of modifying the oral bioavailability of some BCS-III drugs in a sex dependent manner. This mechanism is most likely related to its inhibitory effects over P-gp. A different overall activity of P-gp in the sexes may explain it is only observed in male but not female rats.

The degree to which excipients modulate drug disposition may be modified by sex. It is important that regulatory authorities and pharmaceutical industry take this knowledge into account during the pharmaceutical development stages of their products.

1. INTRODUCTION

Establishing Bioequivalence

According to the European Union Directive 2001/83/EC generic and hybrid drug products can be introduced in the European market without the need for further safety and efficacy studies. Generic products are developed to be the same as a medicine that has already been authorised, the "reference medicine" (EuropeanMedicinesAgency, 2010). To establish whether the proposed product and the reference product can be considered the same, bioequivalence between both should be established, either *in vivo* or *in vitro*. *In vivo* studies are usually two sequence, two period, two treatment crossover clinical studies where the proposed and reference products are administered to a group of healthy volunteers and the pharmacokinetic profile of both products is compared (other study designs may be accepted). *In vitro* work usually comprises a series of dissolution studies across the physiological pH range (pH 1.2 to 6.8) where the composition, manufacturing process, pharmaceutical form and pharmacokinetics of the active substance are critical points to consider. Usually, if excipients with "known effect" on the bioavailability of drugs are present in either the proposed or the reference medicinal products, waiving the requirement for a bioequivalence study is difficult, as both products need to be quantitatively and qualitatively the same. Waiving of bioequivalence studies is usually limited to compounds that are very soluble and with extensive and previously quantified human absorption; compounds with incomplete absorption may also be considered for a waiver but the mechanism behind the limited absorption should be known. Drugs that may be eligible for a waiver are usually grouped within the biopharmaceutical classification system, BCS, classes I and III. This system was developed to classify drugs into four classes according to their intrinsic solubility and their intestinal apparent permeability: Class I (high solubility, high permeability), Class II (low solubility, high permeability), Class III (high solubility, low permeability), and Class IV (low solubility, low permeability) (Chen et al., 2011, Amidon et al., 1995, Benet et al., 2008). Poorly soluble drugs (classes II and IV) cannot be considered for a waiver due to the difficulty of evaluating *in vitro* their *in vivo* behaviour.

Whether bioequivalence is to be established *in vivo* or *in vitro*, understanding the effect of excipients in the bioavailability of drugs is crucial for the formulation process. Also, males and females respond differently to drugs: sex plays a crucial role in pharmacokinetics and pharmacodynamics. Although recent advances were made in our understanding of sex differences in the gastrointestinal tract, sex driven drug effects are still poorly understood (Schiebinger, 2014, Regitz-Zagrosek, 2012). It is also important to note that current European drug Legislation does not factor in sex as a variable for the purposes of establishing bioequivalence. Bioequivalence studies are mainly conducted in men and biowaivers of BCS III compounds do not consider sex. The choice of excipients in a formulation may impact the active substance disposition (Garcia-Arieta, 2014). But the degree to which excipients modulate drug disposition may be modified by sex.

PEG 400 and the bioavailability of BCS-III drugs

Polyethylene glycol 400 (PEG 400) is widely used as a solubilizer in liquid pharmaceutical forms. It may be used not necessarily to solubilise the active substances but to prompt the solubilisation of other formulation components, such as preservatives, stabilizers, antioxidants, flavourings and colourings. Thus, despite the high solubility of BCS-III compounds, PEG 400 may be present in other formulations.

It was previously found that PEG 400 can modulate the bioavailability of different drugs in a sex dependent way: at high doses, PEG 400, acts as an osmotic laxative, reducing gastrointestinal transit time, thus bioavailability, of drugs in the sexes (Basit et al., 2002, McConnell et al., 2008); however, at lower doses, PEG 400 significantly increases the bioavailability of ranitidine and cimetidine (BCS class III drugs) in men but not women (Afonso-Pereira et al., 2017, Ashiru et al., 2008). The mechanisms underlying this are still unclear, but a sex difference has been identified. An animal model using wistar rats was developed to study the effect of PEG 400 in the absorption of BCS class III drugs (Afonso-Pereira et al., 2017, Afonso-Pereira et al., 2016). In male rats low doses of PEG 400 increased the bioavailability of ranitidine and cimetidine in a dose dependent manner (Afonso-Pereira et al., 2016, Afonso-Pereira et al., 2017); however no such effect was observed in females. It is important to understand if this sex-dependent effect of PEG 400 is restricted to ranitidine and cimetidine, two structurally similar compounds, or if other BCS-III drugs can be affected. Understanding this may prove useful in guiding companies and regulators in establishing additional parameters on the investigation of bioequivalence.

BCS class III drugs can be grouped based on the mechanism underlying their poor permeability (Estudante et al., 2013):

- *Intrinsically poorly permeable molecules* – ionized, polarized or simply too large to effectively cross the phospholipid bi-layer;

- *Substrates for efflux transporters* – Molecules that can cross the cell membrane with relative ease but are subjected to efflux transport mechanisms at the apical end of the enterocytes. Once having reached the cytosol, the active substance will be actively transported back into the intestinal lumen, delaying the absorption.

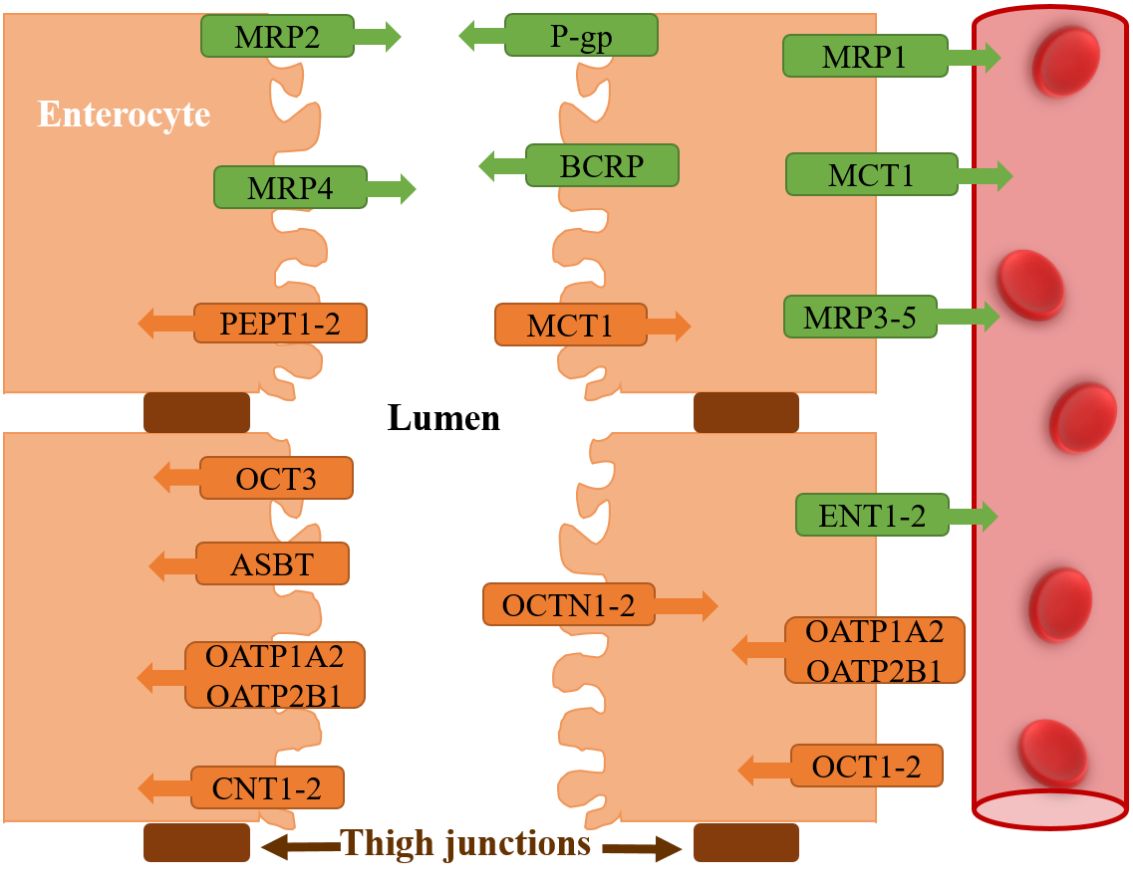
The mechanism behind the enhancement of the bioavailability of ranitidine and cimetidine in male subjects has not yet been elucidated; nor the reasons for the observed sex-determined differences in drug bioavailability explained. However, cimetidine and ranitidine are substrates for influx and efflux transporters that usually play a role in drug disposition (Afonso-Pereira et al., 2017, Ashiru et al., 2008, Afonso-Pereira et al., 2016).

Intestinal transporters

Both uptake and efflux transporters are important in determining oral drug disposition by controlling absorption and, consequently, bioavailability (Shugarts and Benet, 2009).

There are two main groups of intestinal membrane transporters: influx transporters, that bring compounds into the cell; and efflux transporters that secret compounds out of the cell. Depending on their location on the basal or apical end of the enterocyte, these transporters will either favour or hinder drug absorption (Figure 1). The phospholipid bilayer that constitutes the cell membrane is fluid, however, the enterocytes are interconnected by thigh junctions; these allow the cell to be polarised and different transporters are expressed in the apical and basal side of the enterocyte (Goole et al., 2010). As an example, an efflux transporter at the apical end of the enterocyte (P-gp) will secrete compounds back to the intestinal lumen from the cytosol, hindering absorption. However another efflux transporter, but this time at the basal side of the enterocyte (MCT1) will stimulate drug passage to the blood, enhancing absorption.

Figure 1. Enterocyte with intestinal uptake (orange) and efflux (green) transporters expressed at the apical and basal sides. Adapted from (Custodio et al., 2008).



glycoprotein P (P-gp); multidrug resistance associated protein (MRP); breast cancer resistance protein (BCRP); monocarboxylate transporter protein (MCT); peptide transport protein (PEPT); organic anion transporting polypeptide (OATP); organic cation transporter (OCT); apical sodium-dependent bile acid transporter (ASBT); concentrative nucleotide transporter (CNT); electroneutral organic cation transporter (OCTN); equilibrative nucleoside transporter (ENT).

There are two super families of intestinal transporters: solute carrier (SLC) transporters and ATP-binding cassette (ABC) transporters. Although most are capable of bidirectional transport, ABC transporters are considered to be responsible for efflux of substrates, while SLC transporters are responsible for the influx (Roth et al., 2012).

The intestinal influx transporters belong to two families of SLC: SLC and SLCO. In rats the SLC (also known as SLC 21A) mainly transports small organic anions and cations whereas the SLCO (also known as SLC 22A) transports preferably large organic anions (van Montfoort et al., 2001). These transporters work by uniport, symport, or antiport transport mechanisms without consuming ATP (Li et al., 2000, Roth et al., 2012). These work usually in favour of the chemiosmotic gradient created by the translocation of ions across the cellular membrane (Srimaroeng et al., 2008).

The SLC encompasses the organic anion transporters (OAT), the organic cation transporters (OCT, SLC22A), the electroneutral organic cation transporters (OCTN), the equilibrative nucleoside transporters (ENT,PMAT, SLC29), the concentrative nucleoside transporters (CNT, SLC28), the apical Na^+ dependent bile salt transporter (ASBT, SLC10), the monocarboxylate transporters (MCT, SLC16), and the peptide transporters (PEPT, SLC15). The SLCO are the organic anion transporting polypeptides (OATP) (Roth et al., 2012, Shugarts and Benet, 2009, Goole et al., 2010).

The efflux transporters from the ABC family can be subdivided into seven main genetic groups (Goole et al., 2010). The major transporters of this family are: P-glycoprotein (P-gp, ABCB1, MDR1); multidrug resistance proteins (MRP1-6, ABCC1-6), of which MRP2 is the most studied; and breast cancer resistance protein (BCRP, ABCG2) (Shugarts and Benet, 2009, Choudhuri and Klaassen, 2006). These transporters are located at the intestine, liver, kidney and blood-brain barrier. They efflux a wide range of drugs such as statins, antibiotics and chemotherapeutic agents, modulating *in vivo* exposure (Estudante et al., 2013).

Aims of the study

The aims of this work are to understand if different BCS class III compounds are affected by low doses of PEG 400; if the effect of PEG 400 is only prevalent on males (not females); and, in doing so, to gain a better understanding of the mechanisms underlying the bioavailability enhancing effect of PEG 400.

As a result, four BCS-III compounds were selected with different affinities to different intestinal transporters (Table 1): Ranitidine, cimetidine, ampicillin and metformin.

Ranitidine has an oral bioavailability of 60% and 25% of the orally administered dose is excreted unchanged in urine over 24 hours (van Hecken et al., 1982, Miller, 1984). The paracellular route is responsible for 60% of ranitidine bioavailability with 40% being influenced by intestinal membrane transporters (Bourdet et al., 2006). A considerable amount

of ranitidine is secreted into the luminal environment, mainly in the small intestine (Collett et al., 1999). Ranitidine interacts with several transporters as described in Table 1.

The oral bioavailability of cimetidine is approximately 60% with approximately half of the administered dose secreted in urine and metabolism accounting for up to 40% of its elimination (Somogyi and Gugler, 1983). Similarly to ranitidine cimetidine has been reported to be a substrate for multiple intestinal transporters as stated in Table 1.

Ampicillin is poorly bioavailable in human and animals, following oral administration bioavailability is approximately 30–40% (Lafforgue et al., 2008). It is excreted by both renal and hepatic routes subjected to enterohepatic circulation (Galtier and Alvinerie, 1979, Soto et al., 2014). Approximately 15% is excreted unchanged in urine (Desager et al., 1989). Ampicillin is absorbed by the influx transporter PEPT1 in the small intestine (Sala-Rabanal et al., 2008, Bretschneider et al., 1999). However the absorption of ampicillin across the intestinal membrane in a rat model was not found to be concentration-dependent (Lafforgue et al., 2008).

Metformin's oral bioavailability is variable due to the saturable uptake mechanism responsible for its absorption (Proctor et al., 2008). Its oral bioavailability has been calculated as approximately 61% (Pentikäinen et al., 1979) with values between 32% to 61% being reported (Sambol et al., 1996). Metformin is absorbed by passive and active transport from the duodenum (Song et al., 2006). It is excreted in the kidney by glomerular filtration and active tubular secretion with a negligible metabolism and negligible binding to plasma proteins. Metformin is a substrate for several transporters as stated in Table 1.

These drug/transporters combinations were selected as they have previously been reported to being implicated in excipient–transporter (Goole et al., 2010). An *in vivo* animal study was performed, where the compounds were administrated to male and female rats in the presence and absence of PEG 400.

Table 1. Reported interactions of BCS-III drugs with main groups of influx and efflux intestinal membrane transporters.

	Transporter	*Cimetidine*	*Ranitidine*	*Metformin*	*Ampicillin*
Influx	PEPT1	X[abc]	X[abc]	X[abc]	S[q]
	PEPT2	X[abc]	X[abc]	X[abc]	S[q]
	OCT1	I[d]	S[hj]	S[m]	X[ar]
	OCT3	S[e]	S[j]	S[n]	X[ar]
	OCTN1	I[f]	-	S[0]	X[ar]
	OCTN2	S[f]	-	X[0]	X[ar]
Efflux	P-gp	S[i]	S[kl]	X[p]	S[s]
	BCRP	S[g]	X[l]	X[a]	-
	MRP2	-	X[l]	-	-

"I": inhibitor of the transporter; "S": substrate of the transporter; "X": not a substrate; "-": no literature information available; [a](Konig et al., 2013); [b](Leibach and Ganapathy, 1996); [c](Liang et al., 1995); [d](Engel and Wang, 2005); [e](FDA, 2008); [f](Owen et al., 2005); [g](Shimizu et al., 2011); [h](Han et al., 2013); [i] (Dahan and Amidon, 2009); [j](Muller et al., 2005); [k](Bourdet and Thakker, 2006); [l](Collett et al., 1999); [m](Wang et al., 2002); [n](Chen et al., 2010); [0](Nakamichi et al., 2013); [p](Song et al., 2006); [q](Sala-Rabanal et al., 2008); [r](Muller and Fromm, 2011); [s](Siarheyeva, 2006).

2. MATERIALS AND METHODS

2.1 Materials and animals

Ranitidine, cimetidine and polyethylene glycol 400, glacial acetic acid, sodium acetate trihydrate and sodium dodecyl sulfonate were obtained from Sigma Aldrich (Dorset UK). Metformin was obtained from USV Ltd.(Mumbai, India). Ampicillin sodium was obtained from VWR Ltd.(Chicago, USA). The following HPLC grade solvents, water, methanol and acetonitrile were purchased from Fisher Scientific (Loughborough, UK). Sodium dihydrogen phosphate and ammonium acetate were purchased from VWR International LLC(Radnor, Pennsylvania). Male and female Wistar rats (8 weeks old) were purchased from Harlan UK Ltd (Oxfordshire, UK).

2.2 Animal procedures

All the rats were keep at a room temperature of 25°C and on a light-dark cycle of 12h. The rats were caged in a group, allowed to move freely and provided with food and water before the experiment. The rats were fasted for twelve hours in metabolic cages prior to the start of the experiment; food was made available four hours after dosing of the animals.

The animals were administered the active substances with and without 26 mg/kg of PEG 400 via oral gavage in an aqueous solution. The doses of the active substances are as follows: 50 mg/kg of ranitidine, 50 mg/kg of cimetidine, 50 mg/kg of ampicillin, and 20 mg/kg of metformin. The experiments for ranitidine, ampicillin and metformin were conducted with n=6 (6 males and 6 females, with and without PEG 400). For cimetidine, n=5. One of the female rats that was administered ranitidine concomitantly with PEG 400 aspirated the solution and had to be culled prior to any blood sampling taking place, thus for this group data is only presented for 5 animals.

The doses of ranitidine and cimetidine were chosen based on previous work (Afonso-Pereira et al., 2016, Eddershaw et al., 1996), and on the quantification capabilities of the method used. As for ampicillin and metformin, dose escalation studies, with 3 male rats each, were conducted *in-house* in order to determine the lowest doses that could be administered to the rats while being able to describe the pharmacokinetic profile of the active substance. The other doses used in the escalation studies were for metformin, 50 mg/kg and 100 mg/kg; and for ampicillin, 20 mg/kg and 30 mg/kg.

The dose of PEG 400 was selected as the dose that lead to a higher increase in the bioavailability of ranitidine (Afonso-Pereira et al., 2016) and cimetidine in previous work.

After dosing, the rats were placed in individual metabolic cages and were allowed to move freely. Approximately 200 μL of blood were collected from the tail vein into anticoagulant centrifuge tubes (BD Microtainer® K2E Becton, Dickinson and Company, USA) at the following time points: 30 min, 1.25 h, 2 h, 3 h, 4 h, and 6 h. In between sampling, the rats were

placed back in the metabolic cages. Blood volumes were taken in accordance with the project license and were stored on ice until the last collection point. After 8 h, the animals were sacrificed in a CO_2 euthanasia chamber (Schedule 1 method), and approximately 2 mL of blood were obtained via cardiac puncture.

Blood samples were centrifuged at 10000 rpm (930 g) for 10 min on a Centrifuge 5804R (Eppendorf AG, 22331 Hamburg, Germany) within 8h of sampling. 50 µL of the supernatant (plasma) was collected and placed into a 1.5 mL Eppendorf tube, and immediately frozen at -20°C prior to analysis.

2.3 Sample analysis

Plasma samples were thawed and assayed.

Ranitidine and cimetidine: for each sample, 50µL of thawed plasma was mixed with the same volume of acetonitrile in order to precipitate the plasma proteins. After 1 min of vortex-mixing, a further 100µL of HPLC grade water was added to the mixture, and after subsequent vortex-mixing, the samples were centrifuged at 4ºC for 10 min at 10000 rpm. The resulting supernatant was subjected to HPLC-UV analysis using a previously validated method (Ashiru et al., 2007): column 5µm Luna SCX (Phenomenex, UK); mobile phase 20:80 (acetonitrile: 0.1 M sodium acetate pH=5.0); flow rate 2 mL/min; injection volume 40 µL; detection at 320 nm for ranitidine and 288.0 nm for cimetidine.

Ampicillin: for each sample, 100 µL of thawed plasma was mixed with the same volume of methanol in order to precipitate the plasma proteins. The mixture was vortex-mixed for 6 seconds and centrifuged at 10000 rpm for 10 min. The supernatant was collected and analysed in a HPLC-UV: C18 (250 mm×4.6 mm I.D 5 µm); mobile phase of 10 mM sodium dihydrogen phosphate buffer (pH 7.0, 60%); methanol (40%); flow rate 0.6 mL/min; detection 220 nm; injection volume 50 µL.

Metformin: for each sample, 100 µL of thawed plasma was mixed with the same volume of acetonitrile in order to precipitate the plasma proteins. The mixture was vortex-mixed for 6 seconds and centrifuged at 10000 rpm for 10 min. The supernatant was collected and analysed in a HPLC-UV: C18 (250 mm×4.6 mm I.D./5 µm); mobile phase of 10 mM sodium dihydrogen phosphate buffer with 10mM sodium dodecyl sulfonate (pH 7.0, 60%); acetonitrile (40%); flow rate 1.0 mL/min; detection 234 nm; injection volume 50 µL.
Calibration standards were prepared with blank rat plasma samples spiked with drug subjected to the above-mentioned treatment.

The plasma concentration *versus* time profiles were produced for each animal. C_{max} and t_{max} were taken from these profiles. The cumulative area under the plasma concentration versus time curve ($AUC_{0\text{-}480}$) was calculated using the integration method with OriginPro 9.0 (OriginLab, Northampton, MA, USA).

2.4 Statistical analysis

The overall data was analysed by one-way ANOVA, followed by a Tukey post-hoc analysis and by individual t-student comparisons between individual groups and the appropriate controls, with IBM SPSS Statistics 19 (SPSS Inc., Illinois, USA).

3. RESULTS

For each of the four drugs there were four groups of animals. The groups were divided by sex and co-administration, or not, of PEG 400. The animals that were not co-administered PEG 400 are the control groups, whereas the animals that were administered PEG 400 are the test groups.

The results are presented in Tables 2 to 6 and Figures 2 and 3.

In the control groups, the bioavailability of ranitidine, cimetidine ($p \leq 0.05$) and ampicillin ($p \leq 0.05$) was higher in female than male rats (Figure 2). This was not observed with metformin (Tables 2, 4 and 5). The mean C_{max} is also higher in females than males for all the drugs, especially in the case of cimetidine (Table 3).

Following the co-administration of PEG 400 with the drugs, test groups, the AUC_{0-t} of ranitidine, cimetidine and ampicillin increases ($p \leq 0.05$) in males but not in females. The AUC_{0-t} of metformin is not changed by this dose of PEG 400 (Table 6 and Figure 3).

There is a high variability of t_{max} in ranitidine, cimetidine and ampicillin. It is observed from the individual plasma profiles in Figures 4 and 5 that there are two plasma concentration peaks. The reported t_{max} is the median value.

The half-life of cimetidine and metformin are similar in male and female rats. The half-life of ranitidine in male rats is approximately half of that in females, Tables 2 to 5.

The $AUC_{0-\infty}$ is less than 25% of the AUC_{0-t} for ranitidine, cimetidine and metformin. $AUC_{0-\infty}$ was not determined for ampicillin due to the insufficient number of sampling points on the terminal log-linear phase of the plasma concentration curve (EuropeanMedicinesAgency, 2010). The sampling for ampicillin was not sufficient to estimate $AUC_{0-\infty}$ and $t_{1/2}$ and these are not represented in Table 4. According to the plasma concentration curves of ampicillin, additional sampling beyond the 8 hour experiment would be necessary. This was not possible due to limitations of the maximum blood that could be safely collected from the animals in one day.

Table 2. Pharmacokinetic parameters of Ranitidine (non-transformed values; arithmetic mean ± SD, t_{max} median, range) without PEG 400 (control) and with PEG 400 (test)

Treatment	AUC_{0-t} (µg.min/mL)	$AUC_{0-\infty}$ (µg.min/mL)	C_{max} µg/mL	t_{max} min	$t_{1/2}$
Male rats: control	347.6 ± 42.6	349.3 ± 38.3	1.8 ± 0.4	150.0	72.3 ± 26.0
Female rats: control	409.4 ± 175.4	425.0 ± 166.9	2.1 ± 0.7	30.0	123.3 ± 26.7
Male rats with PEG 400: test	517.6 ± 140.3	519.7 ± 126.2	2.4 ± 0.4	150.0	80.7 ± 25.1
Female rats with PEG 400: test	437.6 ± 90.3	445.0 ± 79.2	2.0 ± 0.5	180.0	130.9 ± 84.2

AUC_{0-t} area under the plasma concentration curve from administration to last observed concentration at time t.
$AUC_{0-\infty}$ area under the plasma concentration curve extrapolated to infinite time.
C_{max} maximum plasma concentration
t_{max} time until Cmax is reached
$t_{1/2}$ half life

Table 3. Pharmacokinetic parameters of Cimetidine (non-transformed values; arithmetic mean ± SD, t_{max} median, range) without PEG 400 (control) and with PEG 400 (test)

Treatment	AUC_{0-t} (µg.min/mL)	$AUC_{0-\infty}$ (µg.min/mL)	C_{max} µg/mL	t_{max} min	$t_{1/2}$
Male rats: control	879.3 ± 67.6	913.4 ± 110.4	3.6 ± 0.6	75.0	80.0 ± 57.7
Female rats: control	1585.0 ± 316.3	1603.5 ± 276.2	7.3 ± 1.3	75.0	87.7 ± 23.7
Male rats with PEG 400: test	1203.9 ± 152.1	1215.9 ± 137.0	5.9 ± 0.7	120.0	88.4 ± 23.3
Female rats with PEG 400: test	1625.3 ± 463.4	1650.2 ± 412.1	8.5 ± 3.0	75.0	84.9 ± 31.4

AUC_{0-t} area under the plasma concentration curve from administration to last observed concentration at time t.
$AUC_{0-\infty}$ area under the plasma concentration curve extrapolated to infinite time.
C_{max} maximum plasma concentration
t_{max} time until Cmax is reached
$t_{1/2}$ half life

Table 4. Pharmacokinetic parameters of Ampicillin (non-transformed values; arithmetic mean ± SD, t_{max} median, range) without PEG 400 (control) and with PEG 400 (test)

Treatment	AUC_{0-t} (µg.min/mL)	$AUC_{0-\infty}$ (µg.min/mL)	C_{max} µg/mL	t_{max} h	$t_{1/2}$
Male rats: control	454.1 ± 92.9	NE	2.9 ± 0.3	120.0	NE
Female rats: control	639.9 ± 42.9	NE	3.3 ± 0.6	120.0	NE
Male rats with PEG 400: test	824.0 ± 42.2	NE	5.2 ± 0.7	30.0	NE
Female rats with PEG 400: test	656.4± 79.4	NE	2.6 ± 0.2	120.0	NE

AUC_{0-t} area under the plasma concentration curve from administration to last observed concentration at time t.
$AUC_{0-\infty}$ area under the plasma concentration curve extrapolated to infinite time.
C_{max} maximum plasma concentration
t_{max} time until Cmax is reached
NE not estimated due to the lack of a minimum of three samples during the terminal log-linear phase

Table 5. Pharmacokinetic parameters of Metformin (non-transformed values; arithmetic mean ± SD, t_{max} median, range) without PEG 400 (control) and with PEG 400 (test)

Treatment	AUC_{0-t} (µg.min/mL)	$AUC_{0-\infty}$ (µg.min/mL)	C_{max} $\mu g_{/mL}$	t_{max} h	$t_{1/2}$
Male rats: control	36.2 ± 4.8	44.5 ± 5.0	0.132 ± 0.025	180.0	265.3 ± 112.2
Female rats: control	39.7 ± 10.2	52.4 ± 6.6	0.171 ± 0.095	75.0	272.9 ± 177.1
Male rats with PEG 400: test	34.2 ± 10.8	45.9 ± 8.7	0.135 ± 0.071	120.0	272.3 ± 105.2
Female rats with PEG 400: test	47.5 ± 7.4	68.3 ± 16.9	0.166 ± 0.022	180.0	326.8 ± 156.7

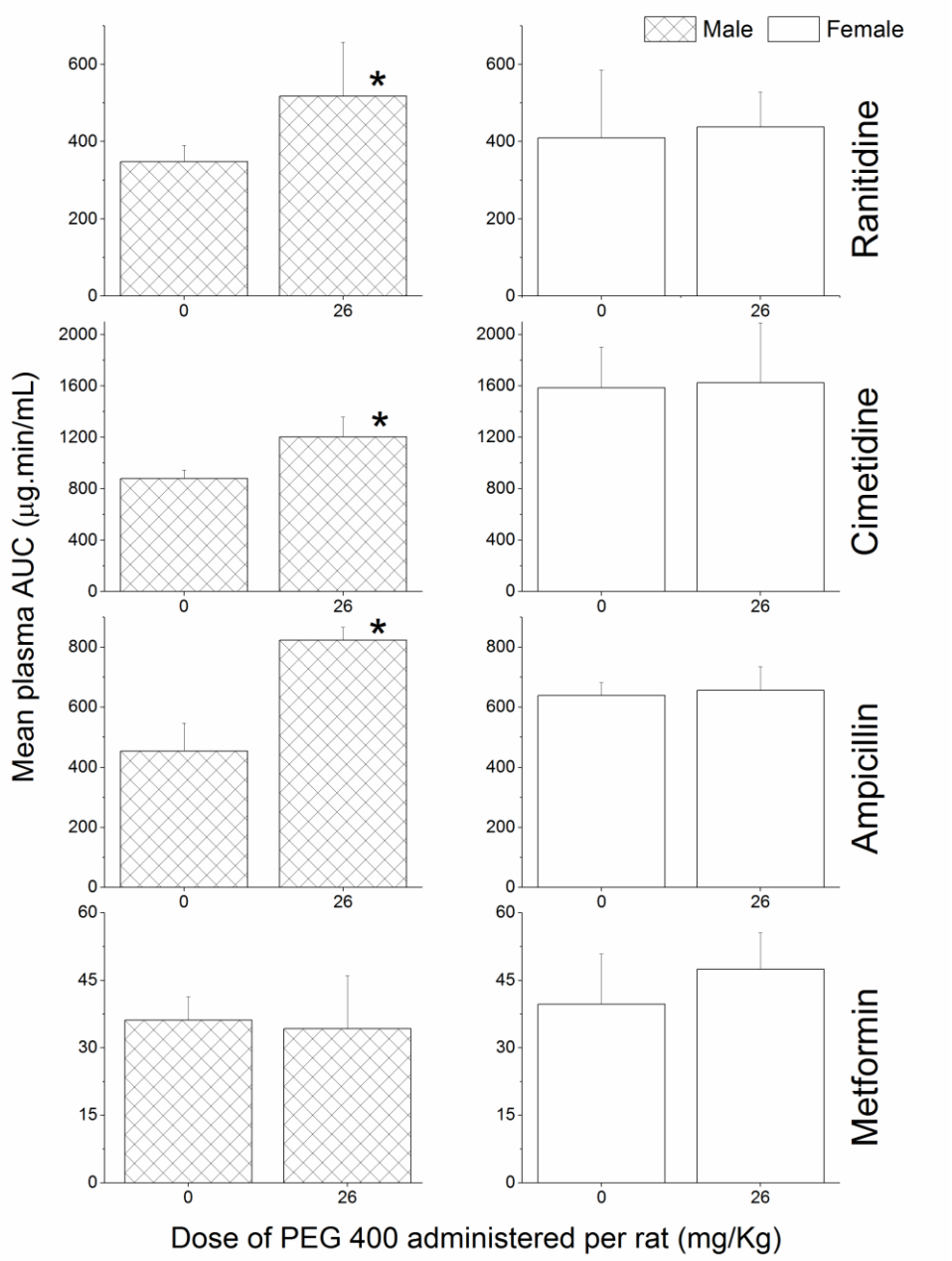
AUC_{0-t} area under the plasma concentration curve from administration to last observed concentration at time t.
$AUC_{0-\infty}$ area under the plasma concentration curve extrapolated to infinite time.
C_{max} maximum plasma concentration
t_{max} time until Cmax is reached
$t_{1/2}$ half life

Table 6. Percentage variation of the AUC_{0-480} of ranitidine, cimetidine, ampicillin and metformin between the control (drug administered as an oral solution without PEG 400) and respective test groups (same drug administered as an oral solution with 26 mg/kg of PEG 400).

Drug	Ranitidine	Cimetidine	Ampicillin	Metformin
Male rats change in AUC between test and control (%)	+49%*	+37%*	+81%*	-5%
Female rats change in AUC between test and control (%)	+7%	+3%	+3%	+20%

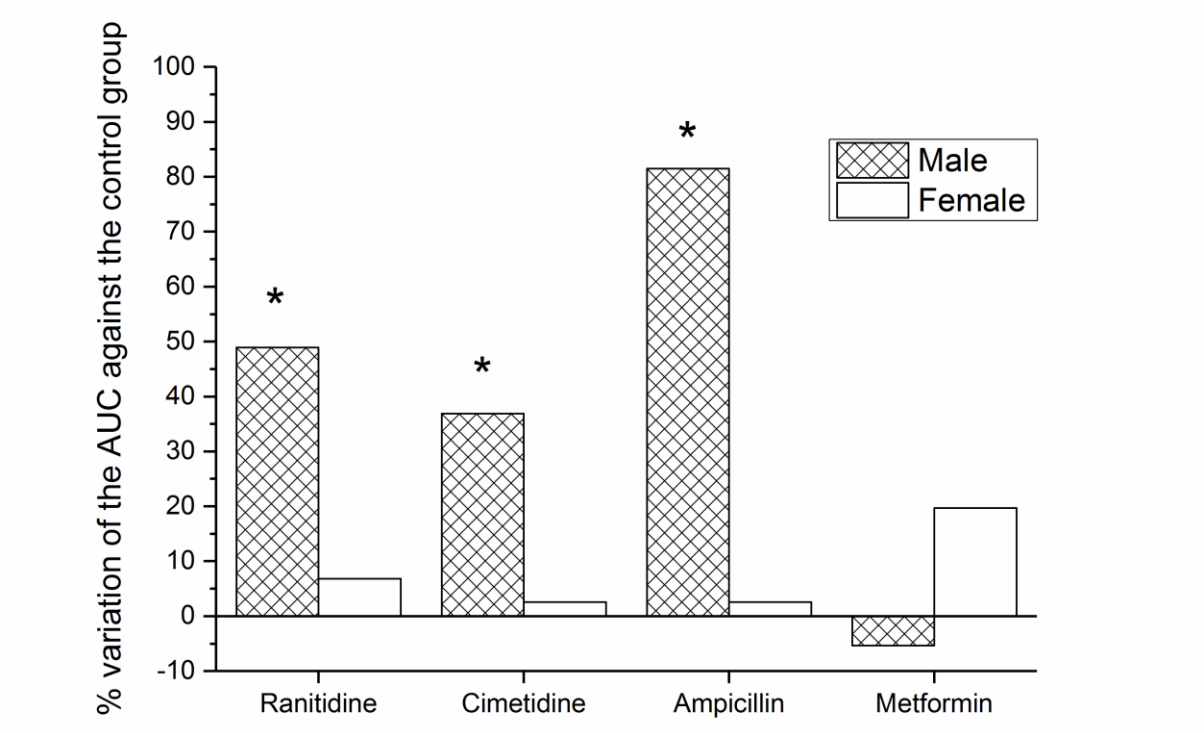
***** Indicates statistical difference ($p<0.05$) from the control group.

Figure 2. Mean AUC_{0-480} of the animal groups of ranitidine, cimetidine, ampicillin and metformin.



*Denotes statistically significant difference compared to the control group (0 mg/kg of PEG 400) ($p<0.05$).

Figure 3. Percentage variation of the AUC_{0-480} of the BCS III model drugs between a dose of PEG 400 of 26 mg/kg and the control group (0 mg/kg of PEG 400) in male and female Wistar rats.



*Denotes statistically significant difference compared to the control group (0 mg/kg of PEG 400) ($p<0.05$).

4. DISCUSSION

There is a very high variability the t_{max} of the different drugs. This is due partially to interindividual variability and to the existence of two maxima in the plasma concentrations of the drugs. This is more evident in the cases of ranitidine, cimetidine and ampicillin. It is also observed that in some cases the first t_{max} coincides with the first blood collection time point, 30 minutes (Tables 2 and 4). The second t_{max} is usually located around the 180 minutes time point. Ideally sampling schedule should include frequent sampling around the predicted t_{max} to provide a reliable estimate of peak exposure. In particular, the sampling schedule should be planned to avoid C_{max} being the first point of a concentration time curve (EuropeanMedicinesAgency, 2010). However, the total amount of blood that can be collected from the rats over a 24h period is limited by the animal size. The total number of samples is thus limited by the volume of each sample. 200 μL of blood are required per sample for processing; as a result only a maximum of 6 samples could be safely collected (1993). A more frequent sampling schedule was not achievable. There is the risk that both the first and second C_{max} values may have been missed in some animals with the subsequent underestimation of the AUC_{0-480}. Furthermore, for ampicillin, not enough sampling points were available on the log linear part of the plasma concentration curve, preventing the PK parameters from being estimated (Table 4). In this work the potential for PEG 400 to increase the bioavailability (represented by the AUC_{0-480}) of BCS-III drugs in male but not female rats is being investigated. Even if the actual C_{max} are slightly missed, overall AUC comparisons between the groups are still acceptable and a statistical analysis of the effect of PEG 400 can be performed.

The existence of two plasma peaks for cimetidine and ranitidine may have two main explanations: enterohepatic recycling and a discontinuous absorption in the gut. Enterohepatic recycling was dismissed (Pedersen and Miller, 1980, Mummaneni and Dressman, 1994). It was observed that, when cimetidine was administered in fasted men a marked secondary plasma peak was observed. This secondary peak was not observed when cimetidine was administered IM or IV. This suggests that following its absorption, cimetidine is not secreted back to the luminal environment in the bile. Ranitidine has also been reported to be mainly eliminated unchanged in urine, with hepatic clearance accounting for a very small fraction of its elimination (0.7 – 2.6%) (van Hecken et al., 1982, Richards, 1983, Garg et al., 1983, Miller, 1984, Klotz and Walker, 1990).

Figure 4. Individual plasma profiles for each male rat dosed with ranitidine, cimetidine, ampicillin and metformin. The Control groups were not co-administered PEG 400; the Test groups were co-administered 26 mg/kg of PEG 400.

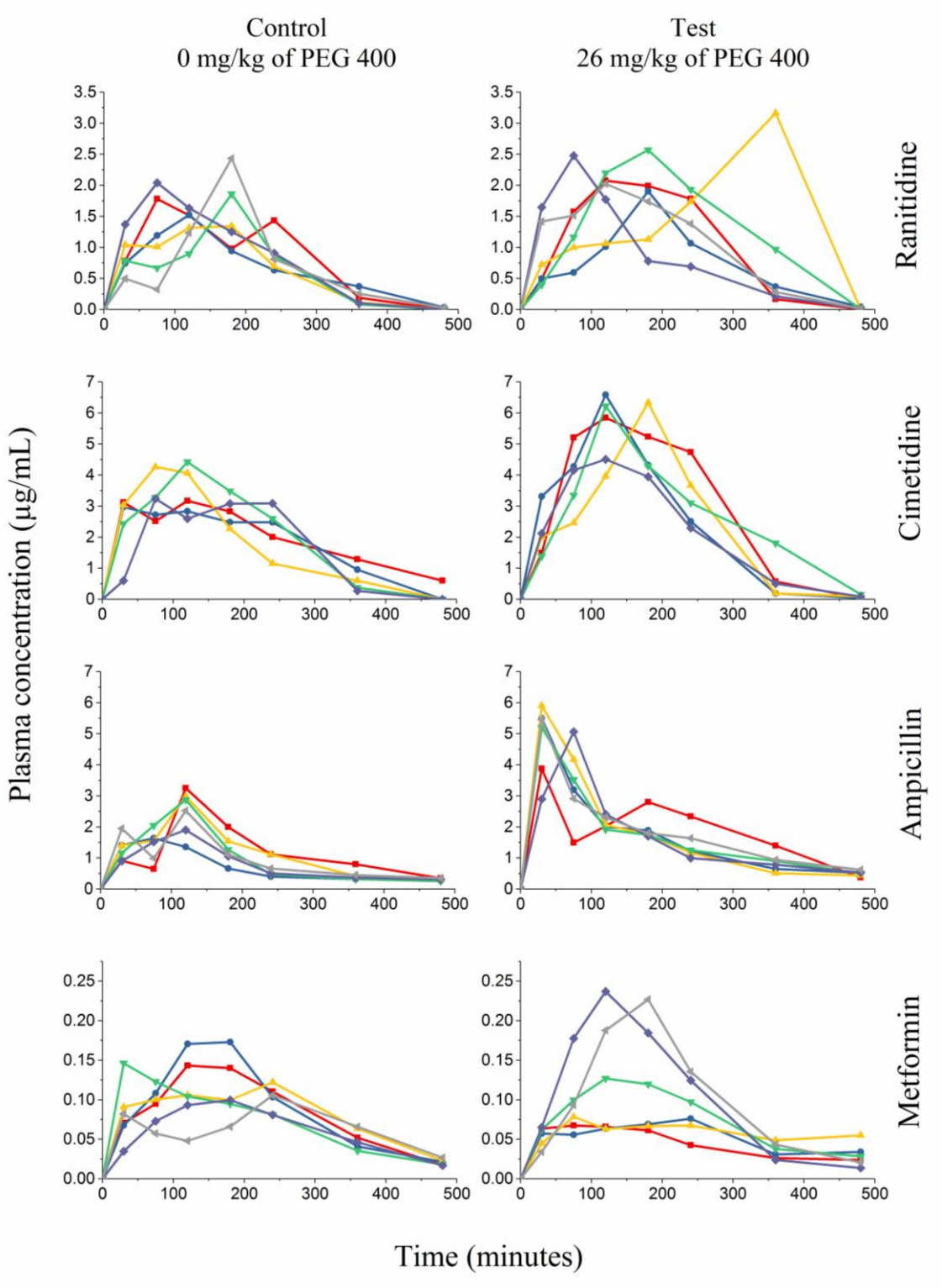
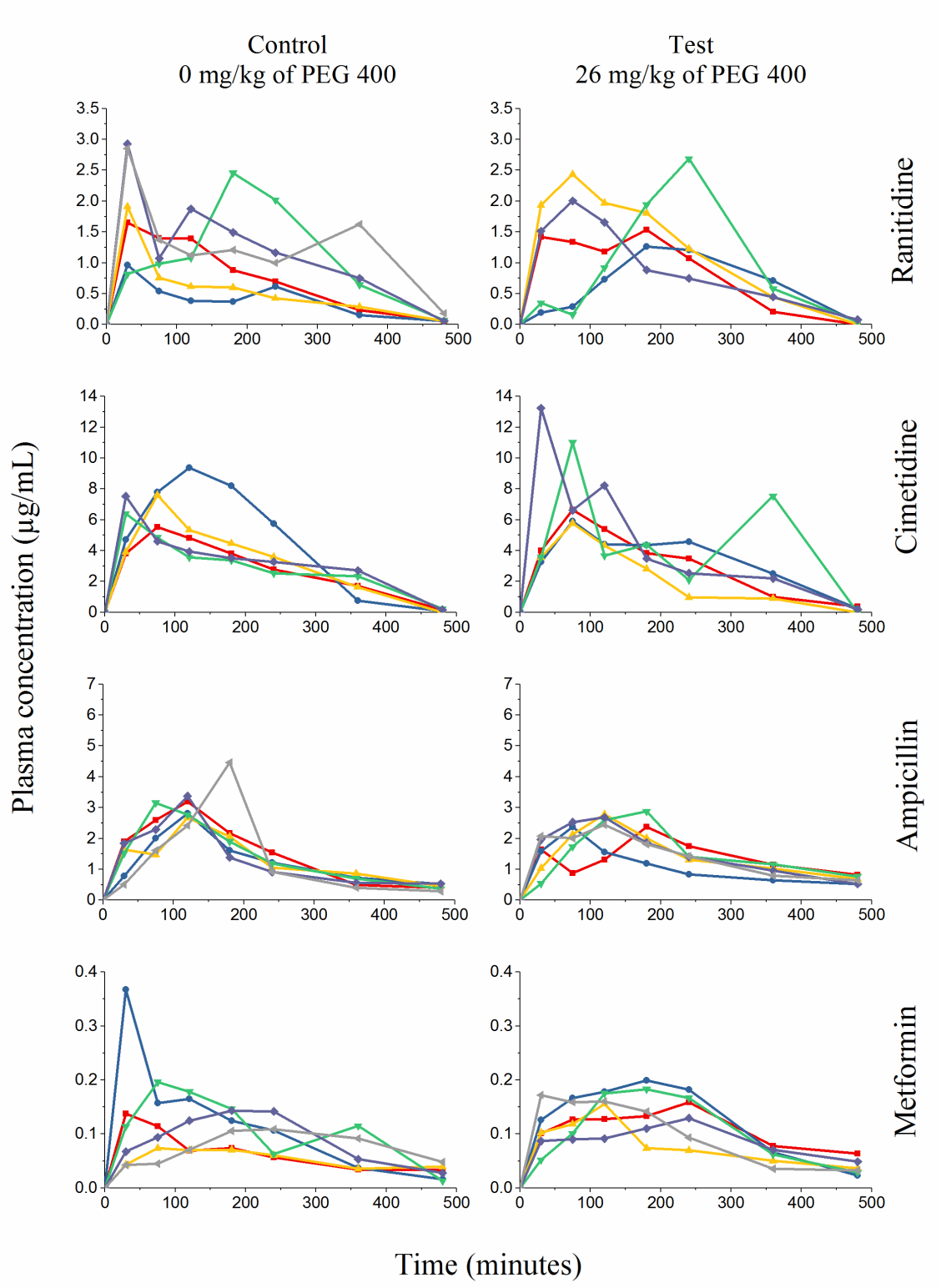


Figure 5. Individual plasma profiles for each female rat dosed with ranitidine, cimetidine, ampicillin and metformin. The Control groups were not co-administered PEG 400; the Test groups were co-administered 26 mg/kg of PEG 400.



The existence of two maxima in the plasma concentration curves of ranitidine (Richards, 1983, Miller, 1984) and cimetidine (Richards, 1983) can be explained by a bimodal absorption pattern of the drug in the gut. In fact ranitidine and cimetidine were found to be more permeable in the rat's duodenum and ileum than in the jejunum. Several reasons were put forward for this discontinuous absorption of ranitidine and cimetidine, such as solubility of the active substance, pH of the intestinal luminal environment or uptake transporters (Mummaneni and Dressman, 1994). The authors did not find a suitable explanation for this phenomena. At the time of the previous research, 1994, there had not been reports of the potential of ranitidine to interact with intestinal "secretive" transporters, mainly efflux ones such as BCRP, P-gp and MRP. These were demonstrated to affect drugs by secreting them back into the intestinal lumen from the enterocyte cytosol, reducing the amount of drug that is absorbed (Garcia-Arieta, 2014, Goole et al., 2010). A heterogeneous distribution of these transporters in the gut could lead to "preferred" regions of absorption of certain actives. P-gp was found to increase distally in the rat gut (MacLean et al., 2008). And inhibiting P-gp lead to an increase of fexofenadine, but only distally, where P-gp is more abundant (MacLean et al., 2010).

Furthermore, studies which have identified regional differences in the absorption of ranitidine from the small intestine (Mummaneni and Dressman, 1994) suggest that the effects of PEG 400 are not necessarily homogeneous throughout the gastrointestinal tract. Regionally differing permeation can be associated with the combination of different transporters (influx and efflux) with regional differences in their expression levels in the small intestine (Kagan et al., 2010, Lindell et al., 2003).

It was also observed that in the control groups, the bioavailability of ranitidine, cimetidine ($p \leq 0.05$) and ampicillin ($p \leq 0.05$) was higher in female than male rats (Figure 2), but not for metformin. This may be related to the mechanism underlying the sex difference in the bioavailability enhancing effect of PEG 400 and is discussed in the next section.

There are three major findings in this study:

- PEG 400, in small amounts, increases the oral bioavailability of BCS-III drugs, ranitidine, cimetidine and ampicillin in male rats.

- Despite being a BCS-III drug, the bioavailability of metformin was not changed by PEG 400 in rats.

- The bioavailability enhancing effect of PEG 400, in the considered doses, is sex-dependent; it is only observed in male but not female rats.

PEG 400 increases the oral bioavailability of ranitidine, cimetidine and ampicillin but not metformin

Several mechanisms could be hypothesised to explain why PEG 400 increases the bioavailability of ranitidine, cimetidine and ampicillin but not metformin. One explanation alone may not be sufficient to fully account for a mechanistic explanation and further studies may be required.

- PEG 400 increases the solubility of the active substances;

  All these active substances were chosen because of their biopharmaceutical classification category: BCS III. These are very soluble substances that have been reported to have incomplete absorptions. Because these are already very soluble compounds, it is unlikely any change in the solubility of the active substances due to a solubilising agent would have any effect on their bioavailability. Furthermore all active substances were administered in an aqueous solution and not in a solid oral dosage form. The effect of the solubility of the active substances in the respective dissolution rates would not apply for oral solutions. The active is already dissolved and available to be absorbed. The rate limiting step of the absorption of these drugs (BCS III) is the permeation through the gastrointestinal wall and not their release from the formulation (an oral solution in this case). In addition, metformin was not affected by PEG 400. The effect of PEG 400 as a solubilising agent can be discarded as a possible cause.

- PEG 400 inhibits the metabolism and / or excretion of the active substances;

  PEG 400 has been extensively reported to interact with several transporters in different locations of the organism (Ashiru-Oredope et al., 2011, Collnot et al., 2007, Johnson et al., 2002, Li et al., 2012, Li et al., 2011, Mai et al., 2017, Shen et al., 2008, Wang et al., 2004, Bobby and Sitar, 2001). Several excretion and metabolic mechanisms are potentiated by membrane transporters, as discussed in the introduction. It could be plausible to assume that PEG 400, by inhibiting or stimulating certain transporters in the liver and kidney, could modulate metabolism and excretion. However, it has been reported that PEG 400 is not very well absorbed in the rat with only half of the orally administered dose being absorbed (Lin and Hayton, 1983) and as a result it is considered that its effect is mainly at a local level in the gut and has a limited effect in inhibiting metabolism or inducing excretion. Furthermore, as explained in the introduction, the four model drugs have various degrees and different mechanisms of metabolism and excretion that would exclude a significant effect of PEG 400 in their metabolism and excretion.

- PEG 400 stimulates the absorption of the active substances by:

    - Increasing the intrinsic permeability of the intestinal membrane;

      PEG 400 could function as a permeation enhancer across the intestinal membrane by disarranging the enterocytes tight junctions. Some studies report that PEG 400 does not affect the permeation of drugs that permeate exclusively via the paracellular route (Johnson et al., 2002, Rege et al., 2001). However, it was recently demonstrated that a very high concentration of PEG 400 (50%) was capable of reversibly opening intestinal paracellular tight junctions and increase paracellular permeation by 6 fold in mice. Concentrations of PEG 400 up to 15% had no effect in the permeability of the considered compound(Ma et al., 2017). In our study, the concentration of PEG 400 in the oral solution was approximately of 1.2%, well below the concentration identified as interfering with intestinal tight junctions.

    - Inducing absorptive transporters of the intestinal membrane or inhibiting secretive transporters at the intestinal membrane;

      Absorption would also be increased if PEG 400 was capable of inhibiting intestinal secretive transporters or stimulating intestinal uptake transporters.

      PEPTs, OCTs, OCTNs were identified as influx transporters and P-gp, BCRP and MRPs as efflux transporters that could be related to the absorption or excretion of the four model active substances (table 1). In Figure 1 the location of the main intestinal transporters in the apical or basolateral side of the enterocyte can be consulted.

      PEPTs are intestinal influx transporters that play a major role in the absorption of ampicillin (Sala-Rabanal et al., 2008, Bretschneider et al., 1999). However, they were ruled out as interacting with ranitidine, cimetidine and metformin (Table 1). So it is unlikely that this member of the peptide transporter family for the cellular uptake of di and tripeptides are related to the effect of PEG 400 observed in our study. PEG 400 increased the bioavailability of ranitidine and cimetidine that are not substrates for PEPTs.

      The organic cation transporters (OCT) and the electroneutral organic cation transporters (OCTN) were found to interact with ranitidine, cimetidine and metformin, but these were demonstrated no to interact with ampicillin (Table1). OCT1 and 2 are influx transporters at the basal side of the enterocyte. However, there is also literature to suggest these are located at the apical side (Han et al., 2013). Thus it is theoretically capable of limiting absorption by hindering the passage of compounds from the basal side of the enterocyte to the blood or lymph. It would increase the concentration of a drug in the cytosol, reducing the

concentration gradient from the intestinal lumen and as a result limiting absorption. However, if OCT-1/2 were to be inhibited, the uptake at the basal side of the enterocyte would not occur and bioavailability could possibly be increased. Other organic cation transporters are located at the apical side of the enterocyte and would stimulate absorption if their activity increased or hinder it if their activity was reduced. It is known that ranitidine is a substrate for the organic cation uptake transporters (OCTs) (Ming et al., 2009; Han et al., 2013). However it is also known that cimetidine is an inhibitor of OCT1 (basolateral side) and OCTN2 (apical side) but a substrate for OCT3 and OCTN2 (at the apical side of the enterocyte). Ampicillin on the other hand has no known interaction with these OCT/OCTN transporters (Konig et al., 2013, Muller and Fromm, 2011).

Metformin is also a substrate of the OCTs (Proctor et al., 2008) and for the proton-coupled transporter, the plasma-membrane mono-amine transporter (PMAT), located at the membrane of epithelial cells, facilitating its absorption (Zhou et al., 2007). PMAT is expressed in the human intestine, and may play a role in the intestinal absorption of metformin and possibly other cationic drugs (Zhou and Wang, 2006, Zhou et al., 2007). Nevertheless metformin is not influenced by PEG 400 whereas cimetidine and ranitidine are. Also, ampicillin has its oral bioavailability increased by PEG 400 and is not a substrate to the OCT/OCTN transporters. As a result OCTs may not be the reason for why PEG 400 enhances the bioavailability of three BCS III drugs but not of metformin in male rats.

The major influx transporters were ruled out as possible causes for the effect of PEG 400. However, efflux transporters located at the apical side of the enterocytes, could, if inhibited, stop transporting active substances from the cytosol back to the intestinal lumen. Thus, the amount of active substance that would be absorbed would increase, as a consequence bioavailability would increase. Cimetidine is a substrate for the efflux transporters P-gp (Collett et al., 1999) and BCRP (Pavek et al., 2005). BCRP has greater expression 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). However, ranitidine is only a substrate for P-gp (Collett et al., 1999, Pavek et al., 2005). This may allow us to exclude BCRP as the main reason for the bioavailability enhancing effect of PEG 400 on cimetidine in males and not in females.

It is known that ranitidine is a substrate for the efflux transporter P-glycoprotein (P-gp) (Cook and Hirst, 1994, Collett et al., 1999). And so is cimetidine(Collett et al., 1999) and ampicillin (Siarheyeva et al., 2006) (Table 1).

Metformin was reported to inhibit the expression of the gene MDR1, responsible for the expression of P-gp by activation of the AMP-activated

protein kinase (AMPK) and inhibition of the activation of NF-kB and cAMP-responsive element binding protein (Kim et al., 2011). But it is not a substrate for P-gp itself, as the concomitant intestinal perfusion of metformin with verapamil, a well-established P-gp inhibitor, did not affect the permeation of metformin through the intestinal membrane (Song et al., 2006). Furthermore, in the eight hour experiment the inhibitory effects in the MDR1 gene expression would not affect the overall amount of P-gp (Kim et al., 2011).

The PEG 400 increased the oral bioavailability of ranitidine, cimetidine and ampicillin, but not metformin. Out of the identified transporters, the only major transporter that was identified as interacting with ranitidine, cimetidine and ampicillin, but not metformin and whose inhibition would lead to an increase of the bioavailability of these drugs is P-gp. PEGs are known to inhibit P-gp in a concentration-dependent manner from 0.1 to 20% (w/v) (Hugger et al., 2002, Shen et al., 2006). PEG 400 at concentrations of 1% (w/v) and 2% (w/v) is capable of blocking P-gp in a caco-2 cell line model (Hodaei et al., 2015).The presence of PEG at 1%, 5%, and 20% (w/v) reduced efflux of digoxin (by 47%, 57%, and 64%, respectively, when compared to control (Johnson et al., 2002). Also, the P-gp inhibitor cyclosporine A, when orally administered to male and female rats led to an increase of the bioavailability of ranitidine and ampicillin but not metformin. Males had an increase of over 100% in the $AUC_{0\text{-}480}$ in the cyclosporine A treated animals when compared to the control (Mai et al., 2017). In our oral solution PEG 400 was used in a concentration approaching 1.2%, which would, according to the in vitro work above, lead to an inhibition of approximately 50% on the activity of P-gp. It is thus plausible to assume that P-gp plays a major role in the effect of PEG 400 in BCS-III drugs subjected to efflux transporters at the intestinal lumen.

The bioavailability enhancing effect of PEG 400, in the considered doses, is sex-dependent; it is only observed in male but not female rats

Despite having ruled out OCT transporters as the possible cause for the bioavailability enhancing effect of PEG 400 of ranitidine, cimetidine and ampicillin, the fact that there are sex differences in the expression and distribution of these transporters is illustrative that male and females are indeed different and drug disposition may be affected. This may help us understand why males seem to be affect by low doses of PEG 400 whereas females are not. For example, ranitidine and cimetidine, are known substrates for OCT/OCAT in both the kidney (OCT2) and the liver (OCT1) (Kimura et al., 2005, Bourdet et al., 2006). OCAT expression in rat kidneys was found to be much higher in males than in females (Urakami et al., 1999). Also, differences have been reported in the renal and hepatic expression of organic anion transporters in male and female mice (Buist et al., 2002).More details on hepatic and renal sex differences in OATs are reviewed elsewhere (VanWert et al., 2010).

If sex differences exist in the amount and distribution of some cellular transporters the possibility of differences also occurring with P-gp must be considered.

The activity and expression of P-gp has been reported to be different in males and females (Mariana et al., 2011).

It was demonstrated that the different sex hormones could, over time, change the amount of P-gp expressed in different systemic organs (Bebawy and Chetty, 2009). For example, female rats, when given testosterone had the amount of P-gp reduced in the liver, with a corresponding reduction of biliary clearance of doxorubicin (Suzuki et al., 2006). Other studies have identified sex differences in hepatic P-gp expression (Schuetz et al., 1995, Cummins et al., 2002). In humans, it has been reported that enteric sex differences were present: women had a lower enterocyte P-gp content than men (Potter et al., 2004), but other work suggested that no sex differences were present in the proximal small intestine (Paine et al., 2005). It is known that regional differences in the expression of P-gp may be present it may be that in certain regions of the small intestine there are differences in the relative amount of P-gp. Until a complete mapping of the human intestine is available the hypothesis of different amount of the transporter in the sexes cannot be discarded. In rats, however, a scan of the intestinal expression of P-gp found that P-gp expression increased distally in the sexes but not gender specific differences were seen (MacLean et al., 2008). Expression was evaluated and not the activity of P-gp. It may be that the relative amount of P-gp is similar in the sexes but the degree of activity differs. It was found that testosterone is a potent inhibitor of P-gp (twice as much as progesterone) (Hamilton et al., 2001, Dey et al., 2004). It may be that the amount of P-gp in male and females is the same, but the males are permanently partially inhibited due to circulating testosterone. In our study, we found that PEG 400 increased the bioavailability of ranitidine, cimetidine and ampicillin in male but not in female rats. This difference is related to the efficiency of the P-gp mechanism in the gut. Males have a higher sensitivity to the inhibitory effects of PEG 400 over P-gp than females. The exact mechanism behind this is unclear and further studies should be conducted.

5. CONCLUSION

PEG 400, in small amounts, increases the oral bioavailability of BCS-III drugs, ranitidine, cimetidine and ampicillin in male rats. Despite also being a BCS-III drug, the bioavailability of metformin was not changed by PEG 400 in rats. This finding allowed us to narrow down the possible causes for the bioavailability enhancing effect of PEG 400. The effect of PEG 400 is most likely related to its inhibitory effects over P-gp, an efflux pump, at the intestinal epithelium. A different overall activity of P-gp in the sexes may explain that the bioavailability enhancing effect of PEG 400, in the considered doses, is sex-dependent; it is only observed in male but not female rats.

It is clear that there are sex differences in how the organisms react to excipients administered orally. The degree to which excipients modulate drug disposition may be modified by sex. It is important that regulatory authorities and pharmaceutical industry take this knowledge into account during the pharmaceutical development stages of their products. When conducting BE studies, if excipients that are likely to interact with biological transporters in the gut are present, applicants and authorities should consider the possibility of the sexes responding differently and should design their studies and define their target populations accordingly.

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7. REFERENCES

1993. Removal of blood from laboratory mammals and birds. First report of the BVA/FRAME/RSPCA/UFAW Joint Working Group on Refinement. *Laboratory animals,* 27**,** 1-22.

AFONSO-PEREIRA, F., ASHIRU, D., PATEL, R., MURDAN, S. & BASIT, A. W. 2017. Sex differences in the gut: influence of an excipient on cimetidine bioavailability in humans and rats. UCL School of Pharmacy, University College London.

AFONSO-PEREIRA, F., MURDAN, S., SOUSA, J., VEIGA, F. & BASIT, A. W. 2016. Sex differences in excipient effects: Enhancement in ranitidine bioavailability in the presence of polyethylene glycol in male, but not female, rats. *Int J Pharm,* 506**,** 237-41.

AMIDON, G. L., LENNERNAS, H., SHAH, V. P. & CRISON, J. R. 1995. A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability. *Pharm Res,* 12**,** 413-20.

ASHIRU-OREDOPE, D. A., PATEL, N., FORBES, B., PATEL, R. & BASIT, A. W. 2011. The effect of polyoxyethylene polymers on the transport of ranitidine in Caco-2 cell monolayers. *International Journal of Pharmaceutics,* 409**,** 164-8.

ASHIRU, D. A., PATEL, R. & BASIT, A. W. 2007. Simple and universal HPLC-UV method to determine cimetidine, ranitidine, famotidine and nizatidine in urine: application to the analysis of ranitidine and its metabolites in human volunteers. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences,* 860**,** 235-40.

ASHIRU, D. A., PATEL, R. & BASIT, A. W. 2008. Polyethylene glycol 400 enhances the bioavailability of a BCS class III drug (ranitidine) in male subjects but not females. *Pharm Res,* 25**,** 2327-33.

BASIT, A. W., PODCZECK, F., NEWTON, J. M., WADDINGTON, W. A., ELL, P. J. & LACEY, L. F. 2002. Influence of polyethylene glycol 400 on the gastrointestinal absorption of ranitidine. *Pharmaceutical Research,* 19**,** 1368-74.

BEBAWY, M. & CHETTY, M. 2009. Gender differences in p-glycoprotein expression and function: effects on drug disposition and outcome. *Current drug metabolism,* 10**,** 322-8.

BENET, L. Z., AMIDON, G. L., BARENDS, D. M., LENNERNAS, H., POLLI, J. E., SHAH, V. P., STAVCHANSKY, S. A. & YU, L. X. 2008. The use of BDDCS in classifying the permeability of marketed drugs. *Pharm Res,* 25**,** 483-8.

BOBBY, B. & SITAR, D. S. 2001. The effect of lactate on sex differences in rat renal tubular energy-dependent transport of the organic cation amantadine. *Pharmacology,* 62**,** 188-192.

BOURDET, D. L., POLLACK, G. M. & THAKKER, D. R. 2006. Intestinal absorptive transport of the hydrophilic cation ranitidine: a kinetic modeling approach to elucidate the role of uptake and efflux transporters and paracellular vs. transcellular transport in Caco-2 cells. *Pharmaceutical Research,* 23**,** 1178-87.

BOURDET, D. L. & THAKKER, D. R. 2006. Saturable absorptive transport of the hydrophilic organic cation ranitidine in Caco-2 cells: role of pH-dependent organic cation uptake system and P-glycoprotein. *Pharm Res,* 23**,** 1165-77.

BRETSCHNEIDER, B., BRANDSCH, M. & NEUBERT, R. 1999. Intestinal transport of beta-lactam antibiotics: analysis of the affinity at the H+/peptide symporter (PEPT1), the uptake into Caco-2 cell monolayers and the transepithelial flux. *Pharm Res,* 16**,** 55-61.

BUIST, S. C. N., CHERRINGTON, N. J., CHOUDHURI, S., HARTLEY, D. P. & KLAASSEN, C. D. 2002. Gender-specific and developmental influences on the expression of rat organic anion transporters. *Journal of Pharmacology and Experimental Therapeutics,* 301**,** 145-151.

CHEN, L. G., PAWLIKOWSKI, B., SCHLESSINGER, A., MORE, S. S., STRYKE, D., JOHNS, S. J., PORTMAN, M. A., CHEN, E., FERRIN, T. E., SALI, A. & GIACOMINI, K. M. 2010. Role of organic cation transporter 3 (SLC22A3) and its missense variants in the pharmacologic action of metformin. *Pharmacogenetics and Genomics,* 20**,** 687-699.

CHEN, M. L., AMIDON, G. L., BENET, L. Z., LENNERNAS, H. & YU, L. X. 2011. The BCS, BDDCS, and regulatory guidances. *Pharm Res,* 28**,** 1774-8.

CHOUDHURI, S. & KLAASSEN, C. D. 2006. Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters. *Int J Toxicol,* 25**,** 231-59.

COLLETT, A., HIGGS, N. B., SIMS, E., ROWLAND, M. & WARHURST, G. 1999. Modulation of the permeability of H2 receptor antagonists cimetidine and ranitidine by P-glycoprotein in rat intestine and the human colonic cell line Caco-2. *J Pharmacol Exp Ther,* 288**,** 171-8.

COLLNOT, E. M., BALDES, C., WEMPE, M. F., KAPPL, R., HUTTERMANN, J., HYATT, J. A., EDGAR, K. J., SCHAEFER, U. F. & LEHR, C. M. 2007. Mechanism of inhibition of P-glycoprotein mediated efflux by vitamin E TPGS: influence on ATPase activity and membrane fluidity. *Mol Pharm,* 4**,** 465-74.

COOK, M. J. & HIRST, B. H. 1994. Transepithelial Secretion of the Histamine H-2-Receptor Antagonist Ranitidine in Human Intestinal Epithelial Caco-2 Monolayers Is Mediated by P-Glycoprotein. *Journal of Physiology-London,* 479P**,** P103-P103.

CUMMINS, C. L., WU, C. Y. & BENET, L. Z. 2002. Sex-related differences in the clearance of cytochrome P450 3A4 substrates may be caused by P-glycoprotein. *Clin Pharmacol Ther,* 72**,** 474-89.

CUSTODIO, J. M., WU, C. Y. & BENET, L. Z. 2008. Predicting drug disposition, absorption/elimination/transporter interplay and the role of food on drug absorption. *Adv Drug Deliv Rev,* 60**,** 717-33.

DAHAN, A. & AMIDON, G. L. 2009. Segmental Dependent Transport of Low Permeability Compounds along the Small Intestine Due to P-Glycoprotein: The Role of Efflux Transport in the Oral Absorption of BCS Class III Drugs. *Molecular Pharmaceutics,* 6**,** 19-28.

DESAGER, J. P., COSTERMANS, J., VAN NIEUWENHUYZE, Y. & HARVENGT, C. 1989. Oral bioavailability of ampicillin and amoxycillin alone and bound in fixed proportions to sulbactam and clavulanic acid. *J Int Med Res,* 17**,** 532-8.

DEY, S., GUNDA, S. & MITRA, A. K. 2004. Pharmacokinetics of erythromycin in rabbit corneas after single-dose infusion: role of P-glycoprotein as a barrier to in vivo ocular drug absorption. *J Pharmacol Exp Ther,* 311**,** 246-55.

EDDERSHAW, P. J., CHADWICK, A. P., HIGTON, D. M., FENWICK, S. H., LINACRE, P., JENNER, W. N., BELL, J. A. & MANCHEE, G. R. 1996. Absorption and disposition of ranitidine hydrochloride in rat and dog. *Xenobiotica,* 26**,** 947-56.

ENGEL, K. & WANG, J. 2005. Interaction of organic cations with a newly identified plasma membrane monoamine transporter. *Molecular Pharmacology,* 68**,** 1397-407.

ESTUDANTE, M., MAYA, M., MORAIS, J. G., SOVERAL, G. & BENET, L. Z. 2013. Effect of P-Glycoprotein on the Rat Intestinal Permeability and Metabolism of the BDDCS Class 1 Drug Verapamil. *Mol Pharm,* 10**,** 4038-45.

EUROPEANMEDICINESAGENCY 2010. GUIDELINE ON THE INVESTIGATION OF BIOEQUIVALENCE: CPMP/EWP/QWP/1401/98 Rev. 1/ Corr ** *In:* HTTP://WWW.EMA.EUROPA.EU/DOCS/EN_GB/DOCUMENT_LIBRARY/SCIENTIFIC_GUIDELINE/2010/01/WC500070039.PDF (ed.).

FDA, U. S. F. A. D. A. C. 2008. Drug Information: Drug Development and Drug Interactions: Table of Substrates, Inhibitors and Inducers http://www.fda.gov/Cder/drug/drugInteractions/ tableSubstrates.htm# (accessed 12/17/2008), part of U.S. *Food and Drug Administration Center for Drug Evaluation and Research.http://www.fda.gov/Cder/(accessed 12/11/2008).*

GALTIER, P. & ALVINERIE, M. 1979. Enhancement of ampicillin bioavailability in pigs. *Journal of Veterinary Pharmacology and Therapeutics,* 2**,** 181-186.

GARCIA-ARIETA, A. 2014. Interactions between active pharmaceutical ingredients and excipients affecting bioavailability: impact on bioequivalence. *Eur J Pharm Sci,* 65**,** 89-97.

GARG, D. C., WEIDLER, D. J. & ESHELMAN, F. N. 1983. Ranitidine bioavailability and kinetics in normal male subjects. *Clin Pharmacol Ther,* 33**,** 445-52.

GOOLE, J., LINDLEY, D. J., ROTH, W., CARL, S. M., AMIGHI, K., KAUFFMANN, J.-M. & KNIPP, G. T. 2010. The effects of excipients on transporter mediated absorption. *International Journal of Pharmaceutics,* 393**,** 17-31.

HAMILTON, K. O., YAZDANIAN, M. A. & AUDUS, K. L. 2001. Modulation of P-glycoprotein activity in Calu-3 cells using steroids and beta-ligands. *Int J Pharm,* 228**,** 171-9.

HAN, T. K., EVERETT, R. S., PROCTOR, W. R., NG, C. M., COSTALES, C. L., BROUWER, K. L. & THAKKER, D. R. 2013. Organic cation transporter 1 (OCT1/mOct1) is localized in the apical membrane of Caco-2 cell monolayers and enterocytes. *Molecular Pharmacology,* 84**,** 182-9.

HODAEI, D., BARADARAN, B., VALIZADEH, H. & ZAKERI-MILANI, P. 2015. Effects of polyethylene glycols on intestinal efflux pump expression and activity in Caco-2 cells. *Brazilian Journal of Pharmaceutical Sciences,* 51**,** 745-753.

HUGGER, E. D., AUDUS, K. L. & BORCHARDT, R. T. 2002. Effects of poly(ethylene glycol) on efflux transporter activity in Caco-2 cell monolayers. *Journal of Pharmaceutical Sciences,* 91**,** 1980-1990.

JOHNSON, B. M., CHARMAN, W. N. & PORTER, C. J. 2002. An in vitro examination of the impact of polyethylene glycol 400, Pluronic P85, and vitamin E d-alpha-tocopheryl polyethylene glycol 1000 succinate on P-glycoprotein efflux and enterocyte-based metabolism in excised rat intestine. *AAPS PharmSci,* 4**,** E40.

KAGAN, L., DREIFINGER, T., MAGER, D. E. & HOFFMAN, A. 2010. Role of p-glycoprotein in region-specific gastrointestinal absorption of talinolol in rats. *Drug Metab Dispos,* 38**,** 1560-6.

KIM, H. G., HIEN, T. T., HAN, E. H., HWANG, Y. P., CHOI, J. H., KANG, K. W., KWON, K. I., KIM, B. H., KIM, S. K., SONG, G. Y., JEONG, T. C. & JEONG, H. G. 2011. Metformin inhibits P-glycoprotein expression via the NF-kappaB pathway and CRE transcriptional activity through AMPK activation. *Br J Pharmacol,* 162**,** 1096-108.

KIMURA, N., MASUDA, S., TANIHARA, Y., UEO, H., OKUDA, M., KATSURA, T. & INUI, K. 2005. Metformin is a superior substrate for renal organic cation transporter OCT2 rather than hepatic OCT1. *Drug Metab Pharmacokinet,* 20**,** 379-86.

KLOTZ, U. & WALKER, S. 1990. Biliary excretion of H2-receptor antagonists. *European Journal of Clinical Pharmacology,* 39**,** 91-92.

KONIG, J., MULLER, F. & FROMM, M. F. 2013. Transporters and drug-drug interactions: important determinants of drug disposition and effects. *Pharmacological Reviews,* 65**,** 944-66.

LAFFORGUE, G., ARELLANO, C., VACHOUX, C., WOODLEY, J., PHILIBERT, C., DUPOUY, V., BOUSQUET-MELOU, A., GANDIA, P. & HOUIN, G. 2008. Oral absorption of ampicillin: role of paracellular route vs. PepT1 transporter. *Fundam Clin Pharmacol,* 22**,** 189-201.

LEIBACH, F. H. & GANAPATHY, V. 1996. Peptide transporters in the intestine and the kidney. *Annu Rev Nutr,* 16**,** 99-119.

LI, K. K., CONCEPCION, R. Y., LEE, H., CARDINAL, B. J., EBBECK, V., WOEKEL, E. & READDY, R. T. 2012. An examination of sex differences in relation to the eating habits and nutrient intakes of university students. *J Nutr Educ Behav,* 44**,** 246-50.

LI, L., MEIER, P. J. & BALLATORI, N. 2000. Oatp2 mediates bidirectional organic solute transport: a role for intracellular glutathione. *Mol Pharmacol,* 58**,** 335-40.

LI, M., SI, L., PAN, H., RABBA, A. K., YAN, F., QIU, J. & LI, G. 2011. Excipients enhance intestinal absorption of ganciclovir by P-gp inhibition: assessed in vitro by everted gut sac and in situ by improved intestinal perfusion. *Int J Pharm,* 403**,** 37-45.

LIANG, R., FEI, Y. J., PRASAD, P. D., RAMAMOORTHY, S., HAN, H., YANG-FENG, T. L., HEDIGER, M. A., GANAPATHY, V. & LEIBACH, F. H. 1995. Human intestinal H+/peptide cotransporter. Cloning, functional expression, and chromosomal localization. *J Biol Chem,* 270**,** 6456-63.

LIN, C.-F. & HAYTON, W. L. 1983. Absorption of polyethylene glycol 400 administered orally to mature and senescent rats. *AGE,* 6**,** 52-56.

LINDELL, M., KARLSSON, M. O., LENNERNAS, H., PAHLMAN, L. & LANG, M. A. 2003. Variable expression of CYP and Pgp genes in the human small intestine. *Eur J Clin Invest,* 33**,** 493-9.

MA, B.-L., YANG, Y., DAI, Y., LI, Q., LIN, G. & MA, Y.-M. 2017. Polyethylene glycol 400 (PEG400) affects the systemic exposure of oral drugs based on multiple mechanisms: taking berberine as an example. *RSC Advances,* 7**,** 2435-2442.

MACLEAN, C., MOENNING, U., REICHEL, A. & FRICKER, G. 2008. Closing the gaps: a full scan of the intestinal expression of p-glycoprotein, breast cancer resistance protein, and multidrug resistance-associated protein 2 in male and female rats. *Drug Metab Dispos,* 36**,** 1249-54.

MACLEAN, C., MOENNING, U., REICHEL, A. & FRICKER, G. 2010. Regional absorption of fexofenadine in rat intestine. *European Journal of Pharmaceutical Sciences,* 41**,** 670-674.

MAI, Y., AFONSO-PEREIRA, F., MURDAN, S. & BASIT, A. W. 2017. Excipient-mediated alteration in drug bioavailability in the rat depends on the sex of the animal. *European Journal of Pharmaceutical Sciences,* 107**,** 249-255.

MARIANA, B., ADRIAN, L., GUILLERMO, V., JUAN, S., LAURA, M. & CARLOS, L. 2011. Gender-related differences on P-glycoprotein-mediated drug intestinal transport in rats. *Journal of Pharmacy and Pharmacology,* 63**,** 619-626.

MCCONNELL, E. L., FADDA, H. M. & BASIT, A. W. 2008. Gut instincts: explorations in intestinal physiology and drug delivery. *Int J Pharm,* 364**,** 213-26.

MERINO, G., VAN HERWAARDEN, A. E., WAGENAAR, E., JONKER, J. W. & SCHINKEL, A. H. 2005. Sex-dependent expression and activity of the ATP-binding cassette transporter breast cancer resistance protein (BCRP/ABCG2) in liver. *Mol Pharmacol,* 67**,** 1765-71.

MILLER, R. 1984. Pharmacokinetics and bioavailability of ranitidine in humans. *Journal of Pharmaceutical Sciences,* 73**,** 1376-1379.

MULLER, F. & FROMM, M. F. 2011. Transporter-mediated drug-drug interactions. *Pharmacogenomics,* 12**,** 1017-37.

MULLER, J., LIPS, K. S., METZNER, L., NEUBERT, R. H., KOEPSELL, H. & BRANDSCH, M. 2005. Drug specificity and intestinal membrane localization of human organic cation transporters (OCT). *Biochem Pharmacol,* 70**,** 1851-60.

MUMMANENI, V. & DRESSMAN, J. B. 1994. Intestinal uptake of cimetidine and ranitidine in rats. *Pharmaceutical Research,* 11**,** 1599-604.

NAKAMICHI, N., SHIMA, H., ASANO, S., ISHIMOTO, T., SUGIURA, T., MATSUBARA, K., KUSUHARA, H., SUGIYAMA, Y., SAI, Y., MIYAMOTO, K., TSUJI, A. & KATO, Y. 2013. Involvement of carnitine/organic cation transporter OCTN1/SLC22A4 in gastrointestinal absorption of metformin. *J Pharm Sci,* 102**,** 3407-17.

OWEN, R. P., GRAY, J. H., TAYLOR, T. R., CARLSON, E. J., HUANG, C. C., KAWAMOTO, M., JOHNS, S. J., STRYKE, D., FERRIN, T. E. & GIACOMINI, K. M. 2005. Genetic analysis and functional characterization of polymorphisms in the human concentrative nucleoside transporter, CNT2. *Pharmacogenet Genomics,* 15**,** 83-90.

PAINE, M. F., LUDINGTON, S. S., CHEN, M. L., STEWART, P. W., HUANG, S. M. & WATKINS, P. B. 2005. Do men and women differ in proximal small intestinal CYP3A or P-glycoprotein expression? *Drug Metab Dispos,* 33**,** 426-33.

PAVEK, P., MERINO, G., WAGENAAR, E., BOLSCHER, E., NOVOTNA, M., JONKER, J. W. & SCHINKEL, A. H. 2005. Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine. *J Pharmacol Exp Ther,* 312**,** 144-52.

PEDERSEN, P. V. & MILLER, R. 1980. Pharmacokinetics and bioavailability of cimetidine in humans. *J Pharm Sci,* 69**,** 394-8.

PENTIKÄINEN, P. J., NEUVONEN, P. J. & PENTTILÄ, A. 1979. Pharmacokinetics of metformin after intravenous and oral administration to man. *European Journal of Clinical Pharmacology,* 16**,** 195-202.

POTTER, J. M., MCWHINNEY, B. C., SAMPSON, L. & HICKMAN, P. E. 2004. Area-under-the-curve monitoring of prednisolone for dose optimization in a stable renal transplant population. *Ther Drug Monit,* 26**,** 408-14.

PROCTOR, W. R., BOURDET, D. L. & THAKKER, D. R. 2008. Mechanisms underlying saturable intestinal absorption of metformin. *Drug Metab Dispos,* 36**,** 1650-8.

REGE, B. D., YU, L. X., HUSSAIN, A. S. & POLLI, J. E. 2001. Effect of common excipients on Caco-2 transport of low-permeability drugs. *J Pharm Sci,* 90**,** 1776-86.

REGITZ-ZAGROSEK, V. 2012. Sex and gender differences in health: Science & Society Series on Sex and Science. *EMBO Reports,* 13**,** 596-603.

RICHARDS, D. A. 1983. Comparative pharmacodynamics and pharmacokinetics of cimetidine and ranitidine. *J Clin Gastroenterol,* 5 Suppl 1**,** 81-90.

ROTH, M., OBAIDAT, A. & HAGENBUCH, B. 2012. OATPs, OATs and OCTs: the organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *British Journal of Pharmacology,* 165**,** 1260-1287.

SALA-RABANAL, M., LOO, D. D., HIRAYAMA, B. A. & WRIGHT, E. M. 2008. Molecular mechanism of dipeptide and drug transport by the human renal H+/oligopeptide cotransporter hPEPT2. *Am J Physiol Renal Physiol,* 294**,** F1422-32.

SAMBOL, N. C., CHIANG, J., O'CONNER, M., LIU, C. Y., LIN, E. T., GOODMAN, A. M., BENET, L. Z. & KARAM, J. H. 1996. Pharmacokinetics and pharmacodynamics of metformin in healthy subjects and patients with noninsulin-dependent diabetes mellitus. *J Clin Pharmacol,* 36**,** 1012-21.

SCHIEBINGER, L. 2014. Scientific research must take gender into account. *Nature,* 507**,** 9.

SCHUETZ, E. G., FURUYA, K. N. & SCHUETZ, J. D. 1995. Interindividual variation in expression of P-glycoprotein in normal human liver and secondary hepatic neoplasms. *J Pharmacol Exp Ther,* 275**,** 1011-8.

SHEN, F., CHU, S., BENCE, A. K., BAILEY, B., XUE, X., ERICKSON, P. A., MONTROSE, M. H., BECK, W. T. & ERICKSON, L. C. 2008. Quantitation of doxorubicin uptake, efflux, and modulation of multidrug resistance (MDR) in MDR human cancer cells. *J Pharmacol Exp Ther,* 324**,** 95-102.

SHEN, Q., LIN, Y., HANDA, T., DOI, M., SUGIE, M., WAKAYAMA, K., OKADA, N., FUJITA, T. & YAMAMOTO, A. 2006. Modulation of intestinal P-glycoprotein

function by polyethylene glycols and their derivatives by in vitro transport and in situ absorption studies. *International Journal of Pharmaceutics,* 313**,** 49-56.
SHIMIZU, T., SUGIURA, T., WAKAYAMA, T., KIJIMA, A., NAKAMICHI, N., ISEKI, S., SILVER, D. L. & KATO, Y. 2011. PDZK1 Regulates Breast Cancer Resistance Protein in Small Intestine. *Drug Metabolism and Disposition,* 39**,** 2148-2154.
SHUGARTS, S. & BENET, L. Z. 2009. The Role of Transporters in the Pharmacokinetics of Orally Administered Drugs. *Pharmaceutical Research,* 26**,** 2039-2054.
SIARHEYEVA, A., LOPEZ, J. J. & GLAUBITZ, C. 2006. Localization of multidrug transporter substrates within model membranes. *Biochemistry,* 45**,** 6203-11.
SIARHEYEVA, J. J. L., CLEMENS GLAUBITZ 2006. Localization of Multidrug Transporter Substrates within Model Membranes. *Biochemistry,* 45**,** 6203-6211.
SOMOGYI, A. & GUGLER, R. 1983. Clinical pharmacokinetics of cimetidine. *Clin Pharmacokinet,* 8**,** 463-95.
SONG, N. N., LI, Q. S. & LIU, C. X. 2006. Intestinal permeability of metformin using single-pass intestinal perfusion in rats. *World Journal of Gastroenterology,* 12**,** 4064-4070.
SOTO, E., SHOJI, S., MUTO, C., TOMONO, Y. & MARSHALL, S. 2014. Population pharmacokinetics of ampicillin and sulbactam in patients with community-acquired pneumonia: evaluation of the impact of renal impairment. *Br J Clin Pharmacol,* 77**,** 509-21.
SRIMAROENG, C., PERRY, J. L. & PRITCHARD, J. B. 2008. Physiology, structure, and regulation of the cloned organic anion transporters. *Xenobiotica,* 38**,** 889-935.
SUZUKI, T., ZHAO, Y. L., NADAI, M., NARUHASHI, K., SHIMIZU, A., TAKAGI, K., TAKAGI, K. & HASEGAWA, T. 2006. Gender-related differences in expression and function of hepatic P-glycoprotein and multidrug resistance-associated protein (Mrp2) in rats. *Life Sci,* 79**,** 455-61.
URAKAMI, Y., NAKAMURA, N., TAKAHASHI, K., OKUDA, M., SAITO, H., HASHIMOTO, Y. & INUI, K. 1999. Gender differences in expression of organic cation transporter OCT2 rat kidney. *Febs Letters,* 461**,** 339-342.
VAN HECKEN, A. M., TJANDRAMAGA, T. B., MULLIE, A., VERBESSELT, R. & DE SCHEPPER, P. J. 1982. Ranitidine: single dose pharmacokinetics and absolute bioavailability in man. *Br J Clin Pharmacol,* 14**,** 195-200.
VAN MONTFOORT, J. E., MULLER, M., GROOTHUIS, G. M., MEIJER, D. K., KOEPSELL, H. & MEIER, P. J. 2001. Comparison of "type I" and "type II" organic cation transport by organic cation transporters and organic anion-transporting polypeptides. *J Pharmacol Exp Ther,* 298**,** 110-5.
VANWERT, A. L., GIONFRIDDO, M. R. & SWEET, D. H. 2010. Organic Anion Transporters: Discovery, Pharmacology, Regulation and Roles in Pathophysiology. *Biopharmaceutics & Drug Disposition,* 31**,** 1-71.
WANG, D. S., JONKER, J. W., KATO, Y., KUSUHARA, H., SCHINKEL, A. H. & SUGIYAMA, Y. 2002. Involvement of organic cation transporter 1 in hepatic and intestinal distribution of metformin. *J Pharmacol Exp Ther,* 302**,** 510-5.
WANG, S. W., MONAGLE, J., MCNULTY, C., PUTNAM, D. & CHEN, H. M. 2004. Determination of P-glycoprotein inhibition by excipients and their combinations using

an integrated high-throughput process. *Journal of Pharmaceutical Sciences,* 93**,** 2755-2767.

ZAMBER, C. P., LAMBA, J. K., YASUDA, K., FARNUM, J., THUMMEL, K., SCHUETZ, J. D. & SCHUETZ, E. G. 2003. Natural allelic variants of breast cancer resistance protein (BCRP) and their relationship to BCRP expression in human intestine. *Pharmacogenetics,* 13**,** 19-28.

ZHOU, M. Y., XIA, L. & WANG, J. A. 2007. Metformin transport by a newly cloned proton-stimulated organic cation transporter (plasma membrane monoamine transporter) expressed in human intestine. *Drug Metabolism and Disposition,* 35**,** 1956-1962.

Chapter 6 - In vivo inhibition of P-glycoprotein enhances the bioavailability of ranitidine: a mechanistic study

ABSTRACT

The pharmaceutical excipient, polyethylene glycol 400 (PEG 400), unexpectedly alters the bioavailability of the BCS class III drug ranitidine in a sex-dependent manner. As ranitidine is a substrate for the efflux transporter P-glycoprotein (P-gp), we hypothesized that the sex-related influence could be due to interactions between PEG 400 and P-gp. In this study, we tested this hypothesis.

PEG 400 increases the oral bioavailability of ranitidine in male but not female rats; Verapamil increases the oral bioavailability of ranitidine in both sexes. However, the increase is much higher in male (+252%) than in female rats (+140%); PEG 400 does not affect the oral bioavailability of ranitidine in the sexes if P-gp is blocked by verapamil; A double plasma concentration peak is observed when ranitidine is administered orally in both sexes but disappears if ranitidine is given intravenously; If ranitidine is administered intravenously the route of administration of PEG 400 (oral or IV) does not bear an impact on the oral bioavailability of ranitidine.

These results prove the hypothesis that the sex-specific effect of PEG 400 on the bioavailability of certain drugs is due to the interaction of PEG 400 with the efflux transporter P-gp.

1. INTRODUCTION

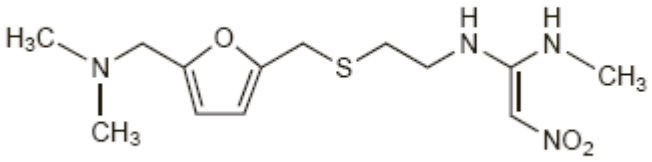
Polyethylene glycol 400 (PEG 400) is widely known as an osmotically active excipient. By retaining water in the gastrointestinal (GI) lumen it exerts a laxative effect, reducing GI transit time, thus reducing the oral bioavailability of some active substances (Basit et al., 2001, Basit et al., 2002, Schulze et al., 2003, Schulze et al., 2005). However, PEG 400, in small amounts, is capable of having the opposite effect: it was found that PEG 400 significantly increased the oral bioavailability of ranitidine (Ashiru et al., 2008), cimetidine and ampicillin in males but not females (Afonso-Pereira et al., 2017a, Afonso-Pereira et al., 2016, Afonso-Pereira et al., 2017b). Metformin, on the other hand, was not affected by dosing with PEG 400 in either sex (Afonso-Pereira et al., 2017b). Ranitidine, cimetidine and ampicillin are mainly absorbed via the paracellular route and are subjected to secretive transport at the intestinal lumen by P-glycoprotein (P-gp) (Afonso-Pereira et al., 2017b). Whereas metformin is not a P-gp substrate and its oral bioavailability is not affected by PEG 400. This led us to hypothesise that PEG 400 could be inhibiting intestinal P-gp, reducing the amount of the active substances that would be secreted back into the lumen, improving drug absorption.

It is important to have a mechanistic understanding of the oral bioavailability enhancing effect of PEG 400 of certain drugs. If intestinal transporters are shown to be influenced by excipients, such that the bioavailability of certain drugs can be modified in a sex dependent manner, formulators, pharmaceutical industries and regulatory authorities may need to revise their approach to excipients. To better understand the forces at play a model drug, ranitidine, was chosen. The wistar rat animal model was previously established (Afonso-Pereira et al., 2016) and a P-gp inhibitor, verapamil, was selected.

Ranitidine

Ranitidine (Figure 1) was first authorised in the UK in 1981 as oral tablets and in 1993 as an oral syrup. Ranitidine is an antagonist of the H-2 histamine receptors and inhibits the gastric secretion of acid, increasing the pH thus ameliorating the symptoms and causes of pathologies caused by gastric acid, such as ulcers and gastric oesophageal reflux (Medicines & Healthcare products Regulatory Agency, 2017).

Figure 1. Structural formula of ranitidine



Ranitidine targets parietal cells in the gastric mucosa inhibiting secretion of gastric acid without any significant H1-antagonist, anticholinergic or ß-adrenergic activity. Ranitidine has a relatively long duration of action supressing gastric acid secretion for up to twelve hours (van Hecken et al., 1982, Richards, 1983, Garg et al., 1983, Miller, 1984, Lipsy et al., 1990).

Ranitidine is absorbed from the small intestine, specifically from the rat terminal ileum (Suttle and Brouwer, 1995, Mummaneni and Dressman, 1994, Eddershaw et al., 1996) The maximum plasma concentration after an oral dose has been reported in rats to be 1-2 hours (Siew et al., 2012) however a second peak is frequently observed around 5-6h (Schaiquevich et al., 2002).

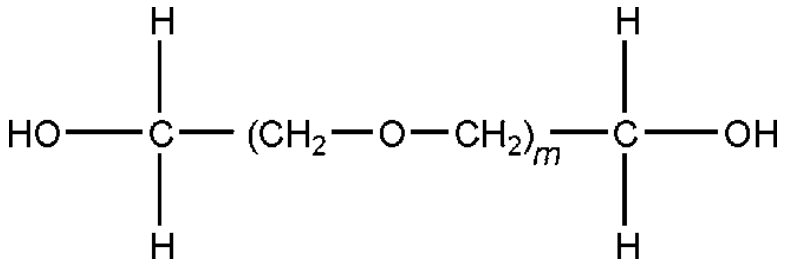
The second plasma peak has been attributed to the separation of the drug mass into multiple boluses and regional differences in uptake rate (Suttle and Brouwer, 1994, Suttle and Brouwer, 1995). Uptake tends to be greatest in the ileum, similar in duodenum and jejunum, and lowest in the colon (Mummaneni and Dressman, 1994). Ranitidine has an oral bioavailability of 60% and 25% of the orally administered dose is excreted unchanged in urine over 24 hours (van Hecken et al., 1982, Miller, 1984). The paracellular route is responsible for 60% of ranitidine bioavailability with 40% being influenced by intestinal membrane transporters (Bourdet et al., 2006). A considerable amount of ranitidine is secreted into the luminal environment, mainly in the small intestine (Collett et al., 1999). This secretion has been found to be possibly related to P-gp (Lee et al., 2002).

PEG 400

The USP32–NF27 describes polyethylene glycol as being an addition polymer of ethylene oxide and water. Polyethylene glycol grades 200–600 are liquids; grades 1000 and above are solids at ambient temperatures. Liquid grades (PEG 200–600) occur as clear, colourless or slightly yellow-coloured, viscous liquids. They have a slight but characteristic odour and a bitter, slightly burning taste (Rowe et al., 2006). Liquid PEGs are water soluble and hygroscopic (Europarat, 2016).

Polyethylene glycols are used in a variety of pharmaceutical formulations, including parenteral, topical, ophthalmic, oral, and rectal preparations (Mohl and Winter, 2004). PEGs are considered non-toxic and non-irritant (Smyth et al., 1950, Smyth et al., 1955). However the WHO recommends a maximum daily dose of 10 mg/Kg (Joint et al.).

Figure 2. Structural formula of Polyethylene glycol; m – average number of oxyethylene groups. For PEG 400, m= 8.7



PEGs have been known to inhibit P-gp in a concentration-dependent manner from 0.1 to 20% (w/v) (Hugger et al., 2002, Shen et al., 2006). PEG 400 at concentrations of 1% (w/v) and 2% (w/v) is capable of blocking P-gp in a caco-2 cell line model (Hodaei et al., 2015).The presence of PEG at 1%, 5%, and 20% (w/v) reduced efflux of digoxin (by 47%, 57%, and 64%, respectively, when compared to control (Johnson et al., 2002). Also, the P-gp inhibitor

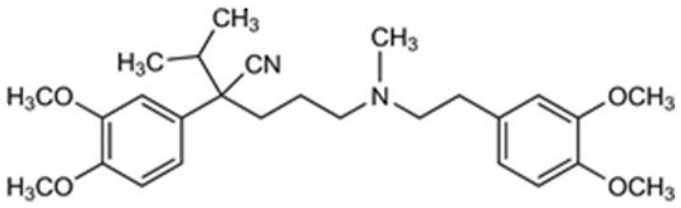
cyclosporine A, when orally administered to male and female rats led to an increase of the bioavailability of ranitidine and ampicillin but not metformin (Mai et al., 2017).

Polymers are hypothesised to be able to inhibit membrane transport by the following mechanisms: inhibition by ATP depletion, by binding to ATP for example; inhibition mediated by interactions with the cell membrane, destabilising the cellular membrane may render the transporters ineffective; inhibition by the formation of a drug-polymer conjugates; inhibition mediated by interfering with ATP-binding sites and blocking of drug binding sites or other sites within the trans-membrane domains (Werle, 2008). It may be that PEG 400 inhibits P-gp by a combination of these mechanisms. It is plausible to consider some degree of steric hindering, membrane interactions and even some degree of binding to one of the active sites of P-gp.

Verapamil

Verapamil, Figure 3, is a phenylalkylamine calcium-channel blocker. Approximately 90% of verapamil oral dose is absorbed from the gastrointestinal tract. However it is extensively metabolised in the liver (Manitpisitkul and Chiou, 1993).

Figure 3. Structural formula of Verapamil



Verapamil has bi- or tri-phasic elimination kinetics and is reported to have a terminal plasma half-life of 2 to 8 hours after a single oral dose or after intravenous dosage. Verapamil acts within 5 minutes when given intravenously and within 1 to 2 hours when given orally; peak plasma concentrations occur 1 to 2 hours after an oral dose. There is considerable interindividual variation in plasma concentrations (Kelly and O'Malley, 1992).

Verapamil is about 90% bound to plasma proteins. It is extensively metabolised in the liver to at least 12 metabolites of which norverapamil has been shown to be active. About 70% of a dose is excreted by the kidneys in the form of its metabolites but about 16% is excreted in the bile into the faeces. Less than 4% is excreted unchanged. Verapamil crosses the placenta and is distributed into breast milk (Tracy et al., 2001, Kelly and O'Malley, 1992). In rats it was found that the pharmacokinetic profile of verapamil was similar to humans. Rats had a shorter half-life and a faster elimination than the humans (Manitpisitkul and Chiou, 1993).

P-glycoprotein (P-gp)

P-gp is codified by the MDR1 gene (ABCB1 gene) and is a member of the ATP-binding cassette (ABC) family of efflux transporters (Estudante et al., 2013, Mizuno et al., 2003).

There are several hypothesis to explain how P-gp is capable of effluxing substrates (Figure 4). These are mainly divided in direct and indirect transport. The indirect transport mechanism advocates that P-gp, instead of transporting the substrates themselves, reduces drug concentration indirectly by altering the cell's electrical membrane potential, by changing cellular pH or even by transporting drug molecules out of the cell via an ATP electrochemical gradient. These hypothesis are unlikely to explain the high level of resistance observed in MDR cell lines, and most of the experimental data support a direct mechanism of drug transport by P-glycoprotein (Germann and Chambers, 1998). According to an extensive literature review conducted by Ursula Germann, P-glycoprotein has been shown to directly bind drug analogues; the activity of P-gp has been described as similar to that of known active transporters against a concentration gradient and has even been identified as an active transporter (Germann and Chambers, 1998).

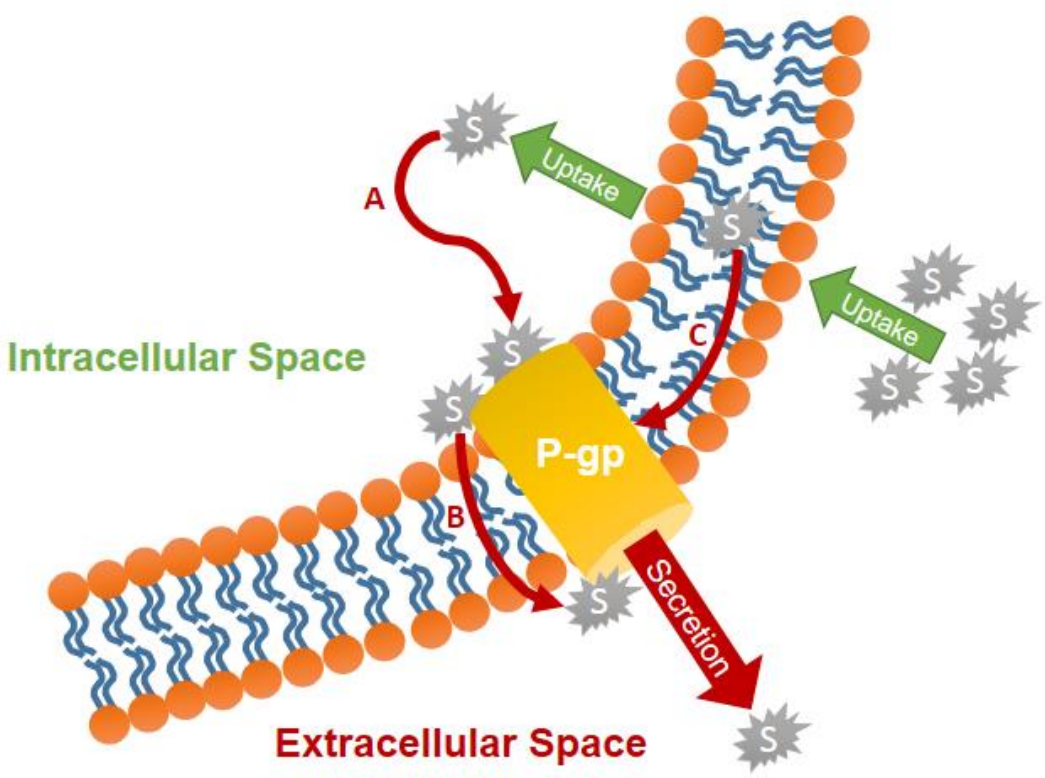
The most common hypothesis for the mechanism of action of P-gp are as described in Figure 4:

- Formation of hydrophobic channel (pore) between the intracellular and extracellular space where compounds can exit the cell.

- Flippase activity – the drug is flipped from the inner leaflet to the outer leaflet of the cell membrane. After reaching the outer leaflet, substrates would then either passively diffuse into the extracellular aqueous phase (a very fast process), or move back to the inner leaflet by spontaneous flip–flop. In order to maintain a substrate concentration gradient across the membrane, the rate of passive transbilayer flip–flop of substrate would need to be slower than the rate of P-gp mediated flipping, so that its concentration remains higher in the outer leaflet (Sharom, 2014). As a conclusion the flippase model involves delivery of drug to the outer leaflet, followed by rapid partitioning into the extracellular medium.

- "Vacuum cleaner model" in which drug interacts with P-gp in the lipid bilayer and is subsequently secreted back into the extracellular space. P-gp may interact with its substrates within the membrane and subsequently efflux them to the extracellular medium (Higgins and Gottesman, 1992). In the vacuum cleaner model, drug is delivered to the extracellular medium, followed by rapid partitioning into the outer leaflet.

Figure 4. Proposed mechanisms by which P-gp secretes substrates. Passive drug uptake across cell membrane. (A) Formation of hydrophobic channel (pore) between the intracellular and extracellular space. (B) Flippase activity the drug is flipped from the inner leaflet to the outer leaflet of the cell membrane. (C) "Vacuum cleaner model" in which drug interacts with P-gp in the lipid bilayer and is subsequently secreted back into the extracellular space.



P-glycoprotein can be found in the membranes of different cells that can be considered as a shield for external substances by actively pushing substances and drugs out of cells (Mizuno et al., 2003, Lin and Yamazaki, 2003). It is expressed in the luminal membrane of the small intestine (Figure 5) and blood–brain barrier, and in the apical membranes of excretory cells such as hepatocytes (Figure 6) and kidney proximal tubule epithelia (Figure 7) (Giacomini et al., 2010). As a result, it can have an impact on the extent of drug absorption (in the intestine), distribution (to the brain, lymphocytes, testes, or placenta) and elimination (in the urine and bile). By ejecting already absorbed drug molecules back into the intestinal lumen P-gp in the enterocyte can reduce the extent of absorption. P-gp is also usually over expressed in some cancer cells making them resistant to antitumor drugs (Germann and Chambers, 1998).

Figure 5. Schematic of role of P-gp intestinal disposition of substrate. (S)P-gp substrate; (A) Absorption of P-gp substrate from intestinal lumen into enterocyte and into the circulation. (3M) Metabolism of substrate in the enterocyte. (E) Secretion of substrate back into the intestinal lumen facilitated by P-gp.

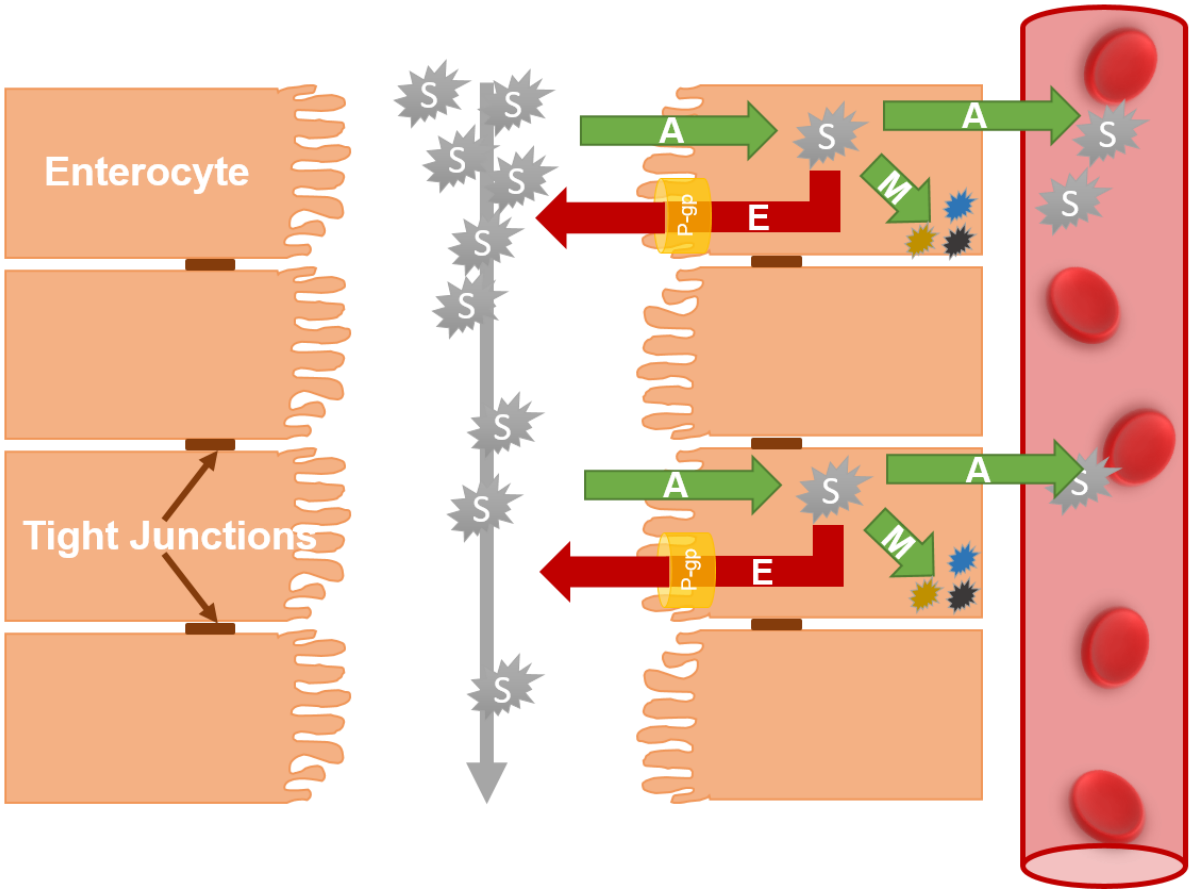


Figure 6. Schematic of role of P-gp in hepatobiliary disposition of substrate. (1) Uptake of P-gp substrate from blood into hepatocyte. (2) Subsequent metabolism, sequestration, or trafficking of substrate. (3) Secretion into bile by P-gp.

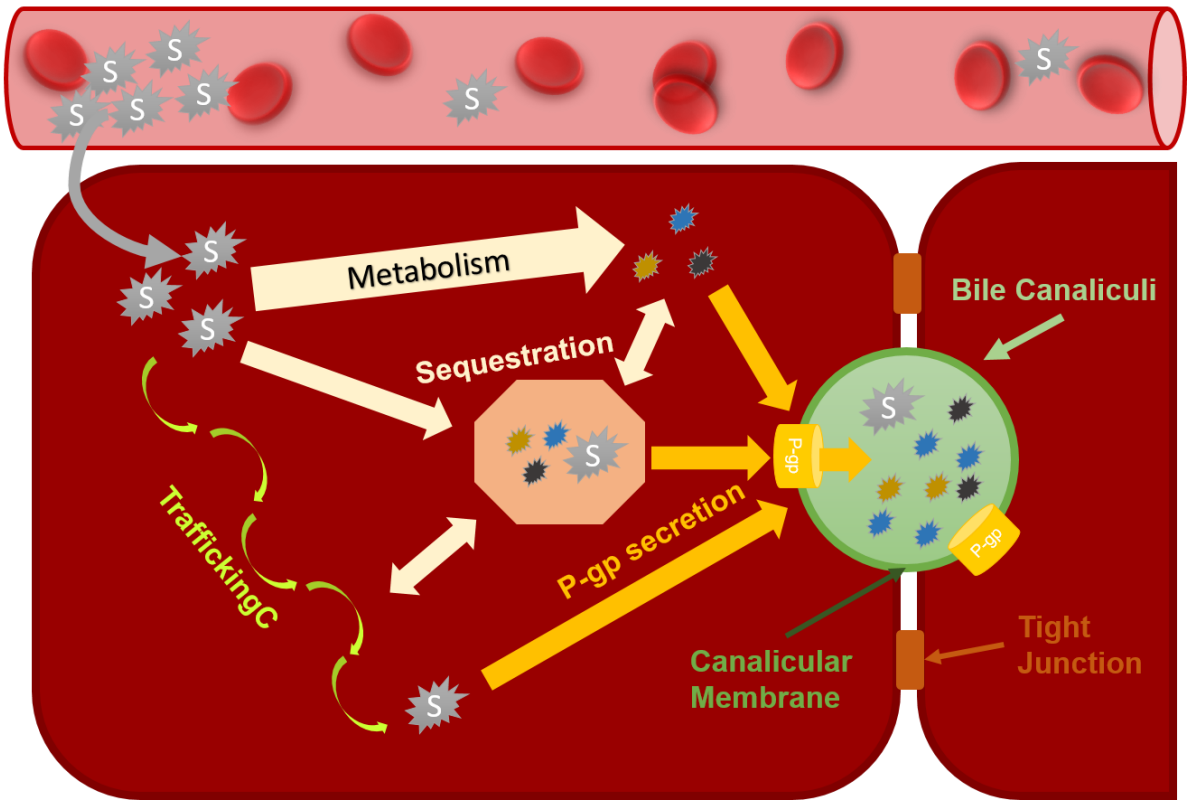
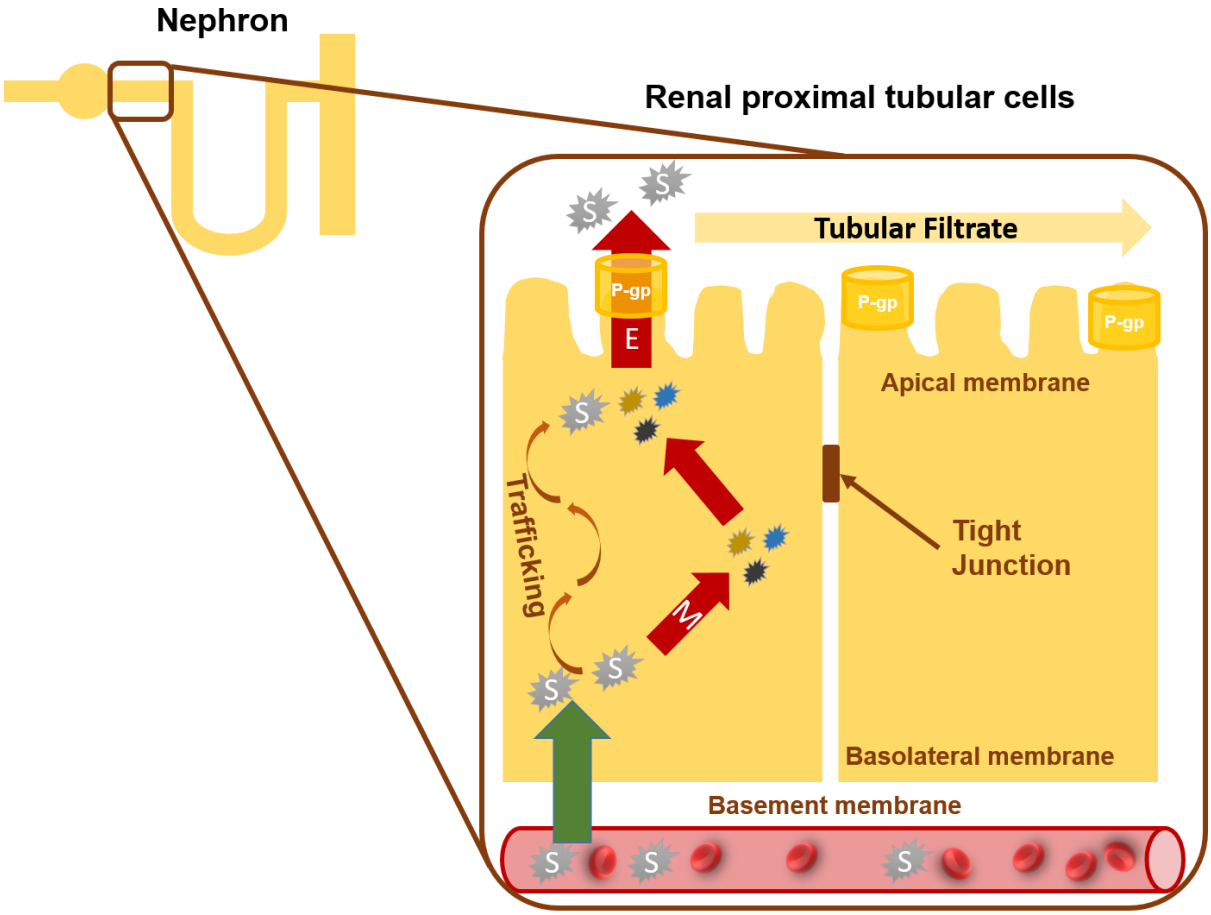


Figure 7. Schematic of role of P-gp in hepatobiliary disposition of substrate. (1) Uptake of P-gp substrate from blood into hepatocyte. M – Metabolism E – Excretion (2) Subsequent metabolism, sequestration, or trafficking of substrate. (3) Secretion into urine by P-gp.



According to the previous findings, if P-gp is inhibited in the gut it could maximise the amount of active substances that are absorbed. As such, a well-established in-vivo P-gp inhibitor was required. Verapamil was chosen as a positive control.

Aims

The aims of this work are:

- To understand if the P-gp inhibitor, verapamil, affects the oral bioavailability of ranitidine in a similar manner to Peg 400. With this experiment it is also possible to register if there are sex differences in the effect of verapamil.
- To understand if food plays a role in the bioavailability enhancing effect of PEG 400 in male rats.
- To determine the absolute bioavailability of ranitidine in male and female rats. Male and female rats were administered ranitidine intravenously (IV).
- To understand if PEG 400 exerts its action by influencing drug absorption, metabolism and/or excretion of ranitidine.

2. MATERIALS AND METHODS

2.1 Materials and animals

Ranitidine and polyethylene glycol 400, glacial acetic acid, sodium acetate trihydrate and sodium chloride were obtained from Sigma Aldrich (Dorset UK). The following HPLC grade solvents, water and acetonitrile were purchased from Fisher Scientific (Loughborough, UK). Male and female Wistar rats (8 weeks old) were purchased from Harlan UK Ltd (Oxfordshire, UK).

2.2 Animal procedures

The animal work was conducted according to the provisions of The Animals (Scientific Procedures) Act 1986 under PPL 70/7421 and PIL 70/24575.

All the rats were keep at a room temperature of 25°C and on a light-dark cycle of 12h. The rats were caged in a group, allowed to move freely and provided with food and water before the experiment. Fasted rats: the rats were fasted for twelve hours in metabolic cages prior to the start of the experiment; food was made available four hours after dosing of the animals. Fed rats: rats had free access to food and water.

All the animals were dosed with 50 mg/kg of ranitidine. The route of administration was either oral (oral gavage) or IV (tail vein). Some animals were coadminestered 40 mg/kg of verapamil via the oral route and some others were coadminestered with 26 mg/kg of PEG 400 via the oral route (gavage) or IV (tail vein). The vehicle for the oral doses was water and the vehicle for the IV doses was 0.9% NaCl. The dose of ranitidine was chosen based on previous work (Afonso-Pereira et al., 2016, Eddershaw et al., 1996), and on the quantification capabilities of the method used. The dose of PEG 400 was selected as the dose that lead to a higher increase in the bioavailability of ranitidine (Afonso-Pereira et al., 2016) and cimetidine in previous work.

The dosing regimen and route of administration of the test products can be found in Table 1.

Table 1. Grouping and treatment of animals where the amount of ranitidine is quantified in the plasma

Group	***Sex***	***Number of rats***	***Fasted/Fed***	***Ranitidine 50 mg/kg***	***PEG 400 26 mg/kg***	***Verapamil 40 mg/kg***
A (Oral Control)	Male	6	Fasted	Oral	-	-
B	Male	6	Fasted	Oral	Oral	-
C	Male	5	Fasted	Oral	-	Oral
D	Male	4	Fasted	Oral	Oral	Oral
E	Male	5	Fed	Oral	-	-
F	Male	4	Fed	Oral	Oral	-
G	Male	6	Fasted	Oral	I.V.	-
H (I.V. Control)	Male	4	Fasted	I.V.	-	-
I	Male	5	Fasted	I.V.	Oral	-
J	Male	5	Fasted	I.V.	I.V.	-
K (Oral Control)	Female	6	Fasted	Oral	-	-
L	Female	5	Fasted	Oral	Oral	-
M	Female	5	Fasted	Oral	-	Oral
N	Female	5	Fasted	Oral	Oral	Oral
O (I.V. Control)	Female	5	Fasted	I.V.	-	-

During the animal experiments there protocol deviations that are highlighted in Table 2. Some of the animals either aspirated the oral solutions or, after dosing and prior to the first blood sampling, showed signs of cardiac distress and had to be culled. As a result, the number of animals in each experiment is not the same. Please note that some experiments were designed for n = 5 while others for n = 6. This difference was due to limitations in the total number of animals we were allowed to use.

Table 2. Protocol deviations to the experiments

Group	***Protocol n***	***Experimental n***	***Reason Comment***
D	5	4	Aspirated oral solution – 1 animal culled
F	5	4	Aspirated oral solution – 1 animal culled
H	5	4	Cardiac distress – 1 animal culled
I	6	5	Aspirated oral solution – 1 animal culled
J	6	5	Cardiac distress – 1 animal culled
L	6	5	Aspirated oral solution – 1 animal culled
O	6	5	Cardiac distress – 1 animal culled

After dosing, the rats were placed back in individual metabolic cages and were allowed to move freely.

Plasma Study

Approximately 200 µL of blood were collected from the tail vein into anticoagulant centrifuge tubes (BD Microtainer® K2E Becton, Dickinson and Company, USA) at the following time points: 30 min, 1.25 h, 2 h, 3 h, 4 h, and 6 h. In between sampling, the rats were placed back in the metabolic cages. Blood volumes were taken in accordance with the project license and were stored on ice until the last collection point. After 8 h, the animals were sacrificed in a CO_2 euthanasia chamber (Schedule 1 method), and approximately 2 mL of blood were obtained via cardiac puncture. Blood samples were centrifuged at 10000 rpm (930 g) for 10 min on a Centrifuge 5804R (Eppendorf AG, 22331 Hamburg, Germany) within 8h of sampling. 50 µL of the supernatant (plasma) was collected and placed into a 1.5 mL Eppendorf tube, and immediately frozen at -20°C prior to analysis.

2.3 Sample analysis

Plasma samples were thawed and assayed.

50µL of thawed plasma was mixed with the same volume of acetonitrile in order to precipitate the plasma proteins. After 1 min of vortex-mixing, a further 100µL of HPLC grade water was added to the mixture, and after subsequent vortex-mixing, the samples were centrifuged at 4ºC for 10 min at 10000 rpm.

HPLC analysis

The resulting supernatants (from the plasma and urine samples) were subjected to HPLC-UV analysis using a previously validated method (Ashiru et al., 2007): column 5µm Luna SCX (Phenomenex, UK); mobile phase 20:80 (acetonitrile: 0.1 M sodium acetate pH=5.0); flow rate 2 mL/min; injection volume 40 µL; detection at 320 nm.

Calibration standards were prepared with blank rat plasma and urine samples spiked with drug subjected to the above-mentioned treatment.

The plasma concentration *versus* time profiles were produced for each animal. C_{max} and t_{max} were taken from these profiles. The cumulative area under the plasma concentration versus time curve ($AUC_{0\text{-}480}$) was calculated using the integration method with OriginPro 9.0 (OriginLab, Northampton, MA, USA).

2.4 Statistical analysis

The overall data was analysed by one-way ANOVA, followed by a Tukey post-hoc analysis and by individual t-student comparisons between individual groups and the appropriate controls, with IBM SPSS Statistics 19 (SPSS Inc., Illinois, USA).

3. RESULTS

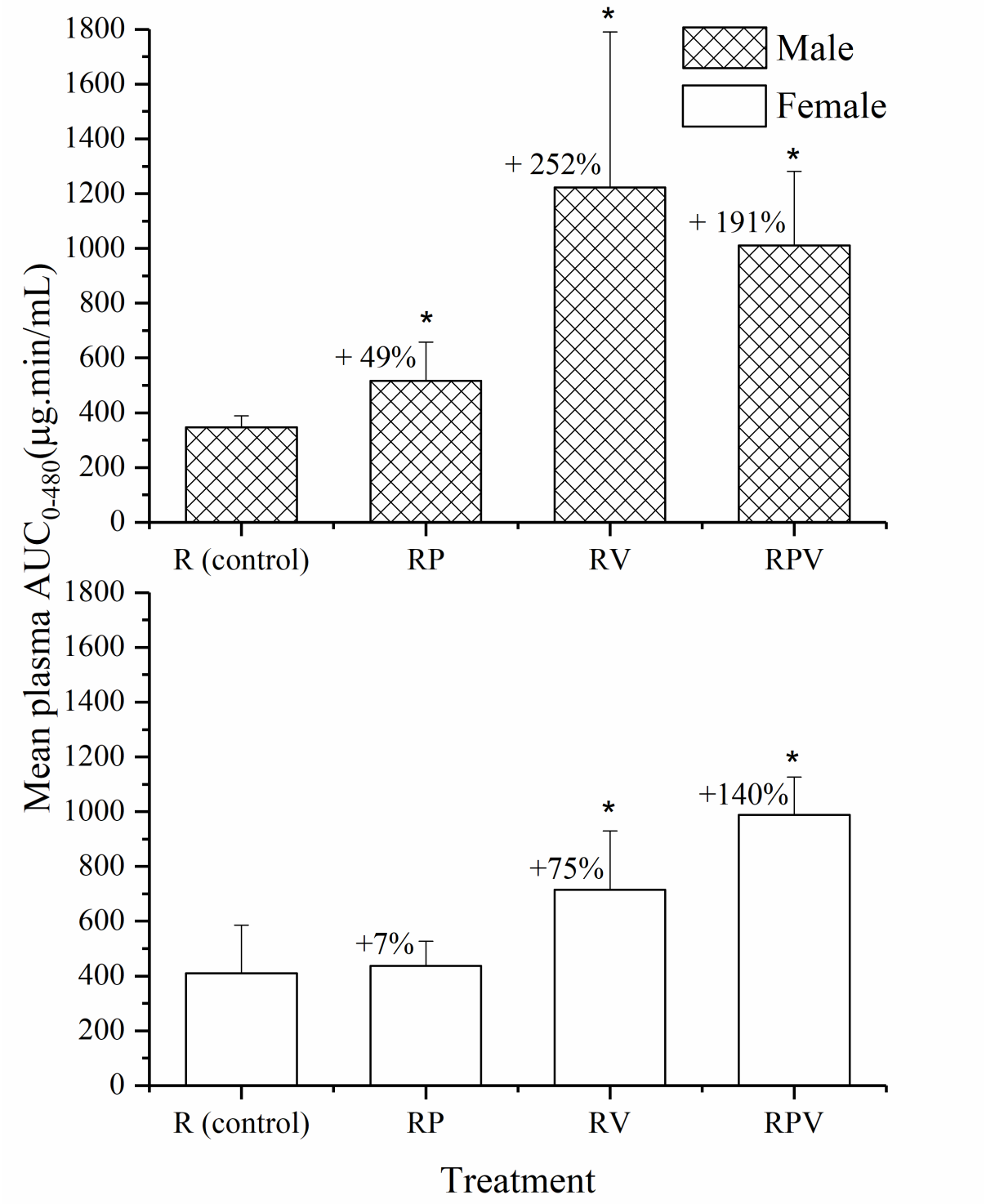
A summary of the results can be found on Table 3.

Table 3. The effects of sex, food, verapamil and the route of administration of PEG 400 and ranitidine in the mean $AUC_{0\text{-}480}$ of ranitidine in Wistar rats; control groups are highlighted in grey. *A* is the control group for male rats and oral ranitidine; *H* is the control group for male rats and IV ranitidine; *K* is the control group for male rats and oral ranitidine; *O* is the control group for male rats and IV ranitidine.

Sex		*Group*	*Treatment*	*Mean $AUC_{0\text{-}480}$ ± SD (µg.min/mL)*	*Change in mean AUC between test and control groups (%)*
♂	Fed	*E*	Ranitidine – Oral	173.7 ± 53.9	-50.2 % *
		F	Ranitidine – Oral PEG 400 – Oral	170.2 ± 34.7	-51.0 % *
	Fasted	*A*	Ranitidine – Oral	347.6 ± 42.5	-
		B	Ranitidine – Oral PEG 400 – Oral	517.6 ± 140.3	+ 48.9 % *
		C	Ranitidine – Oral Verapamil – Oral	1222.5 ± 568.3	+ 251.7 % *
		D	Ranitidine – Oral PEG 400 – Oral Verapamil – Oral	1011.7 ± 270.9	+ 191.0 % *
		G	Ranitidine – Oral PEG 400 – IV	448.6 ± 95.1	+ 29.1 %
		H	Ranitidine - IV	1505.2 ± 524.2	-
		J	Ranitidine – IV PEG 400 – IV	1314.8 ± 475.8	- 12.6 %
		I	Ranitidine – IV PEG 400 – Oral	1514.0 ± 469.3	+ 0.6 %
♀	Fasted	*K*	Ranitidine – Oral	409.8 ± 175.4	-
		L	Ranitidine – Oral PEG 400 – Oral	437.6 ± 90.3	+ 6.8 %
		M	Ranitidine – Oral Verapamil – Oral	714.9 ± 215.9	+ 74.5 % *
		N	Ranitidine – Oral PEG 400 – Oral Verapamil – Oral	987.6 ± 139.7	+ 140.1 % *
		O	Ranitidine - IV	1025.5 ± 568.0	-

* Indicates statistical difference ($p \leq 0.05$) from the respective control group.

Figure 8. Mean AUC_{0-480} of male and female rats dosed with ranitidine, PEG 400 and verapamil under Fasted conditions. The percentage differences in the mean AUC_{0-480} with the control groups are also represented. *Denotes statistically significant difference compared to the control group ($p<0.05$).

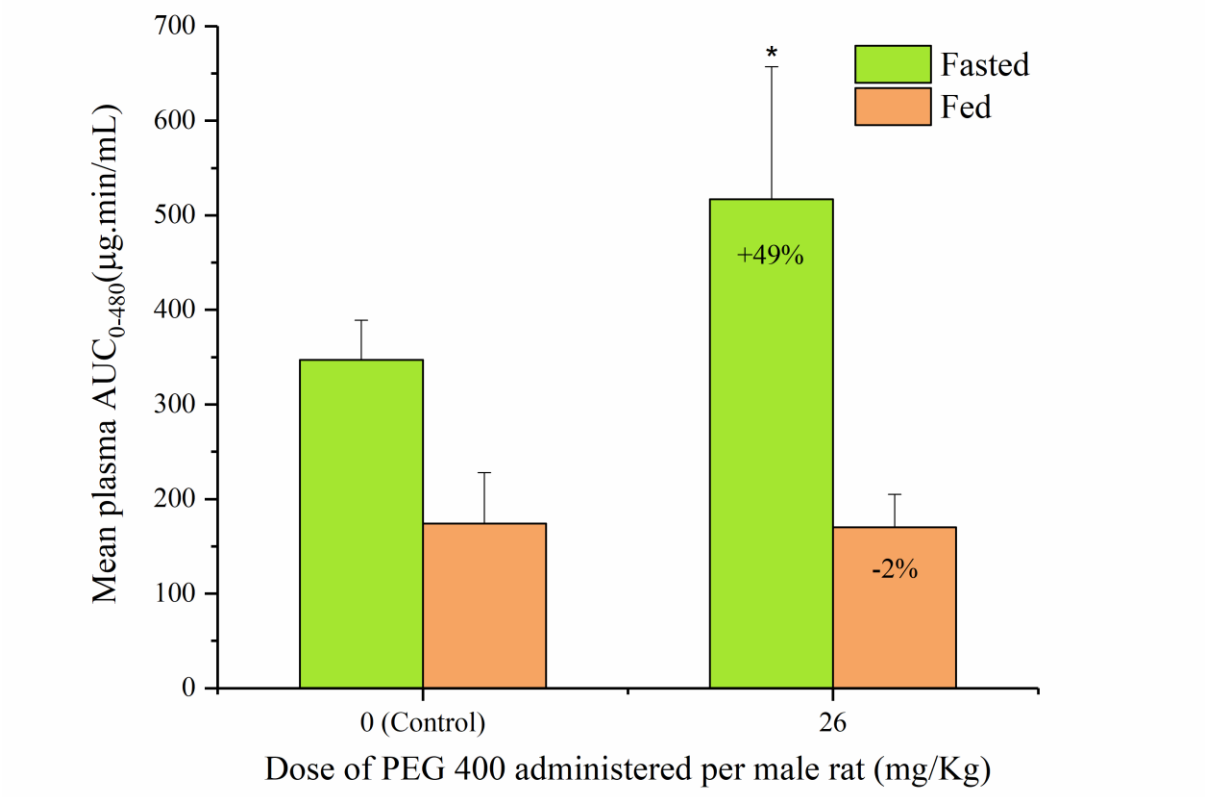


R – Orally administered ranitidine (A & K); RP – orally administered Ranitidine and PEG 400 (B & L)); RV – orally administered ranitidine and verapamil (C & M); RPV – orally administered ranitidine, PEG 400 and verapamil (D & N).

The data found in Figure 8 shows that:

- PEG 400 increases the oral bioavailability of ranitidine in male but not female rats;
- Verapamil increases the oral bioavailability of ranitidine in both sexes. However, the increase is much higher in male (+252%) than in female rats (+140%);
- PEG 400 does not affect the oral bioavailability of ranitidine in the sexes if verapamil is concomitantly administered.

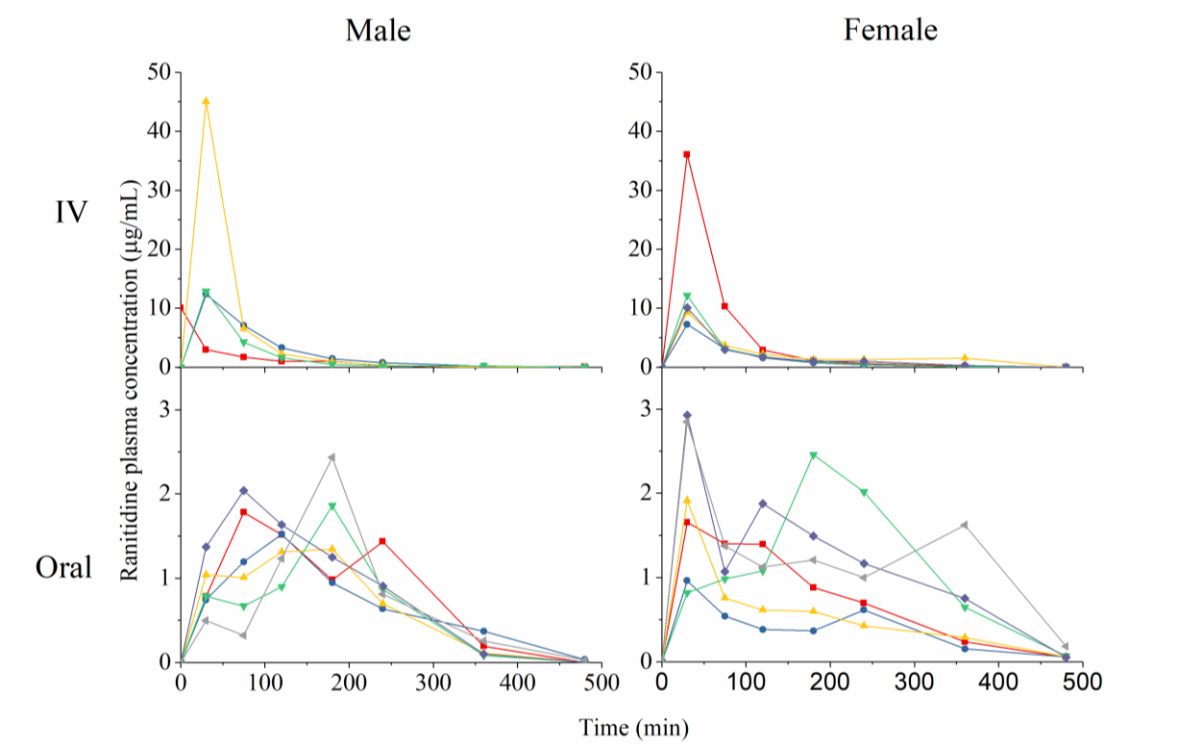
Figure 9. Mean AUC_{0-480} of male rats dosed with ranitidine and ranitidine with PEG 400 under Fasted and Fed conditions. The percentage differences in the mean AUC_{0-480} with the control groups are also represented. *Denotes statistically significant difference compared to the control group ($p<0.05$).



The data found in Figure 9 shows that:

- In male rats, the effect of PEG 400 is nullified by food. While in fasted rats, PEG 400 significantly increases the oral bioavailability of ranitidine, in fed rats the oral bioavailability of ranitidine is not changed by co-administration with PEG 400.

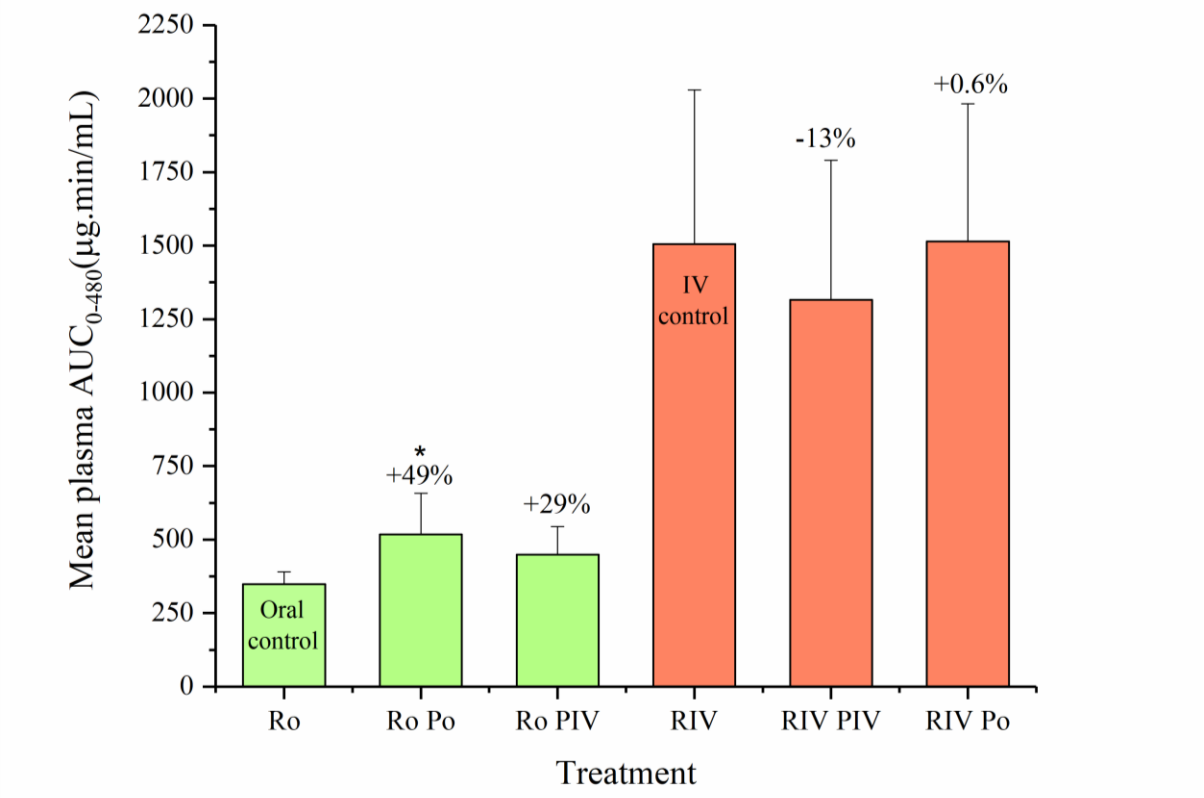
Figure 10. Individual plasma profiles for male and female rats dosed with ranitidine intravenously or orally.



The data found in Figure 10 shows that:

- The oral bioavailability of ranitidine in male and female rats is similar;
- The total bioavailability of ranitidine (defined by the area under the plasma concentration versus time curve) is higher in male than female rats;
- A double plasma concentration peak is observed when ranitidine is administered orally in both sexes;
- The double plasma concentration peak phenomenon is not observed when ranitidine is administered intravenously.

Figure 11. Mean AUC_{0-480} of male rats dosed with ranitidine, verapamil and/or PEG 400 through different routes of administration. The percentage differences in the mean AUC_{0-480} with the control group are also represented.



Ro – ranitidine oral (A); Ro Po – ranitidine oral and PEG 400 oral; PEG 400 (B); Ro PIV – ranitidine oral and PEG 400 IV (G); RIV – ranitidine IV (H); RIV PIV – ranitidine and PEG 400 IV (J); RIV Po – ranitidine IV and PEG 400 oral (I). *Denotes statistically significant difference compared to the control group (only ranitidine was administered) ($p<0.05$).

The data found in Figure 11 shows that:

- Orally administered PEG 400 increases the oral bioavailability of ranitidine
- If PEG 400 is administered intravenously to rats that were orally dosed with ranitidine, the increase in the bioavailability ranitidine is not significant and smaller that when PEG 400 is given orally;
- If ranitidine is administered intravenously the route of administration of PEG 400 (oral or IV) does not bear an impact on the oral bioavailability of ranitidine.

4. DISCUSSION

The P-gp inhibitor, verapamil, causes substantial increases in the oral bioavailability of ranitidine in the male rat. In female rats there is also a significant increase albeit smaller. The overall trend observed previously in the rat (Afonso-Pereira et al., 2017a, Mai et al., 2017, Afonso-Pereira et al., 2017b, Afonso-Pereira et al., 2016) where PEG 400 increases the oral bioavailability of ranitidine in male but not female rats is similar to that of verapamil. The difference is that PEG 400 does not seem to affect female rats whereas verapamil does. However it is also noted that verapamil leads to much higher increases in the oral bioavailability of ranitidine than PEG 400. It is possible that verapamil, by having a much stronger effect, also causes the oral bioavailability of ranitidine to increase in females.

It is known that ranitidine is a substrate for the efflux transporter P-glycoprotein (P-gp) (Cook and Hirst, 1994, Collett et al., 1999). It is also known that PEG 400 is capable of inhibiting P-gp in a concentration-dependent manner (Hugger et al., 2002, Shen et al., 2006). PEG 400 was capable of blocking P-gp in a caco-2 cell line model (Hodaei et al., 2015) and reduced the efflux of digoxin (Johnson et al., 2002). P-gp is located in several organs such as the small intestine (Figure 3), liver (Figure 4) and kidney (Figure 5). An active P-gp will reduce the bioavailability of ranitidine by reducing its absorption, increasing its uptake to the liver; and increasing its tubular secretion in the kidney. However, if P-gp was to be inhibited across the body the intestinal uptake would increase and the liver and kidney uptake would decrease. This would increase the active substance residence time in the blood, thus increasing bioavailability.

From previous studies (Mai et al., 2017, Afonso-Pereira et al., 2017b) it was found that the likely cause for the increase of ranitidine oral bioavailability in the presence of low doses of PEG 400 was related to the inhibition of P-gp. The increase in the bioavailability of certain drugs due to the potent reversible P-gp inhibition by verapamil was observed in irinotecan (Bansal et al., 2009), etoposid (Piao et al., 2008) and digoxin (Collett et al., 2005). Also, the P-gp inhibitor cyclosporine A, when orally administered to male and female rats led to an increase of the oral bioavailability of ranitidine and ampicillin but not metformin. Males had an increase of over 100% in the $AUC_{0\text{-}480}$ in the cyclosporine A treated animals when compared to the control (Mai et al., 2017). It is important to note that both ranitidine and ampicillin are P-gp substrates whereas metformin is not.

It is safe to assume that the inhibition of P-gp increases the oral bioavailability of ranitidine; more so in males than females. This is further confirmed by the fact that, once P-gp is blocked by verapamil, PEG 400 has no effect on the oral bioavailability of ranitidine.

In male rats, the effect of PEG 400 is nullified by food (Figure 9). While in fasted rats PEG 400 significantly increases the oral bioavailability of ranitidine, in fed rats the oral bioavailability of ranitidine is not changed by co-administration with PEG 400. Several foodstuff interactions with P-gp have been reported (Deferme and Augustijns, 2003). Possible mechanisms of action include down regulation of intestinal P-gp expression, competitive inhibition with other P-gp substrates and alteration of the membrane fluidity. Some foods

induce whereas some other inhibit P-gp. In the rats a decrease close to 50% in the oral bioavailability of ranitidine is observed in male fed rats when compared to fasted ones. The fact that PEG 400 did not seem change the oral bioavailability of ranitidine in the fed animals leads us to think that the decrease in the overall bioavailability was related to the fact that food delayed the absorption of ranitidine from the intestine, limiting the total amount of drug that was absorbed. We believe this effect is not related to transporter effects.

Orally administered PEG 400 increases the oral bioavailability of ranitidine. However, when PEG 400 is dosed intravenously to rats that were orally dosed with ranitidine, the change in the oral bioavailability of ranitidine was not significant. Furthermore, when ranitidine was administered intravenously the route of administration of PEG 400 (oral or IV) did not bear an impact on the oral bioavailability of ranitidine (Figure 11). This support our hypothesis that PEG 400 in the systemic circulation has little effect in the oral bioavailability of ranitidine. This further suggests that PEG 400 influences the absorption of ranitidine and not its metabolism and excretion.

Another interesting phenomenon that was observed (Figure 10) is the existence of a double plasma concentration peak when ranitidine is administered orally in both sexes. The double plasma concentration peak phenomenon is not observed when ranitidine is administered intravenously. The existence of two plasma peaks for ranitidine may have two main explanations: enterohepatic recycling and a discontinuous absorption in the gut. Enterohepatic recycling can be safely dismissed because following intravenous administration the double peak phenomenon was not observed. This is further supported in literature where similar behaviour was observed (Pedersen and Miller, 1980, Mummaneni and Dressman, 1994). Ranitidine has also been reported to be mainly eliminated unchanged in urine, with hepatic clearance accounting for a very small fraction of its elimination (0.7 – 2.6%) (van Hecken et al., 1982, Richards, 1983, Garg et al., 1983, Miller, 1984, Klotz and Walker, 1990).

Alternatively, the existence of two plasma concentration maxima (Richards, 1983, Miller, 1984) could be explained by a bimodal absorption pattern of the drug in the gut. Regionally differing permeation can be associated with the combination of different transporters (influx and efflux) with regional differences in their expression levels in the small intestine (Kagan et al., 2010, Lindell et al., 2003). It is possible that a heterogeneous distribution of P-gp or other transporters in the gut could lead to "preferred" regions of absorption of certain actives. P-gp was found to increase distally in the rat gut (MacLean et al., 2008). And inhibiting P-gp lead to an increase of fexofenadine absorption, but only distally, where P-gp is more abundant (MacLean et al., 2010). Also, P-gp mediated efflux appears to be a major factor responsible for GI region-specific absorption of talinolol in the rat (Kagan et al., 2010). Studies have identified regional differences in the absorption of ranitidine from the small intestine. Ranitidine was found to be more permeable in the rat's duodenum than in the jejunum (Mummaneni and Dressman, 1994). It is plausible to assume such an effect could be at play for ranitidine, whereby it is more absorbed in the proximal small intestine due to a smaller activity of P-gp.

In this work we have also seen that verapamil increases the oral bioavailability of ranitidine in both sexes (Figure 8). However, the increase is much higher in male (+252%) than in female rats (+140%). Also, PEG 400 does not seem to affect the oral bioavailability of ranitidine in the sexes if verapamil is concomitantly administered. It was also observed that the oral bioavailability of ranitidine, cimetidine and ampicillin was higher in female than male rats (Afonso-Pereira et al., 2017b). Females appear to be less sensitive to P-gp inhibition than males. At least at an intestinal level, where females would be less capable of effluxing ranitidine back to the intestinal lumen and as such would be more prone to absorbing it. In a toxicological study it was found that that female rats were significantly more sensitive to the toxic effects of ranitidine than male rats. It was observed that females possessed significantly lower LD_{50}s than the males (Tamura et al., 1983). This study supports our hypothesis that male rats are more sensitive to P-gp inhibition. If the males have a more active or more efficient P-gp efflux mechanism, they are more capable of withstanding higher doses of the drug with lower adverse effects than the female rats.

The activity and expression of P-gp has been reported to be different in males and females (Mariana et al., 2011). In humans, it has been reported that enteric sex differences were present: women had a lower enterocyte P-gp content than men (Potter et al., 2004), but other work suggested that no sex differences were present in the proximal small intestine (Paine et al., 2005). It is known that regional differences in the expression of P-gp may be present. In rats, a scan of the intestinal expression of P-gp found that its expression increased distally in the sexes but no gender specific differences were seen (MacLean et al., 2008). In that study, gene expression was evaluated and not the activity of P-gp. It may be that the relative amount of P-gp is similar in the sexes but that the degree of activity differs. It was also found that testosterone inhibits P-gp (twice as much as progesterone) (Hamilton et al., 2001, Dey et al., 2004). It may be that the amount of P-gp in male and females is the same, but the males are permanently partially inhibited due to circulating testosterone. In our study, we found that PEG 400 increased the bioavailability of ranitidine in male but not in female rats and that verapamil increased the oral bioavailability of ranitidine much more in male than female rats. PEG 400 is a weaker inhibitor of P-gp than verapamil. Also the sex difference may be related to the efficiency of the P-gp mechanism in the gut. Males have a higher sensitivity to the inhibitory effects of PEG 400 over P-gp than females. Regional permeability studies, either *ex vivo* or *in vitro*, would be important to map the permeation of ranitidine in the sexes through the different regions of the small intestine in the presence of PEG 400 and a P-gp inhibitor. Information on potential sex and regional differences would be valuable in targeting drug delivery and formulating modified release products.

5. CONCLUSION

The P-gp inhibitor, verapamil, increases the oral bioavailability of ranitidine in male and female rats. This increase is larger in the males. This trend was also observed with cyclosporine A (P-gp inhibitor) in a separate study (Mai et al., 2017). It is safe to assume that the inhibition of P-gp increases the oral bioavailability of ranitidine; more so in males than females. PEG 400 is known to inhibit P-gp in a concentration dependent manner. And ranitidine is known to be a P-gp substrate. An active P-gp will reduce the bioavailability of ranitidine by reducing its absorption, increasing its uptake to the liver; and increasing its tubular secretion in the kidney. However, inhibiting P-gp would increase the intestinal uptake (absorption) and decrease the liver and kidney uptake (metabolism/excretion); ultimately increasing bioavailability.

The hypothesis that PEG 400 increases the bioavailability of ranitidine by inhibiting P-gp is confirmed by the fact that, once P-gp is blocked by verapamil, PEG 400 has no effect on the oral bioavailability of ranitidine.

The increase in the oral bioavailability of ranitidine mediated by PEG is related to the intestinal absorption of the active substance rather than its metabolism or excretion. When PEG 400 is dosed intravenously the change in the oral bioavailability of ranitidine is not significant. PEG 400 in the systemic circulation has little effect in the oral bioavailability of ranitidine.

Male rats are more sensitive to P-gp inhibition than females and this may explain why the increase in oral bioavailability of ranitidine is greater in males. If the males have a more active or more efficient P-gp efflux function, they will absorb less ranitidine than the females. Conversely, if P-gp is inhibited, the backlash will be larger in males than females.

Differences in P-gp have been reported in the sexes but there is also conflicting literature stating the contrary. It may be that the relative amount of P-gp is similar in the sexes but that the degree of activity differs. Hormones may play a role as it is known that testosterone is a potent P-gp inhibitor.

The double peak observed in the plasma concentration versus time curve of ranitidine is most likely due to regional differences in intestinal absorption. The double peak is only observed when ranitidine is administered orally and not intravenously. Ranitidine has been reported to have preferential sites of absorption in the gut. Also, P-gp has been reported to change along the small intestine.

Regional permeability studies, either *ex vivo* or *in vitro*, would be important to map the permeation of ranitidine in the sexes through the different regions of the small intestine in the presence of PEG 400 and a P-gp inhibitor. Information on potential sex and intestinal regional differences on the absorption of ranitidine would be valuable in targeting drug delivery and formulating modified release products. Excipients can affect the activity of transporters, thereby alter drug bioavailability, therapeutic efficacy and adverse side effects, which may be different in males and females.

6. ACKNOWLEDGEMENTS

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7. REFERENCES

AFONSO-PEREIRA, F., ASHIRU, D., PATEL, R., MURDAN, S. & BASIT, A. W. 2017a. Sex differences in the gut: influence of an excipient on cimetidine bioavailability in humans and rats. UCL School of Pharmacy, University College London.

AFONSO-PEREIRA, F., MURDAN, S., SOUSA, J., VEIGA, F. & BASIT, A. W. 2016. Sex differences in excipient effects: Enhancement in ranitidine bioavailability in the presence of polyethylene glycol in male, but not female, rats. *Int J Pharm,* 506**,** 237-41.

AFONSO-PEREIRA, F., YANG, M., MURDAN, S. & BASIT, A. W. 2017b. Establishing Bioequivalence: Can sex play a role in the effect of excipients in the bioavailability of BCS III drugs? . UCL School of Pharmacy.

ASHIRU, D. A., PATEL, R. & BASIT, A. W. 2007. Simple and universal HPLC-UV method to determine cimetidine, ranitidine, famotidine and nizatidine in urine: application to the analysis of ranitidine and its metabolites in human volunteers. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences,* 860**,** 235-40.

ASHIRU, D. A., PATEL, R. & BASIT, A. W. 2008. Polyethylene glycol 400 enhances the bioavailability of a BCS class III drug (ranitidine) in male subjects but not females. *Pharm Res,* 25**,** 2327-33.

BANSAL, T., MISHRA, G., JAGGI, M., KHAR, R. K. & TALEGAONKAR, S. 2009. Effect of P-glycoprotein inhibitor, verapamil, on oral bioavailability and pharmacokinetics of irinotecan in rats. *Eur J Pharm Sci,* 36**,** 580-90.

BASIT, A. W., NEWTON, J. M., SHORT, M. D., WADDINGTON, W. A., ELL, P. J. & LACEY, L. F. 2001. The effect of polyethylene glycol 400 on gastrointestinal transit: implications for the formulation of poorly-water soluble drugs. *Pharmaceutical Research,* 18**,** 1146-50.

BASIT, A. W., PODCZECK, F., NEWTON, J. M., WADDINGTON, W. A., ELL, P. J. & LACEY, L. F. 2002. Influence of polyethylene glycol 400 on the gastrointestinal absorption of ranitidine. *Pharmaceutical Research,* 19**,** 1368-74.

BOURDET, D. L., POLLACK, G. M. & THAKKER, D. R. 2006. Intestinal absorptive transport of the hydrophilic cation ranitidine: a kinetic modeling approach to elucidate the role of uptake and efflux transporters and paracellular vs. transcellular transport in Caco-2 cells. *Pharmaceutical Research,* 23**,** 1178-87.

COLLETT, A., HIGGS, N. B., SIMS, E., ROWLAND, M. & WARHURST, G. 1999. Modulation of the permeability of H2 receptor antagonists cimetidine and ranitidine by P-glycoprotein in rat intestine and the human colonic cell line Caco-2. *J Pharmacol Exp Ther,* 288**,** 171-8.

COLLETT, A., TANIANIS-HUGHES, J., CARLSON, G. L., HARWOOD, M. D. & WARHURST, G. 2005. Comparison of P-glycoprotein-mediated drug–digoxin interactions in Caco-2 with human and rodent intestine: Relevance to in vivo prediction. *European Journal of Pharmaceutical Sciences,* 26**,** 386-393.

COOK, M. J. & HIRST, B. H. 1994. Transepithelial Secretion of the Histamine H-2-Receptor Antagonist Ranitidine in Human Intestinal Epithelial Caco-2 Monolayers Is Mediated by P-Glycoprotein. *Journal of Physiology-London,* 479P**,** P103-P103.

DEFERME, S. & AUGUSTIJNS, P. 2003. The effect of food components on the absorption of P-gp substrates: a review. *J Pharm Pharmacol,* 55**,** 153-62.

DEY, S., GUNDA, S. & MITRA, A. K. 2004. Pharmacokinetics of erythromycin in rabbit corneas after single-dose infusion: role of P-glycoprotein as a barrier to in vivo ocular drug absorption. *J Pharmacol Exp Ther,* 311**,** 246-55.

EDDERSHAW, P. J., CHADWICK, A. P., HIGTON, D. M., FENWICK, S. H., LINACRE, P., JENNER, W. N., BELL, J. A. & MANCHEE, G. R. 1996. Absorption and disposition of ranitidine hydrochloride in rat and dog. *Xenobiotica,* 26**,** 947-56.

ESTUDANTE, M., MAYA, M., MORAIS, J. G., SOVERAL, G. & BENET, L. Z. 2013. Effect of P-Glycoprotein on the Rat Intestinal Permeability and Metabolism of the BDDCS Class 1 Drug Verapamil. *Mol Pharm,* 10**,** 4038-45.

EUROPARAT 2016. *European pharmacopoeia, ninth edition : published in accordance with the Convention on the Elaboration of a European Pharmacopoeia*.

GARG, D. C., WEIDLER, D. J. & ESHELMAN, F. N. 1983. Ranitidine bioavailability and kinetics in normal male subjects. *Clin Pharmacol Ther,* 33**,** 445-52.

GERMANN, U. A. & CHAMBERS, T. C. 1998. Molecular analysis of the multidrug transporter, P-glycoprotein. *Cytotechnology,* 27**,** 31-60.

GIACOMINI, K. M., HUANG, S. M., TWEEDIE, D. J., BENET, L. Z., BROUWER, K. L., CHU, X., DAHLIN, A., EVERS, R., FISCHER, V., HILLGREN, K. M., HOFFMASTER, K. A., ISHIKAWA, T., KEPPLER, D., KIM, R. B., LEE, C. A., NIEMI, M., POLLI, J. W., SUGIYAMA, Y., SWAAN, P. W., WARE, J. A., WRIGHT, S. H., YEE, S. W., ZAMEK-GLISZCZYNSKI, M. J. & ZHANG, L. 2010. Membrane transporters in drug development. *Nat Rev Drug Discov,* 9**,** 215-36.

HAMILTON, K. O., YAZDANIAN, M. A. & AUDUS, K. L. 2001. Modulation of P-glycoprotein activity in Calu-3 cells using steroids and beta-ligands. *Int J Pharm,* 228**,** 171-9.

HIGGINS, C. F. & GOTTESMAN, M. M. 1992. Is the multidrug transporter a flippase? *Trends Biochem Sci,* 17**,** 18-21.

HODAEI, D., BARADARAN, B., VALIZADEH, H. & ZAKERI-MILANI, P. 2015. Effects of polyethylene glycols on intestinal efflux pump expression and activity in Caco-2 cells. *Brazilian Journal of Pharmaceutical Sciences,* 51**,** 745-753.

HUGGER, E. D., AUDUS, K. L. & BORCHARDT, R. T. 2002. Effects of poly(ethylene glycol) on efflux transporter activity in Caco-2 cell monolayers. *Journal of Pharmaceutical Sciences,* 91**,** 1980-1990.

JOHNSON, B. M., CHARMAN, W. N. & PORTER, C. J. 2002. An in vitro examination of the impact of polyethylene glycol 400, Pluronic P85, and vitamin E d-alpha-tocopheryl polyethylene glycol 1000 succinate on P-glycoprotein efflux and enterocyte-based metabolism in excised rat intestine. *AAPS PharmSci,* 4**,** E40.

JOINT, F. A. O. W. H. O. E. C. O. F. A., COMIT. MIXTE, F. A. O. O. M. S. D. E. D. A. A., ORGANISATION MONDIALE DE LA, S. & ORGANISATION DES NATIONS UNIES POUR L'ALIMENTATION ET, L. A. Evaluation of certain

veterinary drug residues in food : eighty-first report of the Joint Fao/Who Expert Committee on Food Additives. 2016 Geneva. World Health Organization.
KAGAN, L., DREIFINGER, T., MAGER, D. E. & HOFFMAN, A. 2010. Role of p-glycoprotein in region-specific gastrointestinal absorption of talinolol in rats. *Drug Metab Dispos,* 38**,** 1560-6.
KLOTZ, U. & WALKER, S. 1990. Biliary excretion of H2-receptor antagonists. *European Journal of Clinical Pharmacology,* 39**,** 91-92.
LEE, K., NG, C., BROUWER, K. L. & THAKKER, D. R. 2002. Secretory transport of ranitidine and famotidine across Caco-2 cell monolayers. *J Pharmacol Exp Ther,* 303**,** 574-80.
LIN, J. H. & YAMAZAKI, M. 2003. Clinical relevance of P-glycoprotein in drug therapy. *Drug Metab Rev,* 35**,** 417-54.
LINDELL, M., KARLSSON, M. O., LENNERNAS, H., PAHLMAN, L. & LANG, M. A. 2003. Variable expression of CYP and Pgp genes in the human small intestine. *Eur J Clin Invest,* 33**,** 493-9.
LIPSY, R. J., FENNERTY, B. & FAGAN, T. C. 1990. Clinical review of histamine2 receptor antagonists. *Arch Intern Med,* 150**,** 745-51.
MACLEAN, C., MOENNING, U., REICHEL, A. & FRICKER, G. 2008. Closing the gaps: a full scan of the intestinal expression of p-glycoprotein, breast cancer resistance protein, and multidrug resistance-associated protein 2 in male and female rats. *Drug Metab Dispos,* 36**,** 1249-54.
MACLEAN, C., MOENNING, U., REICHEL, A. & FRICKER, G. 2010. Regional absorption of fexofenadine in rat intestine. *European Journal of Pharmaceutical Sciences,* 41**,** 670-674.
MAI, Y., AFONSO-PEREIRA, F., MURDAN, S. & BASIT, A. W. 2017. Excipient-mediated alteration in drug bioavailability in the rat depends on the sex of the animal. *European Journal of Pharmaceutical Sciences,* 107**,** 249-255.
MANITPISITKUL, P. & CHIOU, W. L. 1993. Intravenous verapamil kinetics in rats: Marked arteriovenous concentration difference and comparison with humans. *Biopharmaceutics & Drug Disposition,* 14**,** 555-566.
MARIANA, B., ADRIAN, L., GUILLERMO, V., JUAN, S., LAURA, M. & CARLOS, L. 2011. Gender-related differences on P-glycoprotein-mediated drug intestinal transport in rats. *Journal of Pharmacy and Pharmacology,* 63**,** 619-626.
MEDICINES & HEALTHCARE PRODUCTS REGULATORY AGENCY. 2017. *Medicines & Healthcare products Regulatory Agency* [Online]. Available: https://www.gov.uk/government/organisations/medicines-and-healthcare-products-regulatory-agency [Accessed 2017].
MILLER, R. 1984. Pharmacokinetics and bioavailability of ranitidine in humans. *Journal of Pharmaceutical Sciences,* 73**,** 1376-1379.
MIZUNO, N., NIWA, T., YOTSUMOTO, Y. & SUGIYAMA, Y. 2003. Impact of drug transporter studies on drug discovery and development. *Pharmacol Rev,* 55**,** 425-61.
MOHL, S. & WINTER, G. 2004. Continuous release of rh-interferon alpha-2a from triglyceride matrices. *J Control Release,* 97**,** 67-78.

MUMMANENI, V. & DRESSMAN, J. B. 1994. Intestinal uptake of cimetidine and ranitidine in rats. *Pharmaceutical Research,* 11**,** 1599-604.

PAINE, M. F., LUDINGTON, S. S., CHEN, M. L., STEWART, P. W., HUANG, S. M. & WATKINS, P. B. 2005. Do men and women differ in proximal small intestinal CYP3A or P-glycoprotein expression? *Drug Metab Dispos,* 33**,** 426-33.

PEDERSEN, P. V. & MILLER, R. 1980. Pharmacokinetics and bioavailability of cimetidine in humans. *J Pharm Sci,* 69**,** 394-8.

PIAO, Y. J., LI, X. & CHOI, J. S. 2008. Effects of verapamil on etoposide pharmacokinetics after intravenous and oral administration in rats. *Eur J Drug Metab Pharmacokinet,* 33**,** 159-64.

POTTER, J. M., MCWHINNEY, B. C., SAMPSON, L. & HICKMAN, P. E. 2004. Area-under-the-curve monitoring of prednisolone for dose optimization in a stable renal transplant population. *Ther Drug Monit,* 26**,** 408-14.

RICHARDS, D. A. 1983. Comparative pharmacodynamics and pharmacokinetics of cimetidine and ranitidine. *J Clin Gastroenterol,* 5 Suppl 1**,** 81-90.

ROWE, R. C., SHESKEY, P. J., OWEN, S. N. C. & AMERICAN PHARMACISTS, A. 2006. *Handbook of pharmaceutical excipients,* London; Greyslake, IL; Washington, DC, Pharmaceutical Press ; American Pharmacists Association.

SCHAIQUEVICH, P., NISELMAN, A. & RUBIO, M. 2002. Comparison of two compartmental models for describing ranitidine's plasmatic profiles. *Pharmacol Res,* 45**,** 399-405.

SCHULZE, J. D., PETERS, E. E., VICKERS, A. W., STATON, J. S., COFFIN, M. D., PARSONS, G. E. & BASIT, A. W. 2005. Excipient effects on gastrointestinal transit and drug absorption in beagle dogs. *International Journal of Pharmaceutics,* 300**,** 67-75.

SCHULZE, J. D., WADDINGTON, W. A., ELI, P. J., PARSONS, G. E., COFFIN, M. D. & BASIT, A. W. 2003. Concentration-dependent effects of polyethylene glycol 400 on gastrointestinal transit and drug absorption. *Pharmaceutical Research,* 20**,** 1984-8.

SHAROM, F. J. 2014. Complex Interplay between the P-Glycoprotein Multidrug Efflux Pump and the Membrane: Its Role in Modulating Protein Function. *Frontiers in Oncology,* 4**,** 41.

SHEN, Q., LIN, Y., HANDA, T., DOI, M., SUGIE, M., WAKAYAMA, K., OKADA, N., FUJITA, T. & YAMAMOTO, A. 2006. Modulation of intestinal P-glycoprotein function by polyethylene glycols and their derivatives by in vitro transport and in situ absorption studies. *International Journal of Pharmaceutics,* 313**,** 49-56.

SIEW, A., LE, H., THIOVOLET, M., GELLERT, P., SCHATZLEIN, A. & UCHEGBU, I. 2012. Enhanced oral absorption of hydrophobic and hydrophilic drugs using quaternary ammonium palmitoyl glycol chitosan nanoparticles. *Mol Pharm,* 9**,** 14-28.

SMYTH, H. F., JR., CARPENTER, C. P. & WEIL, C. S. 1950. The toxicology of the polyethylene glycols. *J Am Pharm Assoc Am Pharm Assoc,* 39**,** 349-54.

SMYTH, H. F., JR., CARPENTER, C. P. & WEIL, C. S. 1955. The chronic oral toxicology of the polyethylene glycols. *J Am Pharm Assoc Am Pharm Assoc,* 44**,** 27-30.

SUTTLE, A. B. & BROUWER, K. L. 1994. Bile flow but not enterohepatic recirculation influences the pharmacokinetics of ranitidine in the rat. *Drug Metabolism and Disposition,* 22**,** 224.

SUTTLE, A. B. & BROUWER, K. L. 1995. Regional gastrointestinal absorption of ranitidine in the rat. *Pharmaceutical research,* 12**,** 1311-1315.

TAMURA, J., SATO, N., EZAKI, H., MIYAMOTO, H., ODA, S., HIRAI, K., TOKADO, H., MATSUMOTO, M. & SHIRAI, T. 1983. [Acute toxicity of ranitidine and its metabolite in mice, rats and rabbits, and subacute oral toxicity of ranitidine in rats]. *J Toxicol Sci,* 8 Suppl 1**,** 1-24.

VAN HECKEN, A. M., TJANDRAMAGA, T. B., MULLIE, A., VERBESSELT, R. & DE SCHEPPER, P. J. 1982. Ranitidine: single dose pharmacokinetics and absolute bioavailability in man. *Br J Clin Pharmacol,* 14**,** 195-200.

WERLE, M. 2008. Natural and Synthetic Polymers as Inhibitors of Drug Efflux Pumps. *Pharmaceutical Research,* 25**,** 500-511.

Conclusion and Future Work

Pre-clinical testing is important for the improved understanding of the behaviour of orally administered drugs. Despite the lack of direct correlation or physiological compatibility between animals and humans, the usefulness of animal models lies in the estimation of drug behaviour, rather than in providing a physiologically identical paradigm.

The rat was identified as a suitable model to study sex dependent excipient effects in the oral absorption of soluble but poorly permeable drugs (BCS III). Sex differences were identified in the rat gastrointestinal luminal environment. These differences were observed in the pH, osmolality, surface tension and buffer capacity of the luminal fluids and tended to increase distally.

As observed in Humans, in the rat, low doses of PEG 400 were found to be capable of increasing the oral bioavailability of ranitidine and cimetidine in male but not females. In females of both species, low doses of PEG 400 did not have an effect in the oral bioavailability of ranitidine or cimetidine.

The effect of PEG 400 on the oral bioavailability of ranitidine and cimetidine was also found to be dose dependent. A dose of 26 mg/kg was found have the largest effect in the rat.

The effect of PEG 400 is no limited to ranitidine and cimetidine, two structurally similar compounds, but is observed in other BCS III compounds whose bioavailability is limited by intestinal efflux transporters such as ampicillin.

Metformin, which is also subjected to the action of membrane transporters, was not affected by PEG 400 in the sexes. Ranitidine, cimetidine and ampicillin are substrates for the efflux pump, P-gp, whereas metformin is not.

PEG 400, most probably increases the oral bioavailability of ranitidine, cimetidine and ampicillin by inhibiting P-gp. PEG 400 has been reported to inhibit P-gp in a dose dependent manner. This was confirmed by the fact that after knocking out P-gp *in vivo* with verapamil, a P-gp reversible blocker, we found that PEG 400 did not affect the oral bioavailability of ranitidine in male rats.

The inhibition of P-gp that significantly impacts the oral bioavailability of ranitidine occurs at the intestinal membrane level: when ranitidine was administered orally but PEG 400 intravenously the increase in the oral bioavailability of ranitidine was not significant; when ranitidine was administered intravenously the route of administration of PEG 400 (oral or IV) did not bear an impact on the bioavailability of ranitidine.

A double plasma concentration peak was observed when ranitidine was administered orally in both sexes but disappeared if ranitidine was given intravenously, suggesting little enterohepatic

recycling takes place and a possible bimodal absorption pattern. Ranitidine was reported to have preferential absorption sites in the intestine. P-gp was also shown in humans and rats not to be homogeneously distributed. The activity of P-gp tends to increase distally.

Verapamil, was found to also increase the oral bioavailability of ranitidine in female rats, not just the males. Nevertheless, the increase produced in the females was much smaller than in the males. The same finding was reported in other work with a different P-gp blocker, Cyclosporine A. This proves that males are more sensitive to *in vivo* P-gp inhibition than females.

Regional differences in the distribution of P-gp may justify the double peak phenomena and also help explain the reason behind the sex dependent effect of PEG 400. Conflicting literature data exists some suggesting P-gp quantity varies with the sex (differently for each tissue), while some suggests that in the intestine of the rat there are no sex differences in the amount of P-gp. However, it may be that the amount of P-gp in male and female rats is the same, but the males are permanently partially inhibited due to circulating testosterone. Testosterone is a stronger P-gp inhibitor than progesterone and oestrogens.

It is still unclear where in the gut does PEG 400 exert it effect and why are there sex differences. To better understand this further work is required.

It would be helpful to identify in which areas of the small intestine ranitidine is preferentially absorbed. This would be performed either *ex vivo* (perfusion studies) or *in vitro* (with excised intestinal tissue) in permeation chambers. An intestinal map of ranitidine absorption would be available. This would be performed in male and female rats so that sex differences could be identified.

Following the mapping of ranitidine absorption, PEG 400 and a P-gp blocker would be used to map their effects. This would also be conducted in male and female rats.

Having identified the areas of the intestine where PEG 400 and P-gp influence the permeability of ranitidine and where sex differences are observed, research could then focus on these sections to identify biological reasons for the sex difference, such as different amount or activity of P-gp.

The animal model developed could also be useful in assessing if other excipients could have similar effects as PEG 400. Several excipients have been reported to interact with membrane transporters but *in vivo* data is usually lacking.

Excipients are capable of changing drug disposition and their role should not be underestimated. The degree to which excipients modulate drug bioavailability may be modified by sex. It is important that regulatory authorities and pharmaceutical industry take this knowledge into account during the pharmaceutical development stages of pharmaceutical products.