International Journal of Pharmaceutics 629 (2022) 122365

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Sex-specific effects of excipients on oral drug bioavailability

Yang Mai [a,b,1,*], Christine M. Madla [b,1], Haibin Shao [a,1], Yujia Qin [a], Hamid A. Merchant [c], Sudaxshina Murdan [b], Abdul W. Basit [b,*]

[a] School of Pharmaceutical Sciences (Shenzhen), Sun Yat-sen University, Guangzhou 510275, China
[b] UCL School of Pharmacy, University College London, 29 – 39 Brunswick Square, London WC1N 1AX, United Kingdom
[c] Deparment of Pharmacy, School of Applied Sciences, University of Huddersfield, Queensgate, Huddersfield HD1 3DH, United Kingdom

A R T I C L E I N F O

Keywords:
Pharmaceutical excipients
Gastrointestinal tract
Solubilizing agents
Sex differences
Bioavailability
Efflux membrane transporters
Testosterone
Hormones
Nuclear receptors
Personalized medicines
ABCB1
ABCC2
ABCG2

A B S T R A C T

The mechanism of action of excipients eliciting sex differences in drug bioavailability is poorly understood. In this study, the excipients Cremophor RH 40 (PEG 40 hydrogenated castor oil), Poloxamer 188 (2-methyloxirane) and Tween 80 (polyoxyethylene (80) sorbitan monooleate) were screened at 0.07 – 5% concentrations for their effect on ranitidine bioavailability in male and female Wistar rats. We show that all excipient concentrations significantly increased ranitidine bioavailability in male, but not female, rats. The effect of these excipients on the intestinal efflux transporters P-glycoprotein (P-gp), breast cancer resistant protein (BCRP) and multi-drug resistant protein 2 (MRP2) were also monitored. Measured by ELISA assay, in male rats, peak reductions in intestinal P-gp protein expression occurred in the presence of 1% Cremophor RH 40 and Poloxamer 188 and 0.5% Tween 80. In contrast, no distinct changes were observed in female intestinal P-gp expression. Unlike P-gp, all excipients had a positive effect on MRP2 protein expression – albeit only in males – in a concentration-dependent manner. The excipients did not modulate intestinal BCRP protein expression in either sex. Endogenous hormones and a nuclear receptor (testosterone, oestradiol and pregnane X receptor; PXR) that are purported to regulate intestinal efflux membrane transporter expression were also quantified. In the presence of all excipients, testosterone levels significantly elevated in males, although PXR levels reduced at similar rates in both sexes. No significant effects were identified in oestradiol levels in male and female rats. It is clear that excipients are not inert and their pathway for modulating drug response is multi-dimensional and specific between sexes. This study showed that excipients increased drug bioavailability of a P-gp drug substrate due to its reductive effect on intestinal P-gp expression; we propose that this link may be due to the excipients modulating fundamental testosterone levels. Understanding the implication of excipients on intestinal physiology and hormone levels can therefore improve pharmaceutical design, clinical efficacy and instigate next generation personalised, sex-specific formulations.

1. Introduction

Excipients being classed as 'inert' is now being challenged. Emerging research has shown that excipients are able to actively modulate intestinal transporters, thus manipulating drug absorption in the gut (Basit et al., 2002). For example, solubilising excipients such as Vitamin E TPGS (D-α-tocopheryl polyethylene glycol succinate) increased the uptake of P-glycoprotein (P-gp) substrates including doxorubicin, vinblastine, paclitaxel and colchicine through the inhibition of P-gp efflux function (Dintaman and Silverman, 1999), thereby influencing the outcome of oral drug absorption in Caco-2 cell lines (Flanagan, 2019; Martinez et al., 2022; Reker et al., 2019).

Excipients have further been shown to alter baseline expression of transporters. Lipid-based agents from Peceol (glyceryl monooleate), Gelucire 44/14 (lauroyl polyoxyl-31 glycerides), Tween 20 (Polyethylene glycol sorbitan monolaurate), Eudragit S100 to other PEG forms increased the permeability of Rhodamine 123 due to the reduced expression of P-gp in cell lines and pre-clinical animal models (Hodaei et al., 2015; Mohammadzadeh et al., 2014; Sachs-Barrable et al., 2007). However, fundamental research has shown that males and females respond differently to drug products (Madla et al., 2021). More recently, novel research has uncovered that excipients interact differently with

* Corresponding authors.
E-mail addresses: maiy6@mail.sysu.edu.cn (Y. Mai), a.basit@ucl.ac.uk (A.W. Basit).
[1] There authors contributed equally towards this manuscript.

https://doi.org/10.1016/j.ijpharm.2022.122365
Received 26 August 2022; Received in revised form 27 October 2022; Accepted 30 October 2022
Available online 3 November 2022
0378-5173/© 2022 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

gastrointestinal (GI) transporter systems between the sexes (Reker et al., 2020; Yu et al., 2021).

The sex-specific influence of excipients on drug bioavailability was first recognised following a study with a widely-used solubilising agent PEG 400 which was found to increase the oral bioavailability of ranitidine (Ashiru et al., 2008) and cimetidine (Mai et al., 2020). Such enhancements, however, were only reflected in male and not female human volunteers. An animal model was established to investigate the reason for this phenomenon (Afonso-Pereira et al., 2016). Interestingly, the sex-related influence of PEG 400 only occurred for drugs whose absorption is controlled by the P-gp efflux transporter (Mai et al., 2018a; Mai et al., 2018b). Cyclosporine A, a known P-gp inhibitor countered the effect of PEG 400 on the bioavailability of ampicillin and ranitidine in both male and female rats, superseding the sex-specific effect of PEG 400 (Mai et al., 2017). Such a phenomena is not limited to PEG 400 but extended to other polyoxyethylated excipients. PEG 2000, Cremophor RH 40, Poloxamer 188 and Tween 80 also exhibited a sex-specific reduction on P-gp expression and increased the absorption of ranitidine, a known P-gp substrate (Mai et al., 2019). This could be due to the innately differential expression of P-gp across the intestinal tract in male and female Wistar rats (Madla et al., 2022).

The Food and Drug Administration (FDA) have issued a clinical guidance for drug developers to plan and evaluate studies determining interactions with ATP binding cassette (ABC) transporters (FDA, 2020). There are, however, hundreds of clinically important intestinal transporters (International Transporter et al., 2010) mediated by hormones and nuclear receptors in response to the dynamic intestinal environment maintaining homeostasis (Rigalli et al., 2019). Notwithstanding, the effect of formulation design on therapeutic outcomes governed by transporters and innate mechanisms in the sexes remains largely unknown.

This study aimed to understand the fundamental mechanism that elicits sex differences in drug response from excipient exposure. As such, the effect of commonly used pharmaceutical excipients including Cremophor RH 40, Poloxamer 188 and Tween 80 at concentrations from 0.07% to 5% w/v were explored in male and female Wistar rats. Specifically, the study aimed to understand the implications of excipient exposure on three levels; i) *in vivo* ranitidine bioavailability, ii) expression of key intestinal efflux transporters (P-gp, BCRP and MRP2) that influence drug absorption, and iii) endogenous compounds (testosterone, oestradiol and pregnane X receptor, PXR) that are purported to regulate ABC transporter expression in the GI tract.

2. Materials and methods

2.1. Reagents and materials

Ranitidine hydrochloride, glacial acetic acid and sodium acetate trihydrate were obtained from Sigma Aldrich (Dorset, UK). Tween 80 (Polysorbate 80) was purchased from Fluka (Dietikon, Switzerland). Poloxamer 188 and Cremophor RH 40 (PEG 40 castor oil) were obtained from Agenda (Bradford, UK) and BASF (Cheadle, Germany) respectively. HPLC-grade water and acetonitrile were purchased from Fisher Scientific (Loughborough, UK).

2.2. Pharmacokinetic study

2.2.1. Animals

Male (approximately 250 g) and female (approximately 200 g) Wistar rats (8 weeks old) were purchased from Harlan UK Ltd. (Oxfordshire, UK) and the Sun Yat-sen University Laboratory Animal Centre (Guangzhou, China).

2.2.2. Impact of excipient concentrations on ranitidine bioavailability

The stock solutions were prepared by homogenously mixing 100 mL of water containing 2.5 g ranitidine hydrochloride and either 0 (control), 0.07, 0.1, 0.5, 1.0 or 5.0 g excipients (Cremophor RH 40, Poloxamer 188 and Tween 80). This corresponded to concentrations of 0.07%, 0.1%, 0.5%, 1% and 5% w/v respectively. The solutions were kept under magnetic stirring for 10 min.

Animal work for screening the potential sex effect of excipient from 0.07%, 0.1%, 0.5%, 1% and 5% concentrations were conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act, 1986. All rats were housed at room temperature (25°C) and in a light–dark cycle of 12 h. The rats were caged in groups of six of single sex, allowed to move freely and provided with food and water *ad libitum* before experiment. The day before the experiment, the rats were fasted overnight and individually housed in grid-bottom cages, also known as metabolic cages.

On the day of the experiment, each rat was weighed and administered 50 mg/kg ranitidine in the absence or presence of excipients (Cremophor RH 40, Poloxamer 188 and Tween 80) by oral gavage at 0.07%, 0.1%, 0.5%, 1% and 5% concentrations. Male and female rats were given 0.5 mL and 0.4 mL of the control or test oral solutions, respectively. Approximately 250 μL – 300 μL of blood was then collected from the rats' tail vein and transferred to anticoagulant centrifuge tubes (BD Microtainer® K2E Becton, Dickinson and Company, USA) at 0.5, 1.25, 2, 3, 4 and 6 h timepoints. At 8 h post-administration, the rats were killed in a CO_2 euthanasia chamber and approximately 1 mL of blood was taken by cardiac puncture.

2.2.3. Preparation of blood samples

The samples were prepared for further analysis using a previously reported method (Afonso-Pereira et al., 2016). Briefly; all blood samples were centrifuged at 12,000 G for 10 min and the supernatants (plasma samples) were collected into 1.5 mL Eppendorf tubes. 50 μL of the supernatant was mixed with equal volume of acetonitrile in a 1.5 mL Eppendorf tubes to precipitate plasma proteins. After 1 min of vortex-mixing, 100 μL HPLC grade water was added to the mixture, vortex-mixed again for 30 s and centrifuged at 4°C for 10 min at 12,000 G. The clear supernatant was then collected, of which 40 μL was analysed by the HPLC as described below.

2.2.4. HPLC analysis

Chromatographic analysis was performed using a HPLC system (Agilent Technologies, 1260 Infinity) equipped with a pump (model G1311C), autosampler (model G1329B) and a diode array UV detector (model G1314B). The sample was subjected to HPLC-UV analysis using a previously validated method (Ashiru et al., 2007) using 5 μm Luna SCX column (Phenomenex, UK) and eluted using 20:80 (acetonitrile):(0.1 M sodium acetate at pH = 5.0) at 2 mL/min.

2.2.5. Pharmacokinetic analysis

Pharmacokinetic parameters (C_{max}, t_{max}, $AUC_{0\text{-}480}$, $AUC_{0\text{-}\infty}$, Cl, Vd and $t_{½}$) were calculated by non-compartmental analyses using Microsoft Excel add-in PKSolver (Zhang et al., 2010).

2.3. Impact of excipient concentrations on intestinal efflux transporter expression, endogenous sex hormones and nuclear receptors

2.3.1. Animals

Animal work for screening the potential sex-effect of excipient from 0.07%, 0.1%, 0.5%, 1% and 5% concentrations were conducted at the Sun Yat-sen University Laboratory Animal Centre. *In vivo* animal protocols were agreed by the Administrative Committee on Animal Research in Sun Yat-sen University. All rats were housed, prepared and treated as shown in Section 2.2.2.

2.3.2. Sample collection

The rats were sacrificed in a CO_2 euthanasia chamber 1 h post-administration. Approximately 1 mL of blood was taken by cardiac puncture and then transferred to anticoagulant centrifuge tubes (BD

Microtainer® K2E Becton, Dickinson and Company, USA). Blood samples were centrifuged at 4°C, 3600 rpm, 12, 000 G for 10 min and stored at −80°C until used for analyses. The intestines were rapidly removed and as the jejunum is the main site of absorption for immediate-release oral pharmaceutical formulations, it was the only segment of interest chosen for the work herein. As such, the jejunum (10 cm from the ligament of Treitz) was cut, washed with cold Krebs Bicarbonate Ringer's (KBR) solution and put into beakers with KBR solution on ice. This consisted of 10 mM D-glucose, 1.2 mM calcium chloride ($CaCl_2$), 1.2 mM magnesium chloride ($MgCl_2$), 115 mM sodium chloride (NaCl), 25 mM sodium bicarbonate ($NaHCO_3$), 0.4 mM monopotassium phosphate (KH_2PO_4) and 2.4 mM dipotassium phosphate (K_2HPO_4). pH was adjusted to 7.4 using sodium hydroxide (NaOH) or hydrochloric acid (HCl). The tissue was allowed to rest for approximately 20 min to achieve low tissue temperature and minimise potential tissue damage during preparation. Approximately 2–3 cm long pieces from the proximal part of the jejunum were opened along their mesenteric border. The jejunum segments were then gently washed with KBR buffer to remove their contents. To obtain the mucosal tissue, sections were placed on an ice-cold glass plate and the serosa was gently squeezed out with tweezers.

2.3.3. Tissue preparation

The mucosal tissues (approximately 50 mg) from Section 2.3.2 were cut into small pieces and homogenised in 0.5 mL radio-immunoprecipitation assay (RIPA) lysis buffer at 30 Hz for 30 s with a TissueLyser II (QIAGEN, Hilden, Germany). This was repeated twice at intervals of 30 s to ensure complete homogenisation. The tissue homogenates were incubated at 4°C for 2 h and centrifuged at 4°C, 12,000 G for 5 min. The total tissue protein was collected from the supernatants. Concentration was subsequently determined using a BCA Assay Protein kit (Beyotime Biotechnology, Shanghai, China) and performed according to the manufacturer's instructions.

2.3.4. ELISA Assay procedure

To measure the targeted transporter protein and sex hormone levels, P-gp (MM-70245R2), BCRP (MM-0606R2), MRP2 (MM-0607R2), testosterone (MM-0577R1) and oestradiol (MM-0567R2) were quantified by ELISA Assay kits (Meimian Biotech, Guangzhou, China) according to the manufacturer's instructions. Beta-actin was chosen as the internal control protein which was measured by ELISA Assay kit (RTDL00014, Assay Genie, Dublin, Ireland). Briefly, 50 μL of serially titrated standards, diluted samples and blanks were added to the standard wells of the i) Abcb1 (P-gp); ii) Abcg2 (BCRP); iii) Abcc2 (MRP2); iv) testosterone and v) oestradiol microplates in duplicates, respectively. 100 μL of HRP-conjugate reagent was then added to each well apart from the blank wells. The plate was covered with a plate sealer membrane and incubated for 60 min at 37°C. The plate sealer was then removed and the liquid discarded by rigorously flicking into an acceptable waste receptacle. The washing buffer solution provided in the assay kit was diluted 20-fold with distilled water. It was then added to each well, shaken on a plate stirrer for 3 s and drained. This was repeated 5 times and wells were blotted dry using a paper towel to remove any remaining liquid. 50 μL Chromogen Solution A and 50 μL Chromogen Solution B was added to each well, covered and incubated for 15 min at 37°C. 50 μL of the Stop Solution was added to each well; a blue colour change to a yellow solution would have indicated a stop in the reaction. Upon analysis, the blank well is taken as zero. Absorbance was then measured at 450 nm in a plate reader following the addition of the Stop Solution within 15 min.

A linear calibration line was constructed using a i) P-gp; ii) BCRP; iii) MRP2; iv) testosterone and v) oestradiol, and appropriately diluted in 50 mM carbonate buffer (pH 9.5). Tissue supernatants for protein transporter quantification, and plasma supernatants for hormone quantification were also appropriately diluted to 50 mM carbonate buffer (pH 9.5). Absorbance was measured at 450 nm after the reaction and the protein and hormone expression was calculated according to the standard protein calibration curve. Protein and hormone concentrations in all unknown and standard preparations were measured as per instructions of the ELISA kit in duplicate.

2.3.5. Measurement of PXR levels by Real-Time Reverse-Transcription polymerase chain reaction

Following collection (Section 2.3.2), the mucosal tissues were then kept in a RNA later® Stabilization Solution (Thermofisher). Total RNA in each intestinal sample was isolated and purified with PureLink® RNA Mini Kit (Thermofisher), and RNA concentration was measured with Nanodrop 2000 (Thermofisher) according to the manufacturer's instructions.

Subsequently, the quantification of the target RNA was conducted as followed: 1 mg total RNA of each sample was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad). To quantify the levels of *PXR* mRNA real-time reverse-transcription polymerase chain reaction (RT-qPCR) was performed on the 7500 Real Time PCR System (Applied Biosystems, Thermofisher) using the following method described (MacLean et al., 2008). Briefly, 50 μL PCR reaction contained 25 μL of PowerUp™ SYBR Green PCR Master Mix (Thermofisher), 500 nM each of forward and reverse primers, and 1 μg of cDNA. anti-beta actin was used for normalisation and amplification of 1 μg cDNA, respectively. Real-time PCR was carried out in 96 well PCR plates (Thermofisher). The amplification program for all genes consisted of one pre-incubation cycle at 95°C with a 10 min hold, followed by 45 amplification cycles with denaturation at 95°C with a 10 s hold, an annealing temperature of 50°C with a 10 s hold, and an extension at 72°C with a 10 s hold. Amplification was followed by a melting curve analysis which ran for one cycle with denaturation at 95°C with a 1 s hold, annealing at 65°C with a 15 s hold and melting at 95°C with a 1 s hold. Distilled water was included as a negative control in each run to access specificity of primers and possible contaminants. PXR primers (5′ – 3′, ATGGAGGTCTT-CAAATCTG and AATCCCTTACATCCTTCAC, forward and reverse, respectively) were designed by primer-BLAST searching with publicly available sequence information of the GeneBank of the National Center for Biotechnology Information (NCBI) and purchased from Eurofins (Eurofins Genomics, Germany).

Relative expression of *PXR* mRNA in intestine were calculated using 7500 software (version 2.0.6, Thermofisher). The average of the threshold cycle (Ct) values for *PXR* mRNA and the internal control (β-actin) was taken, and then the differences between Ct values for tested genes and internal control (ΔCt) were calculated for all the experimental samples.

2.4. Statistical analysis

2.4.1. Pharmacokinetic statistical analysis

Results from the pharmacokinetic study are expressed as mean ± S.D. ($n = 6$). Mean percentage change was calculated using the test group data (drug and excipient) against the control (drug alone). The control and test group data were analysed by one-way ANOVA followed by post-hoc Tukey analysis with a 95% confidence interval using IBM SPSS Statistics 16 (SPSS Inc., Illinois, USA).

2.4.2. Intestinal transporters, hormones and PXR quantification

All results are expressed as mean ± standard deviation (S.D.) ($n = 5$). To compare the differences in mean value of distributions for the expression of intestinal P-gp, BCRP, MRP2, testosterone, oestradiol and PXR between males and females in the presence of excipients and a range of concentrations, a one-way ANOVA followed by a post-hoc Tukey analysis was performed using Spyder (Python 3.8) with a 95% confidence interval (Spyder Doc Contributors, Massachusetts, USA). Spearman's rank correlation coefficient was used to determine if there was an ordinal relationship between excipient effects on efflux transporters, testosterone, oestradiol and PXR using SciPy package version 1.7.2 and Python 3.8.1. (Dover, DE, USA) on Jupyter Notebook version

6.0.3 (San Diego, CA, USA).

3. Results and discussion

3.1. Pharmacokinetic study

The bioavailability of ranitidine in the absence of excipients presented by the cumulative area under the plasma concentration versus time curve (AUC_{0-480}) in male and female rats was 338 ± 27 µg.min/mL and 451 ± 53 µg.min/mL, respectively. The ranitidine plasma concentration–time profiles in the presence of Cremophor RH 40, Poloxamer 188 and Tween 80 at 0.07%, 0.1%, 0.5%, 1% and 5% concentrations in male and female rats are shown in Fig. 1a,b, Fig. 2a,b and Fig. 3a,b and Tables 1–4 respectively. Fig. 1c, Fig. 2c and Fig. 3c highlight the percentage change in ranitidine bioavailability in the presence of the excipients studied.

Herein, a distinct bell-shaped curve can be described for the influence of all the excipients studied on the bioavailability of ranitidine in males. Ranitidine bioavailability in the presence of Cremophor RH 40 and Poloxamer 188 steadily increased from 18% and 21% at 0.07% to 68% and 53% at 1% excipient concentrations, respectively. A reduction in ranitidine bioavailability occurred when increasing excipient concentrations to 5%, albeit these differences were all still significant in male but not female rats (Fig. 1c and Fig. 2c). A similar bell-shaped curve can be outlined in terms of the modulatory effect of Tween 80 on ranitidine bioavailability, however, peak effects occurred at a lower concentration of 0.5% (49% enhancement in drug bioavailability against the control) when compared to Cremophor RH 40 and Poloxamer 188 (Fig. 3c). Similarly, however, modulatory effects on ranitidine bioavailability decreased following the increase of Tween 80 concentrations from 1% and 5% (Table 4). Higher concentrations of surfactants have been shown to accelerate small intestinal transit time due to the stimulatory effect of increased bulk volume in the lumen via osmosis. This, therefore, limits the time available for drug absorption to occur and consequently leads to reduced drug bioavailability (Basit et al., 2001; Mai et al., 2019; Schulze et al., 2003). As such, the osmotic effect at higher Cremophor RH 40. Poloxamer 188 and Tween 80 concentrations may supersede the mechanistic action of excipients increasing ranitidine bioavailability. Studies have shown that ranitidine is primarily a P-gp drug substrate (Bourdet and Thakker, 2006; Collett et al., 1999) and the *in vivo* response of ranitidine in the presence of these excipients is linked to the modulation of P-glycoprotein. No such enhancement in ranitidine bioavailability in the presence of these solubilising excipients were seen in females.

3.2. Intestinal efflux transporters and endogenous compounds

Several studies have proposed that the underlying mechanism behind excipient effects may be attributed to the sex-specific differential expression of many metabolising enzymes, intestinal membrane transporters and their association with hormones and xenobiotic receptors (Holmstock et al., 2013). To understand the sex-specific effect of excipients on oral drug bioavailability, key efflux membrane transporters that influence the outcome of intestinal absorption were measured (Madla et al., 2022).

Figs. 4–6 summarises the jejunal P-gp, MRP2 and BCRP expression at 1 hr following the oral administration of Cremophor RH 40, Poloxamer 188 and Tween 80 as quantified via ELISA. Supplementary Figures 1 – 3 show a linear calibration curve of P-gp, MRP2 and BCRP standards for ELISA, respectively.

3.2.1. Influence of Cremophor RH 40 on P-gp, MRP2 and BCRP

Cremophor RH 40 at 0.07% – 5% was able to significantly decrease the expression of P-gp protein in male jejunal tissues by −7%, −21%, −26%, −34% and −17%, respectively ($p < 0.05$). In females, however, Cremophor RH 40 was able to significantly increase P-gp expression by 12%, 7%, 14%, 9% and 2%, respectively (Fig. 4a; Supplementary Table 1). This closely reflected *in vivo* drug performance of ranitidine whereby 1% Cremophor RH 40 provoked peak enhancement in drug bioavailability of 68% (Fig. 1c). The opposite influence of excipient effects was demonstrated in the expression of jejunal MRP2. From 0.07% to 5%, Cremophor RH 40 increased the expression of MRP2 in male jejunal tissues at 0.07% by 17%, but statistically by 24%, 37%, 43% and 26% in the presence of 0.1% –5%, respectively, in comparison to the control ($p < 0.05$). MRP2 jejunal expression in females, however, were varied however were not statistically different when compared to the control (Fig. 4b; Supplementary Table 2). Cremophor RH 40 had little to no effect on the expression of BCRP. In female tissues, however, BCRP expression increased by 3% – 11% although not statistically different to its control (Fig. 4c; Supplementary Table 3).

Our results which demonstrate the modulatory effect of Cremophor RH 40 at low doses in male rats reflect similar findings to previous investigations; in a study by Wandel et al., the effects of low concentrations of Cremophor RH 40 (0.001% to 3%) as potential P-gp inhibitors were tested with the use of digoxin transport in Caco-2 cells. It showed that the increase in Cremophor RH 40 concentration inhibited P-gp efflux transporter ability at a greater extent and achieving complete P-gp inhibition following the administration of 3% Cremophor RH 40 (Wandel et al., 2003). However, there is limited knowledge and understanding in the effect of Cremophor RH 40 on drug bioavailability in females. A clear statistical contrast to that seen in male rats ($p < 0.05$) was observed in our study. In females, the bioavailability of ranitidine was not influenced in the presence of Cremophor RH 40 in low concentrations whilst higher dose of Cremophor RH 40 reduced the ranitidine absorption.

3.2.2. Influence of Poloxamer 188 on P-gp, MRP2 and BCRP

In the presence of Poloxamer 188, P-gp expression decreased in males by −4% when orally administered with 0.07% excipient concentrations. A statistical decrease in P-gp expression was seen from 0.1% to 5% Poloxamer 188 concentrations by −20%, −34%, −43% and −18%, respectively ($p < 0.05$). In females, P-gp expression increased by 8% in the presence of 0.07% Poloxamer 188 but statistically increased by 11%, 8%, 8% following oral administration of 0.1%, 0.5% and 1% concentrations. However, P-gp expression in females decreased by 3% in the presence of 5% Poloxamer 18 ($p < 0.05$) (Fig. 5a; Supplementary Table 4). Specifically, a peak reduction in P-gp expression occurred in the presence of 1% Poloxamer 188 (Fig. 5a) which led to the highest increase of ranitidine bioavailability of 53% in male rats (Fig. 2c) with again, a complimentary negative bell-shaped curve.

MRP2 expression followed a similar pattern in the presence of Poloxamer 188 (Fig. 5b; Supplementary Table 5). Male jejunal MRP2 expression increased by 4% and 17% in the presence of 0.07% and 0.1% Poloxamer 188. From 0.5% to 5% however, MRP2 expression statistically increased in male tissues only by 30%, 53% and 46% respectively ($p < 0.05$). MRP2 did not significantly change in the presence of Poloxamer 188 in female jejunal tissues. The presence of Poloxamer 188 increased BCRP expression across all concentrations tested in both male and female jejunal tissues. The largest change in BCRP expression was at 0.1% whereby an increase of 20% and 11% was demonstrated in males and females respectively, however none of the results were statistically significant ($p > 0.05$) (Fig. 5c; Supplementary Table 6).

Previous studies have reported a dose-dependent influence of Poloxamers on intestinal efflux transporters. Although 0.8% Poloxamer 188 was reported to not inhibit intestinal P-gp for the transport of talinolol on male volunteers (Bogman et al., 2005), a significant ($p < 0.05$) difference was later identified in the transport of P-gp substrate diltiazem from the intestinal sacs of male rats, pre-treated with 0.25%, 0.5% and 1% Poloxamer 188 when compared with the control (absence of excipients). This was attributed to the inhibition of P-gp transporter and CYP3A enzyme respectively (Hafsa et al., 2015). In females, however, ranitidine concentration steadily increased in the presence of increasing

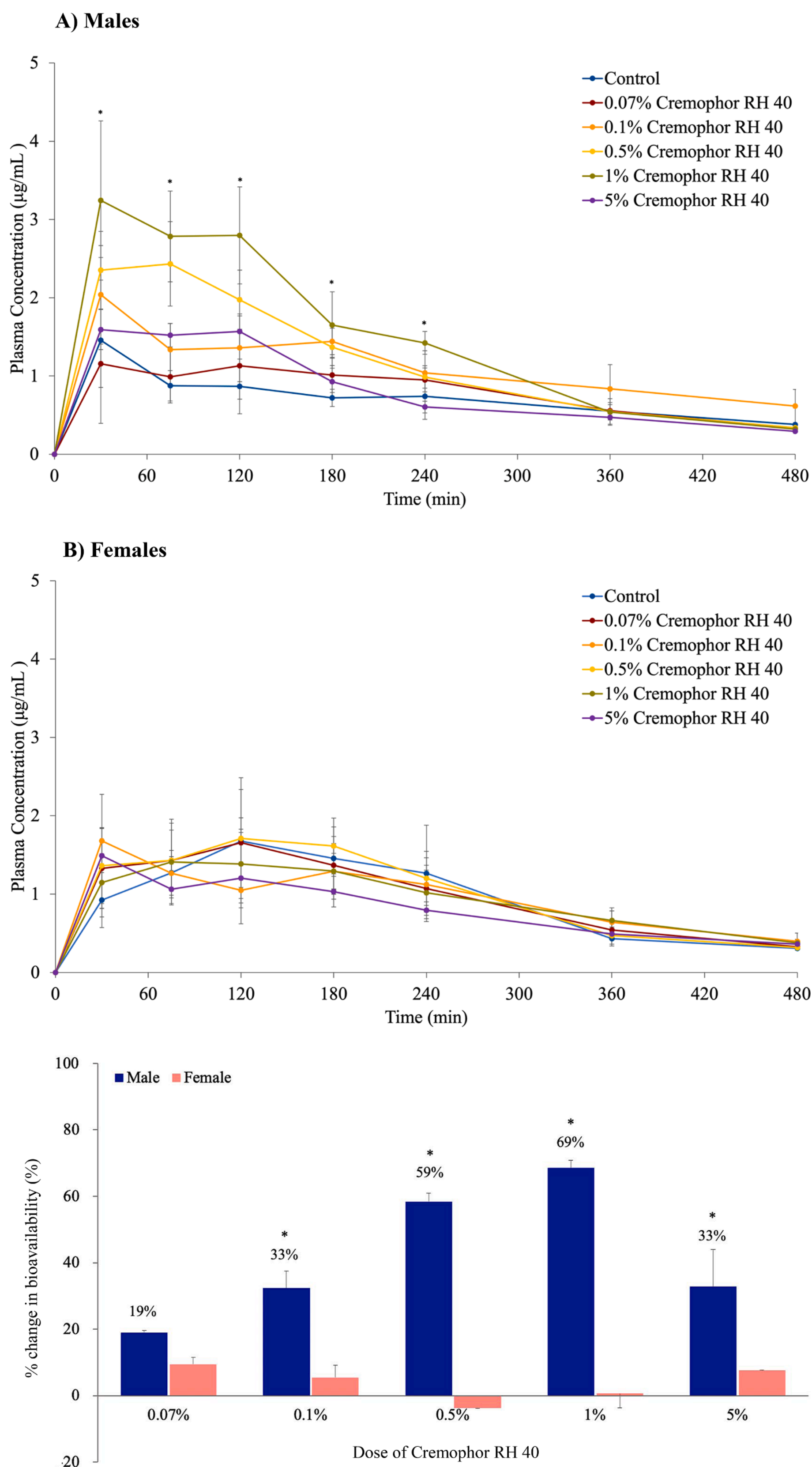

Fig. 1. *In vivo* mean plasma concentration–time curves of ranitidine in the absence and presence of Cremophor RH 40 at 0.07%, 0.1%, 0.5%, 1% and 5% in A) male and B) female rats. C) Mean percentage change (± S.D., n = 6) in the bioavailability of ranitidine in the presence of Poloxamer 188 (0.07% to 5%) in male and female rats. * Values with statistical significance ($p < 0.05$) between the control and Cremophor RH 40.

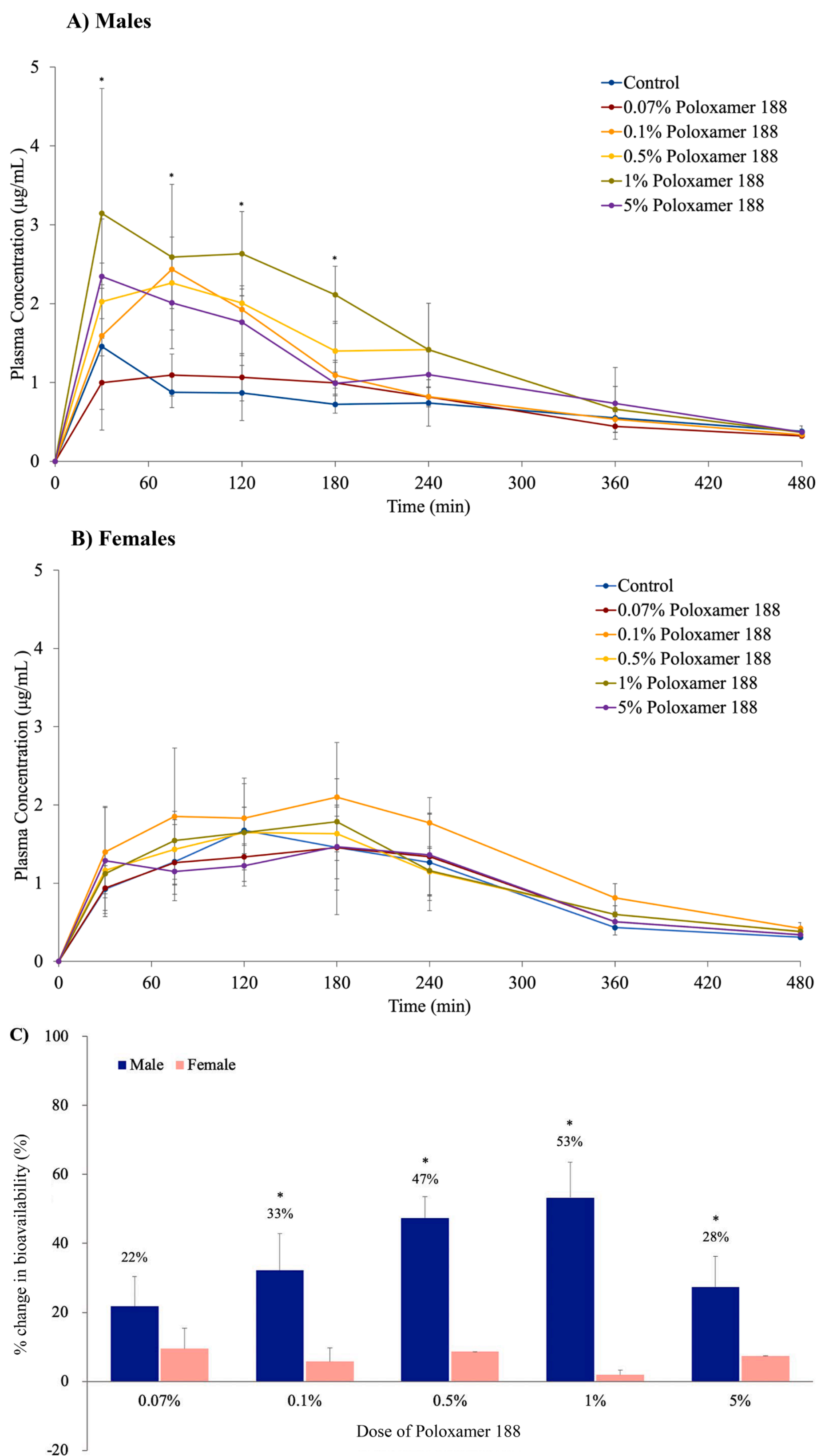

Fig. 2. *In vivo* mean plasma concentration–time curves of ranitidine in the absence and presence of Poloxamer 188 at 0.07%, 0.1%, 0.5%, 1% and 5% in A) male and B) female rats. C) Mean percentage change (± S.D., n = 6) in the bioavailability of ranitidine in the presence of Poloxamer 188 (0.07% to 5%) in male and female rats. * Values are statistically significance ($p < 0.05$) between the control and Poloxamer 188.

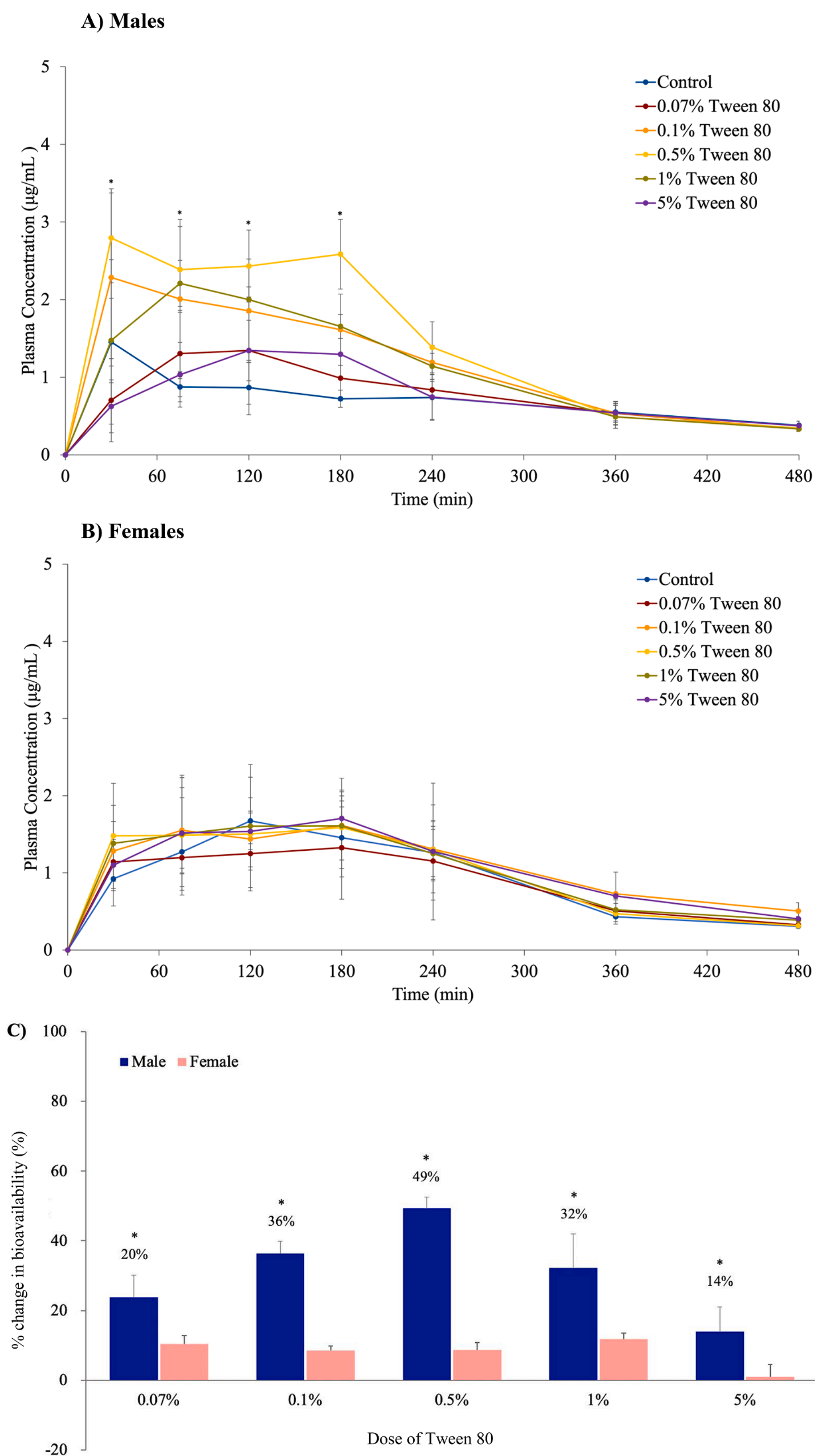

Fig. 3. *In vivo* mean plasma concentration–time curves of ranitidine in the absence and presence of Tween 80 at 0.07%, 0.1%, 0.5%, 1% and 5% in A) male and B) female rats. C) Mean percentage change (± S.D., n = 6) in the bioavailability of ranitidine in the presence of Tween 80 (0.07% to 5%) in male and female rats. * Values are statistically significance ($p < 0.05$) between the control and Tween 80.

Table 1
Effect of Cremophor RH 40 on the pharmacokinetic parameters of ranitidine in male and female Wistar rats (mean ± S.D., n = 6). * Values are statistically different between the control and Cremophor RH 40 groups ($p < 0.05$).

Pharmacokinetic Parameters	Concentrations of Cremophor RH 40 (%)					
	Control	0.07%	0.1%	0.5%	1%	5%
AUC_{0-480} (μg.min/mL)	338 ± 27	403 ± 20	448 ± 32 *	536 ± 47 *	570 ± 88 *	449 ± 53 *
Male						
$AUC_{0-\infty}$ (μg.min/mL)	391 ± 50	462 ± 38	511 ± 29 *	596 ± 41 *	643 ± 85 *	542 ± 46 *
C_{max} (μg/mL)	1.9 ± 0.3	1.9 ± 0.7	3.1 ± 0.4 *	3.4 ± 0.2 *	4.4 ± 0.9 *	2.9 ± 0.3 *
t_{max} (min)	146 ± 79	92 ± 28	101 ± 82	108 ± 57	95 ± 84	110 ± 61
Female						
AUC_{0-480} (μg.min/mL)	451 ± 53	494 ± 82	476 ± 71	434 ± 56	454 ± 30	486 ± 59
$AUC_{0-\infty}$ (μg.min/mL)	507 ± 47	576 ± 43	562 ± 83	523 ± 29	607 ± 100	563 ± 50
C_{max} (μg/mL)	2.4 ± 0.2	2.1 ± 0.3	2.3 ± 0.5	2.2 ± 0.1	2.0 ± 0.06	2.0 ± 0.4
t_{max} (min)	127 ± 75	89 ± 26	125 ± 38	111 ± 32	90 ± 25	118 ± 59

Table 2
Effect of Poloxamer 188 on the pharmacokinetic parameters of ranitidine in male and female Wistar rats (mean ± S.D., n = 6). * Values are statistically different between the control and Poloxamer 188 groups ($p < 0.05$).

Pharmacokinetic Parameters	Concentrations of Poloxamer 188 (%)					
	Control	0.07%	0.1%	0.5%	1%	5%
Male						
AUC_{0-480} (μg.min/mL)	338 ± 27	412 ± 41	447 ± 44 *	498 ± 22 *	518 ± 88 *	431 ± 53 *
$AUC_{0-\infty}$ (μg.min/mL)	391 ± 50	480 ± 57	506 ± 42 *	588 ± 64 *	563 ± 85 *	482 ± 46 *
C_{max} (μg/mL)	1.9 ± 0.3	2.2 ± 0.5	3.3 ± 0.2 *	3.2 ± 0.3 *	4.3 ± 0.9 *	3.2 ± 0.3 *
t_{max} (min)	146 ± 79	100 ± 33	93 ± 59	107 ± 47	95 ± 84	110 ± 61
Female						
AUC_{0-480} (μg.min/mL)	451 ± 53	494 ± 27	477 ± 65	490 ± 71	460 ± 26	485 ± 45
$AUC_{0-\infty}$ (μg.min/mL)	507 ± 47	536 ± 30	538 ± 62	625 ± 99	592 ± 40	580 ± 90
C_{max} (μg/mL)	2.4 ± 0.2	2.2 ± 0.3	1.8 ± 0.3	2.0 ± 0.7	2.1 ± 0.5	2.2 ± 0.3
t_{max} (min)	127 ± 75	114 ± 25	94 ± 50	88 ± 53	100 ± 22	130 ± 84

Poloxamer 188 concentrations. This may be as a result of the longer small intestinal transit time seen in female subjects which therefore, provides an increased contact time between the drug formulation and the intestinal lumen for absorption to occur (Freire et al., 2011). High concentrations may also mimic high fluid load which is known to empty at an accelerated rate when compared with solids (Sadik et al., 2003).

3.2.3. *Influence of Tween 80 on P-gp, MRP2 and BCRP*

Following Tween 80 oral administration, 0.07% concentrations decreased P-gp concentrations in males and females by −6% and −4% respectively. From 0.1% to 5% concentrations, P-gp expression in males statistically decreased by −25%, –32%, –22% and −11% ($p < 0.05$). In females, however, P-gp expression increased by 9%, 9%, 4% and 6% respectively (Fig. 6a and Supplementary Table 7). The greatest reduction in jejunal P-gp expression was seen in the presence of 0.5% Tween 80 (Fig. 6a). Peak enhancement in ranitidine bioavailability by 49% also occurred when co-formulated with 0.5% Tween 80 (Fig. 3c). A negative bell-shaped curve in P-gp expression closely reflected *in vivo* drug performance. A positive bell-shaped curve can be distinguished towards the modulation of MRP2 expression in male rats. Bar 0.07% Tween 80, MRP2 expression statistically increased by 34%, 57%, 45% and 32%, however, this was only demonstrated in male tissues ($p < 0.05$). No significant differences were seen in female jejunal tissues (Fig. 6b; Supplementary Table 8). Although the results were not significant in comparison to the control ($p > 0.05$), Tween 80 also modulated BCRP expression in males and females with a sex difference in response at 0.1%. (Fig. 6c; Supplementary Table 9).

Table 3
Effect of Tween 80 on the pharmacokinetic parameters of ranitidine in male and female Wistar rats (mean ± S.D., n = 6). * Values are statistically different between the control and Tween 80 groups at ($p < 0.05$).

Pharmacokinetic Parameters	Concentrations of Tween 80 (%)					
	Control	0.07%	0.1%	0.5%	1%	5%
Male						
AUC_{0-480} (μg.min/mL)	338 ± 27	406 ± 19 *	461 ± 58 *	505 ± 38 *	447 ± 70 *	385 ± 63 *
$AUC_{0-\infty}$ (μg.min/mL)	391 ± 50	459 ± 24 *	581 ± 98 *	595 ± 61 *	618 ± 94 *	498 ± 69 *
C_{max} (μg/mL)	1.9 ± 0.3	2.2 ± 0.5 *	4.5 ± 1 *	4.3 ± 0.5 *	3.3 ± 0.2 *	2.2 ± 0.5 *
t_{max} (min)	146 ± 79	110 ± 65	120 ± 83	131 ± 57	135 ± 64	152 ± 27
Female						
AUC_{0-480} (μg.min/mL)	451 ± 53	498 ± 67	490 ± 75	490 ± 23	504 ± 55	456 ± 84
$AUC_{0-\infty}$ (μg.min/mL)	507 ± 47	583 ± 80	597 ± 74	566 ± 53	606 ± 60	560 ± 70
C_{max} (μg/mL)	2.4 ± 0.2	1.9 ± 0.5	2.2 ± 0.5	2.1 ± 0.1	2.2 ± 0.2	2.3 ± 0.8
t_{max} (min)	127 ± 75	95 ± 29	143 ± 44	108 ± 29	135 ± 52	150 ± 97

Table 4
Mean percentage change in ranitidine bioavailability in male and female Wistar rats in the presence of pharmaceutical excipients vs control (n = 6). * Values with statistical significance ($p < 0.05$) between the control and excipient groups.

Excipient	Sex	Concentration (%)				
		0.07%	0.1%	0.5%	1%	5%
Cremophor RH 40	Males	+19.2	+32.5 *	+58.6 *	+68.6 *	+32.8 *
	Females	+9.5	+5.5	−3.8	−0.7	+7.8
Poloxamer 188	Males	+21.9	+32.2 *	+47.3 *	+53.3 *	+27.5 *
	Females	+9.5	+5.8	+8.6	+2.0	+7.5
Tween 80	Males	+20.1 *	+36.4 *	+49.4 *	+32.2 *	+13.9 *
	Females	+10.4	+8.6	+8.6	+11.8	+1.1

Tween 80 has been found to enhance the oral bioavailability of drug molecules that are P-gp substrates (Nerurkar et al., 1996). Tween 80 increased the apical-to-basolateral permeability and decreased the basolateral-to-apical permeability of a P-gp substrate Rhodamine 123 in Caco-2 cell monolayers at concentrations ranging from 0.01 mM to 1 mM in a dose-dependent manner (Rege et al., 2002). Secondly, the extent of P-gp inhibition on the cell line by Tween 80 was reported to logarithmically depend on excipient concentration, ranging from 0.5% to 3% (Sun et al., 2004). In an *in vivo* study, 10% concentrations of

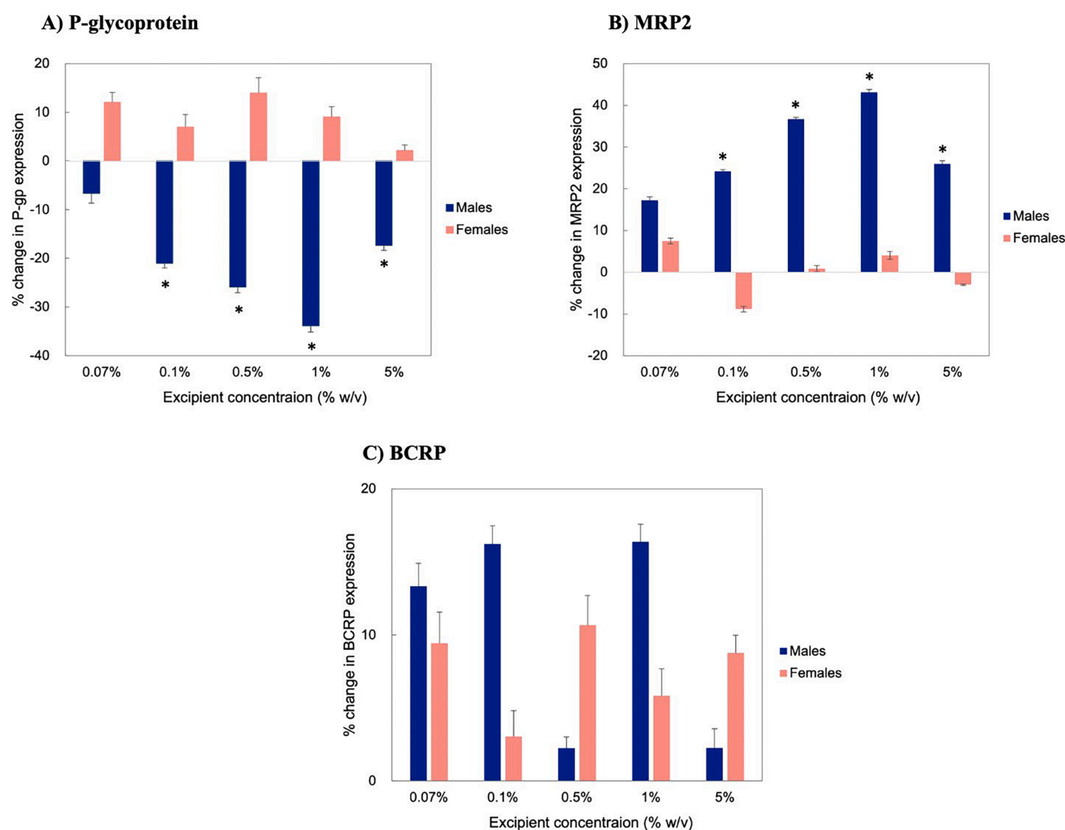

Fig. 4. Percentage change in A) P-gp; B) MRP2 and C) BCRP expression in the absence and presence of Cremophor RH 40 at 0.07% to 5% in male and female jejunal tissues. * Values are statistically different between the control (without excipients) and Cremophor RH 40 groups ($p < 0.05$), and displayed as mean ± S.D., n = 5.

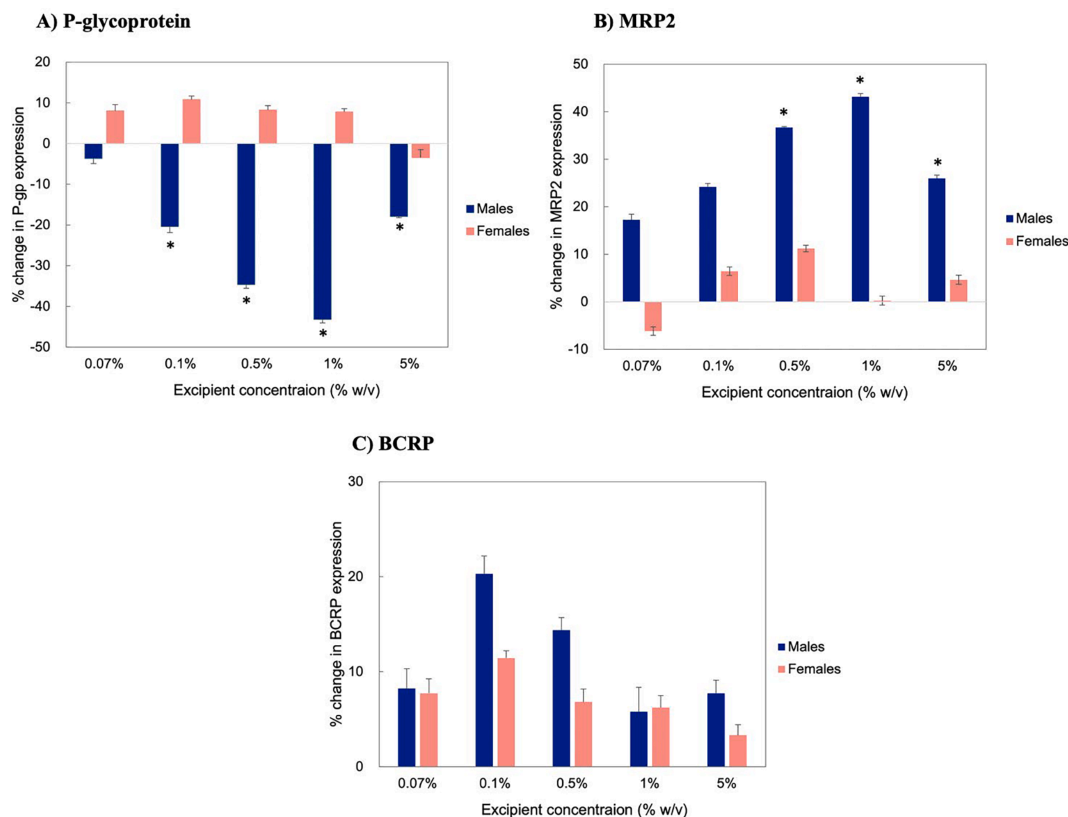

Fig. 5. Percentage change in A) P-gp; B) MRP2 and C) BCRP expression in the absence and presence of Poloxamer 188 at 0.07% to 5% in male and female jejunal tissues. * Values are statistically different between the control (without excipients) and Poloxamer 188 groups ($p < 0.05$), and displayed as mean ± S.D., n = 5.

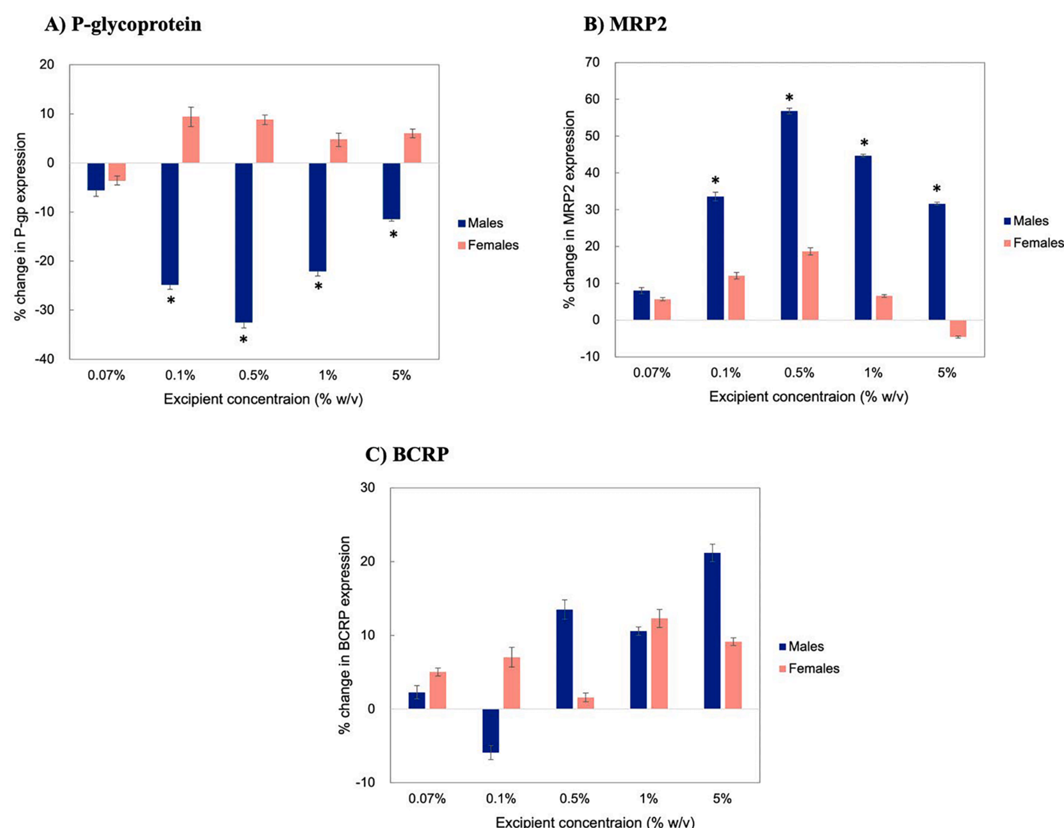

Fig. 6. Percentage change in A) P-gp; B) MRP2 and C) BCRP expression in the absence and presence of Tween 80 at 0.07% to 5% in male and female jejunal tissues. *Values are statistically different between the sexes and control (without excipients) and Tween 80 groups ($p < 0.05$), and displayed as mean ± S.D., n = 5.

Tween 80 elicited a greater impact on the bioavailability of digoxin with a 61% increase in male rats ($p < 0.05$) when compared with 1% Tween which enhanced the drug bioavailability by only 30% (Zhang et al., 2003). This may also be attributed to the diluting effect of the increased fluid load in the gut lumen. Furthermore, a dose-dependent effect of Tween 80 on the function of P-gp could be explained by a clear inhibitory effect of Tween 80 on P-gp ATPase is observed at low concentrations and negated at high concentrations. This is most likely due to a membrane loosening effect at higher concentration of Tween 80 (Li-Blatter et al., 2009).

The effect of the studied excipients and concentrations on efflux transporter expression in the sexes follow the order of magnitude: P-gp > MRP2 > BCRP. Although ranitidine is known to be transported via the paracellular pathway, it is still a substrate of P-gp (Bourdet et al., 2006; Collett et al., 1999). From the inclusion of investigating P-gp expression i) in the sexes and ii) in the presence of excipients, it can be proposed that the P-gp is actively modulated by excipients in a sex- and dose-dependent manner to elicit varying drug response. The increased levels of MRP2, however, showed no effect on *in vivo* ranitidine performance which corroborates the study where MRP2 inhibitors had no effect on H_2-receptor antagonist nizatidine transport, regardless of their concentration (Dahan et al., 2009). This may be due to the differential function of P-gp and MRP2. Specifically, P-gp has been shown to be a primary active transporter of drugs whereas other ATP-dependent transporters including MRP2 efflux xenobiotics through a co-transport mechanism with reduced glutathione (Dahan et al., 2009). BCRP expression resulted to a varied response subject to the presence of excipients with no clear differences between the sexes or the impact of concentration (Fig. 3c, Fig. 5c, Fig. 7c). Like MRP2, inhibition of BCRP has no effect on H_2-receptor antagonist nizatidine transport (Dahan et al., 2009) which closely reflects the lack of influence of varied BCRP expression on ranitidine *in vivo* pharmacokinetics. BCRP, however, demonstrates a narrower range of resistance in comparison to P-gp and MRP2 for a number of anticancer agents including anthracyclines, mitoxantrone and topoisomerase I inhibitors (Dahan et al., 2009).

Through molecular dynamic simulations, a recent study by Moesgaard *et al.* proposed that polysorbates i.e. polyoxyethylated compounds including Cremphor RH 40, Poloxamer 188 and Tween 80, can interact with efflux transporters at a molecular level. Although mechanisms such as the alteration of membrane proteins and depletion of ATP have been previously proposed, molecular dynamic simulations have revealed that PEG chains on polysorbate molecules can orientate themselves around P-gp. For example, PEG chains can assume a 'lollipop' shaped clumps inside the lipid membrane. As such, the fatty acid chains face towards the core of the membrane. At higher concentrations, however, polysorbates can congregate together, forming larger aggregates with PEG chains intertwined (Moesgaard et al., 2022). Further molecular simulations demonstrated that polysorbate-based excipients are able to locate and directly bind to the drug-binding domain of P-gp within a few microseconds, regardless of excipient concentration and P-gp conformation which can lead towards protein inhibition (Moesgaard et al., 2022). A similar mechanism of action of the polyoxyethylated excipients studied may extend its interactions on MRP2.

3.2.4. Effect of excipients on endogenous compounds

Testosterone, oestradiol and pregnane nuclear X receptor (PXR) were also characterised in male and female Wistar rats to understand the influence of excipients on intestinal molecular modulators (Figs. 7–9). Supplementary Figs. 4 and 5 shows a linear calibration curve of testosterone and oestradiol respectively.

Influence of Cremophor RH 40 on testosterone, oestradiol and PXR.

A bell-shaped curve in testosterone expression was achieved following the oral administration of 0.07% – 5% Cremophor RH 40 in male rats (Fig. 7a; Supplementary Table 10). Testosterone expression

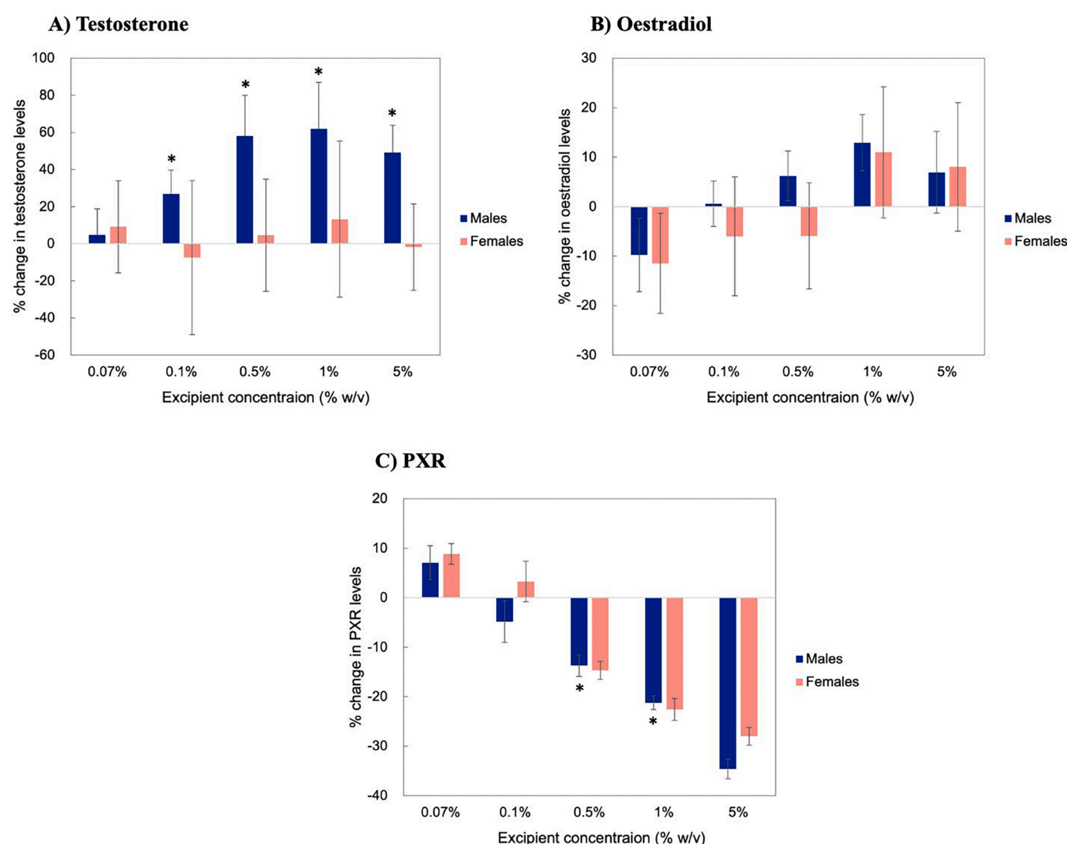

Fig. 7. Percentage change in A) Testosterone; B) Oestradiol and C) PXR expression in the absence and presence of Cremophor RH 40 at 0.07% to 5% in male and female jejunal tissues. *Values are statistically different between the control (without excipients) and Cremophor RH 40 groups ($p < 0.05$), and displayed as mean ± S.D., n = 5.

increased at 0.07% Cremophor RH 40 by 5% but significantly increased by 27%, 58%, 62% and 49% from 0.1% to 5% respectively, in comparison to the control ($p < 0.05$). Little to no difference was seen in testosterone levels in female jejunal tissues. Cremophor RH 40 had minor effects on oestradiol expression. For example, 0.07% concentrations reduced oestradiol levels in males and females by −10% and −11%, respectively. 1%, however, increased oestradiol by 13% and 11% in males and females. Although not statistically significant in comparison to the control, 0.5% Cremophor RH 40 elicited a sex difference between oestradiol levels in male and female rats ($p < 0.05$) (Fig. 7b; Supplementary Table 11). Both male and female rats showed near equal reduction of PXR expression at 0.5% and 1% concentrations ($p < 0.05$) although no sex differences were observed (Fig. 7c; Supplementary Table 12).

3.2.5. *Influence of Poloxamer 188 on testosterone, oestradiol and PXR*

A bell-shaped curve was also demonstrated in testosterone expression in male tissues following Poloxamer 188 oral administration. The highest enhancement in testosterone levels was seen with 1% Poloxamer 188 following a 57% increase, although this was only displayed in males. No significant differences in testosterone levels were seen in female jejunal tissues across all concentrations of Poloxamer 188 (Fig. 8a; Supplementary Table 13). Sex differences in Poloxamer 188 action on oestradiol levels were seen at 0.07% − 0.5% concentrations, although the results were not significantly different to the respective controls (Fig. 8b; Supplementary Table 14). A significant reduction in PXR expression by −18% and −20% was demonstrated in the presence of 0.5 % Poloxamer 188 in male and female rats respectively ($p < 0.05$). Sex differences in the reduction of PXR, however, were seen in the presence of 1% and 5% Poloxamer 188 concentrations whereby PXR in males decreased by −35% versus –23% in females, and −39% versus –32%, respectively ($p < 0.05$) (Fig. 8c; Supplementary Table 15).

3.2.6. *Influence of Tween 80 on testosterone, oestradiol and PXR*

The greatest modulation in testosterone expression was demonstrated following the oral administration of Tween 80 in male rats. Bar 0.07%, Tween 80 from 0.1% to 5% statistically increased testosterone levels by 61%, 70%, 49% and 22% in comparison to the control ($p < 0.05$). Little to no effect was seen in testosterone levels in female jejunal tissues (Fig. 9a; Supplementary Table 16). In comparison to the control, Poloxamer 188 did not statistically affect oestradiol levels in males and females, although a sex difference in excipient effects were seen in the presence of 0.07% and 5% concentrations (Fig. 9b; Supplementary Table 17). Although both PXR expression decreased in both male and female rats in the presence of 0.5 – 5% Tween 80, sex-specific effects were more pronounced in males, reducing by −31%, −38% and −38% respectively (Fig. 9c; Supplementary Table 18).

The effect of the studied excipients and concentrations on the level of endogenous compounds between the sexes follow the order of magnitude: testosterone > PXR > oestradiol. In our study, we found that a bell-shaped curve in testosterone expression was achieved following the oral administration of 0.07% – 5% of all three tested excipients (Cremophor RH 40, Poloxamer 188 and Tween 80) in male rats, which resulted to a negative correlation with their effects on the P-gp expression. Testosterone is reportedly both a substrate and inhibitor of P-gp (Aykan and Seyithanoglu, 2019). A previous study demonstrated that a decrease in the serum testosterone concentration after orchiectomy leads to an increase in P-gp activity and expression on jejunal tissue (Shchulkin et al., 2017). As a P-gp substrate, P-gp could modify the absorption of testosterone. Cremophor RH 40 and Poloxamer 188 had a negative relationship with testosterone levels, whilst the increase in P-gp expression led to increased testosterone levels in the presence of Tween 80. We propose

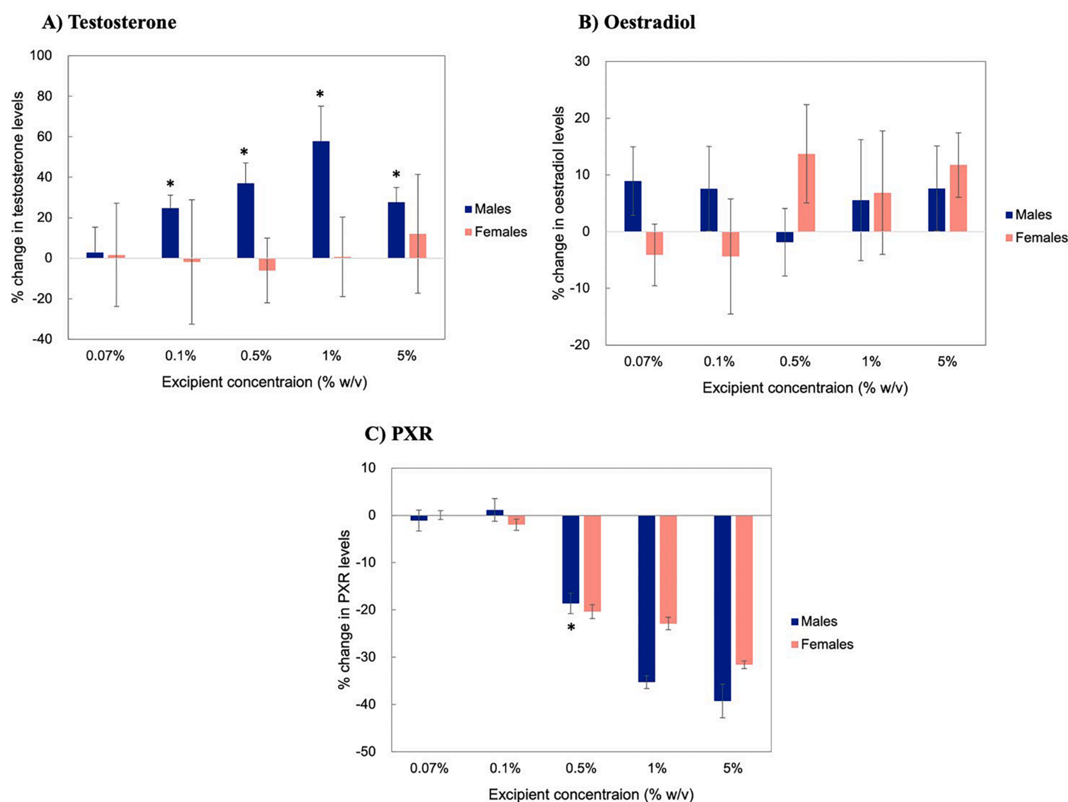

Fig. 8. Percentage change in A) Testosterone; B) Oestradiol and C) PXR expression in the absence and presence of Poloxamer 188 at 0.07% to 5% in male and female jejunal tissues. *Values are statistically different between the control (without excipients) and Poloxamer 188 groups ($p < 0.05$), and displayed as mean ± S.D., n = 5.

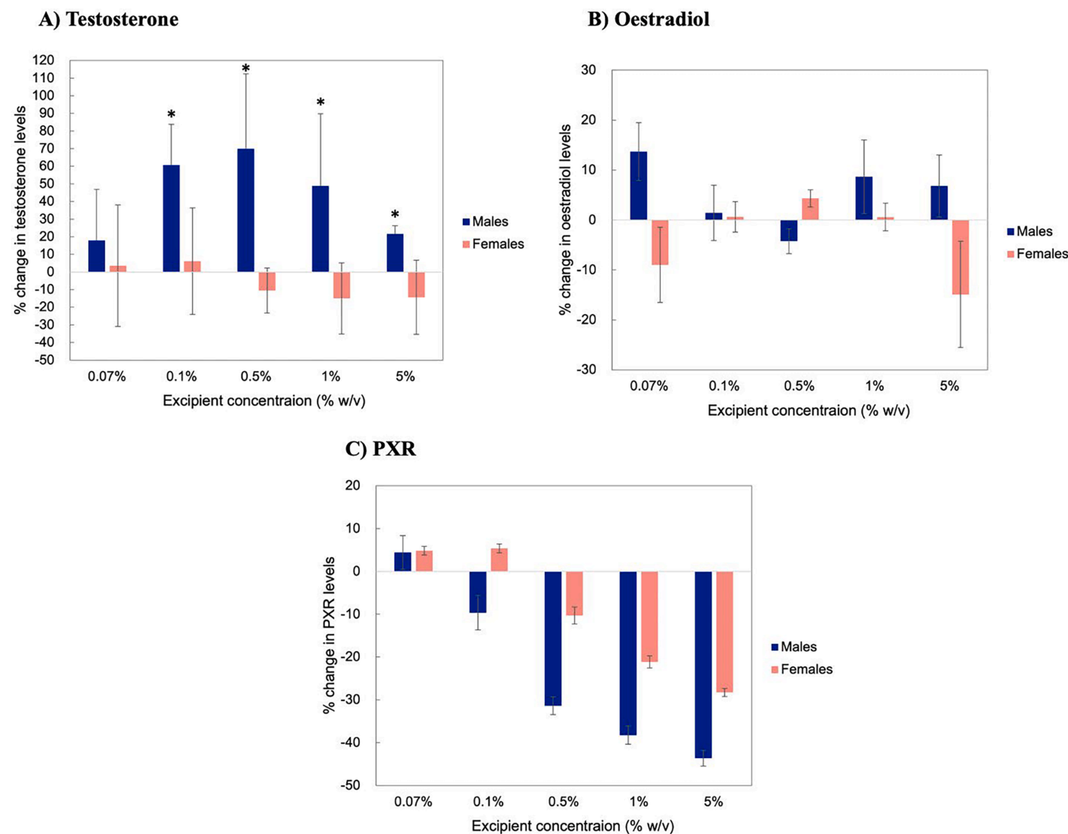

Fig. 9. Percentage change in A) Testosterone; B) Oestradiol and C) PXR expression in the absence and presence of Tween 80 at 0.07% to 5% in male and female jejunal tissues. *Values are statistically different between the control (without excipients) and Tween 80 groups ($p < 0.05$), and displayed as mean ± S.D., n = 5.

that excipients directly alter the P-gp function to modify its substrate testosterone levels or they enhanced the serum concentrations of testosterone resulting in a decrease on jejunal P-gp. Further studies need to illustrate this causality, which might contribute to the mechanism on this sex-specific effect of excipients.

Oestradiol are endogenous oestrogens that are rapidly conjugates with glucuronic acid in intestinal epithelial cells. A study by Järvinen *et al.* identified that oestrogen conjugates are good and specific substrates for BCRP with a higher affinity of transport than MRP2 (Jarvinen et al., 2018). Minimal research has been conducted on the effect of pharmaceutical excipients on intestinal oestradiol levels. Our study has shown no significant effect of Cremophor RH 40, Poloxamer 188 and Tween 80 action on intestinal oestradiol levels in comparison to the control (without the *in vivo* exposure of excipients). However, the regulation of ABC transporters, P-gp in particular, by oestrogen and its metabolites have been previously investigated (Evseenko et al., 2007; Mutoh et al., 2006; Wang et al., 2006). The magnitude of its effect, however, is dependent on hormone concentrations, protein and cell type. A study by Coles et al. revealed that exposure of oestradiol up-regulated the expression of P-gp protein levels, albeit in a concentration-dependent manner. This, however, was conducted on P-gp overexpressing NCI/ADR-RES cells (Coles et al., 2009).

Although research conducted on the interaction between excipients and nuclear receptors is lacking, PXR is well known to induce P-gp by binding to responsive element at a gene-level when activated by PXR ligands (Geick et al., 2001; Kast et al., 2002). In addition, the ability of PXR to cross-react with protein binding sites to govern a bi-directional transcriptional regulatory pathway has been proposed between lipids and P-gp (Barreto-Ojeda et al., 2018). A study found that the excipients glyceryl oleate (Peceol®) and lauroyl PEG-32 glycerides (Gelucire® 44/14) interacted with receptor pathways and effect the efflux of P-gp (Sachs-Barrable et al., 2007). Further studies have hypothesised that inter-individual differences in ABC-transporter expression can be influenced by individual abundance of PXR. As such, this effect can influence the efflux protein transporter on the intestinal membrane differentially in both a sex- and concentration- dependent manner.

3.3. Relationship between efflux transporters, testosterone, oestradiol and PXR expression

Fig. 10 shows the correlation between the expression of P-gp, BCRP and MRP2 in the presence of Cremophor RH 40, Poloxamer 188 and Tween 80 respectively, as well as its impact on testosterone and PXR. The highest positive correlation was for the jejunal expression of MRP2 in the presence of Cremophor RH 40 and Tween 80 in females, and Poloxamer 188 in males ($R^2 = 0.94$) for testosterone levels. This means that the exposure of all excipients respectively induced the expression of MPR2, modulated by the up-regulation of testosterone. A correlation of $r = 0.94$ was garnered for the expression of MRP2 in female rats, modulated by oestradiol in the presence of Tween 80. The highest negative correlation, however, was identified for the relationship between P-gp expression in males and testosterone levels in the presence of Tween 80 ($r = -1$). This demonstrates that the exposure of Tween 80 down-regulated P-gp expression as testosterone levels increased. A negative relationship was demonstrated for the expression of MRP2 in male rats in the presence of Cremophor RH 40 and Poloxamer 188 on PXR levels ($r = -0.77$). The only correlation noteworthy in females was the relationship between MRP2 expression and oestradiol levels in the presence of Tween 80 ($r = 0.94$) (Fig. 10).

4. Conclusion

Excipients are not inert and their active effects extends not only to drug bioavailability modulation. Our results confirmed that excipients interact with the innate expression of efflux transporters and endogenous regulators at a molecular level, and in a sex-specific manner. Cremophor RH 40, Poloxamer 188 and Tween 80 at 0.1% – 5% concentrations elevated the *in vivo* bioavailability of ranitidine. Such enhancement, however, was limited to males and not female Wistar rats and in a concentration-dependent manner. The excipients studied modulated the expression of intestinal transporters in the following magnitude; P-gp > MRP2 > BCRP. This has implications for many drugs that are substrate for these transporters. For instance with ranitidine which is a P-gp substrate, the excipient effects of down-regulating its expression in males resulted to an increase in *in vivo* bioavailability in rats. To further illuminate the mechanistic insights, excipients also affected endogenous compounds expression (testosterone > PXR > oestradiol) that regulate the levels of efflux transporters. Specifically, this study identified that in males, excipients increased drug bioavailability of a P-gp drug substrate by decreasing P-gp intestinal expression. This may be intrinsically modulated by the elevation of testosterone levels. As such, excipient exposure has multi-dimensional effects that

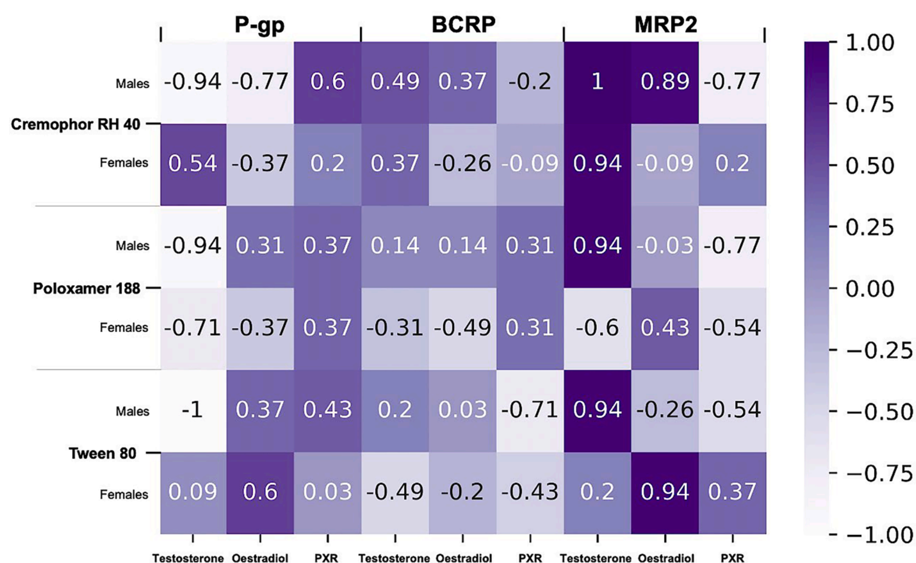

Fig. 10. Correlation between efflux membrane transporters, testosterone and PXR in the presence of Cremophor RH 40, Poloxamer 188 and Tween 80 in male and female rats. Highlighted boxes in yellow show significance levels between excipient, efflux transporter and endogenous parameters ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

can modulate innate intestinal physiology, function and circulatory levels of endogenous compounds. This cascades to differing drug response in the sexes. Therefore, a greater understanding and screening of excipients should be implemented in the formulation design of susceptible drugs. This can lead to a new age of personalised sex-specific pharmaceuticals. Mechanistic understanding of excipient-API and/or excipient-transporter interactions may also guide the development of novel and tailored excipients with bespoke drug modulatory factors to increase its efficacy and safety.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

The authors would like to thank the Engineering and Physical Sciences Research Council UK (EP/L01646X) and the National Natural Science Foundation of China (82003672) for their financial support towards this manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2022.122365.

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 (1-2), 237–241.

Altintas Aykan, D., Seyithanoglu, M., 2019. The effects of administration of Vitamin D, infliximab, and leflunomide on testosterone concentrations in rats under atorvastatin therapy. Eurasian J. Med. 51 (3), 224–227.

Ashiru, D.A.I., 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. J. Chromatogr. B Analyt Technol. Biomed. Life Sci. 860 (2), 235–240.

Ashiru, D.A.I., 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 (10), 2327–2333.

Barreto-Ojeda, E., Corradi, V., Gu, R.X., Tieleman, D.P., 2018. Coarse-grained molecular dynamics simulations reveal lipid access pathways in P-glycoprotein. J. Gen. Physiol. 150, 417–429.

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. Pharm. Res. 18, 1146–1150.

Basit, A.W., Podczeck, F., Newton, J.M., Waddington, W.A., Ell, P.J., Lacey, L.F., 2002. Pharm Res. 19 (9), 1368–1374.

Bogman, K., Zysset, Y., Degen, L., Hopfgartner, G., Gutmann, H., Alsenz, J., Drewe, J., 2005. P-glycoprotein and surfactants: effect on intestinal talinolol absorption. Clin. Pharmacol. Ther. 77 (1), 24–32.

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. Pharm. Res. 23 (6), 1178–1187.

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 (6), 1165–1177.

Coles, L.D., Lee, I.J., Voulalas, P.J., Eddington, N.D., 2009. Estradiol and progesterone-mediated regulation of P-gp in P-gp overexpressing cells (NCI-ADR-RES) and placental cells (JAR). Mol. Pharm. 6 (6), 1816–1825.

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, 171–178.

Dahan, A., Sabit, H., Amidon, G.L., 2009. The H2 receptor antagonist nizatidine is a P-glycoprotein substrate: characterization of its intestinal epithelial cell efflux transport. AAPS J. 11 (2), 205–213.

Dintaman, J.M., Silverman, J.A., 1999. Inhibition of P-glycoprotein by D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS). Pharmaceut Res. 16, 1550–1556.

Evseenko, D.A., Paxton, J.W., Keelan, J.A., 2007. Independent regulation of apical and basolateral drug transporter expression and function in placental trophoblasts by cytokines, steroids, and growth factors. Drug Metab. Dispos. 35 (4), 595–601.

FDA, 2020. In Vitro Drug Interaction Studies - Cytochrome P450 Enzyme- and Transporter-Mediated Drug Interactions. Guidance for Industry. US FDA, Silver Spring, MD.

Flanagan, T., 2019. Potential for pharmaceutical excipients to impact absorption: a mechanistic review for BCS Class 1 and 3 drugs. Eur. J. Pharm. Biopharm. 141, 130–138.

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. Int. J. Pharm. 415, 15–28.

Geick, A., Eichelbaum, M., Burk, O., 2001. Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. J. Biol. Chem. 276, 14581–14587.

Hafsa, R., Sunitha, B., Shravan, K., Avinash, M., 2015. Influence of poloxamer 188 on the intestinal transport of diltiazem hydrochloride. Int. J. Inno Res. Sci. Eng. Tech. 4, 6922–6925.

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. Braz. J. Pharm. Sci. 51, 745–753.

Holmstock, N., Gonzalez, F.J., Baes, M., Annaert, P., Augustijns, P., 2013. PXR/CYP3A4-humanized mice for studying drug-drug interactions involving intestinal P-glycoprotein. Mol. Pharm. 10, 1056–1062.

International Transporter, C., 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, 215–236.

Jarvinen, E., Deng, F., Kidron, H., Finel, M., 2018. Efflux transport of estrogen glucuronides by human MRP2, MRP3, MRP4 and BCRP. J. Steroid Biochem. Mol. Biol. 178, 99–107.

Kast, H.R., Goodwin, B., Tarr, P.T., Jones, S.A., Anisfeld, A.M., Stoltz, C.M., Tontonoz, P., Kliewer, S., Willson, T.M., Edwards, P.A., 2002. Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. J. Biol. Chem. 277, 2908–2915.

Li-Blatter, X., Nervi, P., Seelig, A., 2009. Detergents as intrinsic P-glycoprotein substrates and inhibitors. BBA 1788, 2335–2344.

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 (7), 1249–1254.

Madla, C.M., Gavins, F.K.H., Merchant, H.A., Orlu, M., Murdan, S., Basit, A.W., 2021. Let's talk about sex: differences in drug therapy in males and females. Adv. Drug Deliv. Rev. 175, 113804.

Madla, C.M., Qin, Y., Gavins, F.K.H., Liu, J., Dou, L., Orlu, M., Murdan, S., Mai, Y., Basit, A.W., 2022. Sex Differences in Intestinal P-Glycoprotein Expression in Wistar versus Sprague Dawley Rats. Pharmaceutics 14.

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. Eur. J. Pharm. Sci. 107, 249–255.

Mai, Y., Dou, L., Murdan, S., Basit, A., 2018a. An animal's sex influences the effects of the excipient PEG 400 on the intestinal P-gp protein and mRNA levels, which has implications for oral drug absorption. Eur. J. Pharm. Sci.

Mai, Y., Murdan, S., Awadi, M., Basit, A.W., 2018b. Establishing an in vitro permeation model to predict the in vivo sex-related influence of PEG 400 on oral drug absorption. Int. J. Pharm. 542, 280–287.

Mai, Y., Dou, L., Madla, C.M., Murdan, S., Basit, A.W., 2019. Sex-Dependence in the Effect of Pharmaceutical Excipients: Polyoxyethylated Solubilising Excipients Increase Oral Drug Bioavailability in Male but not Female Rats. Pharmaceutics 11.

Mai, Y., Ashiru-Oredope, D.A.I., Yao, Z., Dou, L., Madla, C.M., Taherali, F., Murdan, S., Basit, A.W., 2020. Boosting drug bioavailability in men but not women through the action of an excipient. Int. J. Pharm. 587, 119678.

Martinez, M.N., Sinko, B., Wu, F., Flanagan, T., Borbas, E., Tsakalozou, E., Giacomini, K. M., 2022. A critical overview of the biological effects of excipients (Part I): impact on gastrointestinal absorption. AAPS J. 24, 60.

Moesgaard, L., Reinholdt, P., Nielsen, C.U., Kongsted, J., 2022. Mechanism behind Polysorbates' Inhibitory Effect on P-Glycoprotein. Mol. Pharm.

Mohammadzadeh, R., Baradaran, B., Valizadeh, H., Yousefi, B., Zakeri-Milani, P., 2014. Reduced ABCB1 expression and activity in the presence of acrylic copolymers. Adv Pharm Bull 4, 219–224.

Mutoh, K., Tsukahara, S., Mitsuhashi, J., Katayama, K., Sugimoto, Y., 2006. Estrogen-mediated post transcriptional down-regulation of P-glycoprotein in MDR1-transduced human breast cancer cells. Cancer Sci. 97, 1198–1204.

Nerurkar, M.M., Burton, P.S., Borchardt, R.T., 1996. The use of surfactants to enhance the permeability of peptides through Caco-2 cells by inhibition of an apically polarized efflux system. Pharm. Res. 13, 528–534.

Rege, B.D., Kao, J.P., Polli, J.E., 2002. Effects of nonionic surfactants on membrane transporters in Caco-2 cell monolayers. Eur. J. Pharm. Sci. 16, 237–246.

Reker, D., Blum, S.M., Steiger, C., Anger, K.E., Sommer, J.M., Fanikos, J., Traverso, G., 2019. "Inactive" ingredients in oral medications. Sci. Transl. Med. 11.

Reker, D., Shi, Y., Kirtane, A.R., Hess, K., Zhong, G.J., Crane, E., Lin, C.H., Langer, R., Traverso, G., 2020. Machine learning uncovers food- and excipient-drug interactions. Cell Rep. 30 (3710–3716), e3714.

Rigalli, J.P., Tocchetti, G.N., Weiss, J., 2019. Modulation of ABC transporters by nuclear receptors: physiological, pathological and pharmacological aspects. Curr. Med. Chem. 26, 1079–1112.

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, 319–331.

Sadik, R., Abrahamsson, H., Stotzer, P.O., 2003. Gender differences in gut transit shown with a newly developed radiological procedure. Scand. J. Gastroenterol. 38, 36–42.

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. Pharm. Res. 20, 1984–1988.

Shchulkin, A.V., Yakusheva, E.N., Chernykh, I.V., Nikiforov, A.A., 2017. The role of testosterone in the regulation of P-glycoprotein functioning. Biol. Bull. 44, 524–530.

Sun, W., Xie, C., Wang, H., Hu, Y., 2004. Specific role of polysorbate 80 coating on the targeting of nanoparticles to the brain. Biomaterials 25, 3065–3071.

Wandel, C., Kim, R.B., Stein, C.M., 2003. "Inactive" excipients such as Cremophor can affect in vivo drug disposition. Clin. Pharmacol. Ther. 73, 394–396.

Wang, H., Zhou, L., Gupta, A., Vethanayagam, R.R., Zhang, Y., Unadkat, J.D., Mao, Q., 2006. Regulation of BCRP/ABCG2 expression by progesterone and 17beta-estradiol in human placental BeWo cells. Am. J. Physiol. Endocrinol. Metab. 290, E798–E807.

Yu, Y.B., Taraban, M.B., Briggs, K.T., Brinson, R.G., Marino, J.P., 2021. Excipient innovation through precompetitive research. Pharm. Res. 38, 2179–2184.

Zhang, Y., Huo, M., Zhou, J., Xie, S., 2010. PKSolver: An add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. Comput. Methods Programs Biomed. 99, 306–314.

Zhang, H., Yao, M., Morrison, R.A., Chong, S., 2003. Commonly used surfactant, Tween 80, improves absorption of P-glycoprotein substrate, digoxin, in rats. Arch. Pharm. Res. 26, 768–772.