Excipient-mediated alteration in drug bioavailability in the rat model depends on the sex of the animal

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1. INTRODUCTION

Pharmaceutical excipients are usually considered as "inactive" ingredients according to pharmaceutical regulations and standards (Bhattacharyya, 2006). However, a number of excipients seem to influence drug absorption. For example, the emulsifying agent cholesterol increases the fluidity of cancer cell membranes (Baggetto and Testa-Parussini, 1990), the suspending agent carrageenan induces inflammatory reactions in rats and mice (Farges et al., 2006, Halici et al., 2007), while the filler mannitol reduces small intestine transit time in a dose-dependent manner (Adkin et al., 1995a, Adkin et al., 1995b). In addition to dose-dependency, the influence of excipients on drug bioavailability can also be sex-dependent. For instance, the absolute bioavailability of the drug celastrol was two-fold higher in the presence of carboxymethylcellulose sodium (CMC-Na) in female rats, while there was no change in male rats (Zhang et al., 2012). In another study, the area under the curve (AUC) of the drug γ-schizandrin following oral administration of pure γ-schizandrin solution (dissolved in water) was 20 times higher in male rats compared to female ones. Surprisingly, an opposite trend was observed when γ-schizandrin was administered as a solid dispersion with PVP K30 or in a capsule prepared in-house, where AUC in female rats was 6-fold higher than in male ones from both γ-schizandrin formulations (Xu et al., 2008, Zhao, 2010). Though the mechanism of this sex-based difference has not been identified, the surprising influence on γ-schizandrin bioavailability could have been caused by the excipients in the formulations, such as PVP K30 in the solid dispersion or starch in the capsule formulation. Clearly, excipients and sex of the organism have a major influence on drug bioavailability and this warrants further investigation.

In our laboratories, we are investigating the sex-based influences of polyethylene glycol 400 (PEG 400) on oral drug bioavailability. PEG 400 is a widely used excipient which is typically employed as a solubility enhancer to improve the dissolution and subsequent bioavailability of poorly-soluble drugs. We have previously shown its sex-related influence on the pharmacokinetics of ranitidine in humans (Ashiru et al., 2008) as well as in rats (Afonso-Pereira et al., 2016), a commonly used animal model. In both humans and rats, PEG 400 had a dose-dependent effect on ranitidine bioavailability in males but not in females.

It is possible that this sex-specific influence of PEG 400 on ranitidine bioavailability could be related to the latter's mechanisms of absorption. Ranitidine is predominantly absorbed via the paracellular pathway and membrane transporters (both influx and efflux transporters are involved) (Bourdet and Thakker, 2006). Therefore, the bioavailability-enhancing effect of PEG 400 in males could be due to the opening of tight junctions by PEG 400 and/or its interactions with membrane transporters. It is known that ranitidine is a substrate for the organic cation uptake transporters (OCTs) (Ming et al., 2009, Han et al., 2013), as well as the efflux transporter P-glycoprotein (P-gp) (Cook and Hirst, 1994, Collett et al., 1999). Meanwhile, 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). Additionally, the activity and expression of P-gp has been reported to be different in males and females (Mariana et al., 2011).

Consequently, we hypothesized that the observed sex-related influence of PEG 400 on the bioavailability of ranitidine could be due to its interaction with the efflux membrane transporter P-gp. The aim of the work discussed in this paper was to test this hypothesis by:

i) determining the influence of PEG 400 on the bioavailability of another P-gp substrate (ampicillin) and a non-P-gp substrate (metformin) in male and female rats,

ii) determining the influence of PEG 400 on drug bioavailability in the presence of a P-gp inhibitor (the immunosuppressive agent cyclosporine A).

Ampicillin, metformin and cyclosporine A have been reported to be a P-gp substrate, a non-P-gp substrate and a P-gp inhibitor, respectively (Siarheyeva, 2006, Song et al., 2006, Liow et al., 2007). Also, considering the PEG 400 inhibition on some uptake transporters such as OATPs (Engel et al., 2012), metformin was chosen because it is also transported via the same uptake transporter, organic cation transporters (OCTs), as ranitidine (Chen et al., 2010). Furthermore, all drugs tested in this study are transported by the paracellular pathway. Thus, any possible influence of PEG 400 on the paracellular pathway would be observed, if applicable. (Lafforgue et al., 2008, Alvi and Chatterjee, 2014) (Details of absorption mechanisms are shown in Table 1).

2. MATERIALS AND METHODS

2.1 Materials and Animals

Metformin hydrochloride and ampicillin sodium were obtained from USV Ltd. (Mumbai, India) and VWR International (Lutterworth, UK), respectively. Cyclosporine A was purchased from Cambridge Bioscience (Cambridge, UK). Ranitidine hydrochloride, polyethylene glycol 400, sodium dodecyl sulfonate and HPLC-grade water were supplied by Sigma-Aldrich (Dorset, UK). HPLC-grade reagents such as acetonitrile, methanol and glacial acetic acid were obtained from Fisher Scientific (Loughborough, UK). Analytical grade reagents such as ammomium acetate and sodium dihydrogen phosphate were procured from VWR International (Lutterworth, UK). Male and female Wistar rats (10 weeks old, approx. 250g and 200g respectively), were purchased from Harlan UK Ltd (Oxfordshire, UK).

2.2 Drug Solution Preparation

Ampicillin and metformin were used at a concentration of 50mg/kg, which was the same dose as ranitidine used in the previous human and rat studies. PEG 400 was used at a dose of 26mg/kg as this caused the greatest enhancement in ranitidine bioavailability in rats (Afonso-Pereira et al., 2016).

Ampicillin, metformin and ranitidine solutions containing 25mg/mL of drugs in the absence or presence of 13mg/mL of PEG 400 were prepared with distilled water. CsA was suspended in water at 25mg/mL.

2.3 Influence of PEG 400 on Drug Bioavailability in the Absence or Presence of P-gp Inhibitor

All the animal work was conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act, 1986. The rats were housed at room temperature (25℃) and in a light-dark cycle of 12h. They were caged in groups of six, allowed to move freely and provided with food and water before the experiment. The day before the experiment, they were fasted overnight and individually housed in metabolic cages.

On the day of the experiment, each rat was weighed and administered an aqueous solution (see details in following sections) by oral gavage. Subsequently, approximately 250μL-300μL of blood was collected from the rats' tail vein into anticoagulant centrifuge tubes (BD Microtainer® K2E Becton, Dickinson and Company, USA) at 0.5, 1.25, 2, 3, 4 and 6h. At 8h post-administration, the rats were killed in a CO_2 euthanasia chamber and about 1mL of blood was taken by cardiac puncture.

2.3.1 Effect of PEG 400 on the Bioavailability of Ampicillin and Metformin in the Absence of P-gp Inhibitor

Each rat was administered an appropriate volume of aqueous solution, corresponding to a dose of 50mg/kg ampicillin or metformin with or without 26mg/kg PEG 400.

2.3.2 Effect of PEG 400 on the Bioavailability of Ampicillin, Metformin and Ranitidine in the Presence of P-gp Inhibitor

The rats were orally administered an appropriate volume of cyclosporine A suspension for a dose of 50mg/kg. Fifteen minutes later, a solution of ampicillin, metformin or ranitidine at a dose of 50mg/kg in the absence or presence of PEG 400 (at 26mg/kg) was administered via oral gavage. After dosing, the rats were placed individually in a metabolic cage and were allowed to move freely until blood collections.

Fifteen minutes before drug administration was chosen as the appropriate time to give the P-gp inhibitor following a study on the influence of timing using ranitidine as the drug. In this study, P-gp inhibition was conducted by orally administering cyclosporine A to animals immediately or 15min, 30min or 60min before dosing with 50mg/kg ranitidine in the absence or presence of 26mg/kg PEG 400. The results (supplementary Figure A) showed the greatest inhibitory influence of CsA on ranitidine bioavailability, when CsA was administered 15min prior to ranitidine administration.

2.4 Preparation of Blood Samples

Blood samples were centrifuged at 10,000rpm for 10min, and the supernatants (plasma samples) were collected into 1.5mL Eppendorf tubes.

For ampicillin and metformin, 100µL of acetonitrile or methanol was added to 100µL of plasma samples respectively, to precipitate the protein. The mixtures were vortex-mixed for 6s and centrifuged in a Centrifuge 5804R (Eppendorf AG, 22331 Hamburg, Germany) at 13,000rpm for 10min. The supernatant was then collected and a 50µL aliquot of each sample was analysed by HPLC.

For ranitidine, the samples were prepared using a reported method (Afonso-Pereira et al., 2016). 50µL of the supernatant was placed into a 1.5mL Eppendorf tube, and the same volume of acetonitrile was added to precipitate the plasma proteins. After 1 min of vortex-mixing, 100µL HPLC grade water was added to the mixture, which was vortex-mixed again for 30s, and centrifuged at 4ºC for 10 min at 10000rpm. The supernatant was collected and 40µL aqueous was analysed by HPLC.

2.5 HPLC Analysis

Chromatographic analysis was performed with a HPLC system (Agilent Technologies, 1260 Infinity) equipped with pump (model G1311C), autosampler (model G1329B), and a diodearray UV detector (model G1314B). The methods were summarized in Table 2.

In the case of ampicillin and metformin, drugs were quantified by HPLC using a Luna C18 (250mm×4.6mm I.D./5µm) column (Phenomenex, UK) and the flow rate was 1mL/min. Ampicillin was quantified using 10mM sodium dihydrogen phosphate buffer (pH 7.0)-methanol (60:40, v/v), while metformin was measured using a mobile phase consisting of 10mM sodium dihydrogen phosphate buffer with 10mM sodium dodecyl sulfonate and acetonitrile (60:40, v/v). The UV detector was set at 220nm (for ampicillin) and at 234nm (for metformin). Linear calibration curves were obtained at concentrations ranges of 20-2000ng/mL for both compounds. The retention times for ampicillin and metformin were 15.3 and 12.6min respectively.

In the case of ranitidine, the sample 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.

2.6 Pharmacokinetic Analysis

Pharmacokinetic parameters, (C_{max}, t_{max}, AUC_{0-480}, AUC_{∞}, CL, Vd and $t_{1/2}$) were calculated by non-compartmental analyses using a free Microsoft Excel add-in, "PKSolver." (Zhang et al., 2010).

2.7 Statistical Analysis

All results are expressed as mean ± SD (n = 6). 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). Repeated measures ANOVA was conducted to assess any statistically significant differences along the absorption profiles in Figure 1 and Figure 3.

3. RESULTS AND DISCUSSION

3.1 Effect of PEG 400 on the Bioavailability of Ampicillin, a P-gp substrate

The influences of PEG 400 on ampicillin absorption in male and female rats are shown in Figure 1-2 and Table 3. It can be seen that ampicillin absorption profiles and pharmacokinetic parameters were similar for males and females in the absence of PEG 400 ($p>0.05$). The presence of PEG 400 caused no changes in the drug absorption in females ($p>0.05$). In contrast, PEG 400 had a considerable influence on ampicillin absorption in male rats ($p<0.05$ for t_{max}, C_{max} and AUC), where ampicillin absorption was more rapid and more extensive compared to the control, such that t_{max} occurred earlier at 38min compared to 120min, C_{max} was almost doubled and the AUC increased by 58%.

This sex-based influence of PEG 400 on ampicillin absorption was the same as that seen with ranitidine, where the latter's bioavailability was increased by 49% in male rats but was unchanged in female ones (Afonso-Pereira et al., 2016). The similar increases in drug bioavailability in male rats for ranitidine and ampicillin indicate that the mechanisms responsible for the sex-specific influence of PEG 400 may be the same. The absorption of ranitidine and ampicillin are known to be controlled by the intestinal efflux transporter P-gp

(Collett et al., 1999, Siarheyeva, 2006), while PEG 400 is also known to influence the P-gp efflux transporter (Cook and Hirst, 1994). From these, we hypothesize that the sex-specific influence of PEG 400 on the absorption of ampicillin and ranitidine is mediated by the influence of PEG 400 on the efflux transporter P-gp. To test this hypothesis, we investigated the sex-based influence of PEG 400 on the absorption of a drug whose absorption is not controlled by P-gp. Metformin (a non-Pgp substrate) was consequently chosen as a model drug and its absorption in the absence and presence of PEG 400 is discussed in the following section.

3.2 Effect of PEG 400 on the Bioavailability of Metformin, a non-P-gp substrate

The influences of PEG 400 on metformin are shown in Figures 3-4 and Table 4. It can be seen that the presence of PEG 400 caused no change in metformin bioavailability in either males or females ($p>0.05$). As expected, any influence of PEG 400 on P-gp had no resulting effect on the absorption of metformin, which is not a substrate for P-gp. In order to further investigate the role of P-gp, a P-gp inhibitor (CsA) was used as discussed in the following section.

3.3 Influence of P-gp Inhibition on the Bioavailability of Ampicillin, Ranitidine and Metformin

We hypothesized that if the sex-related influence of PEG 400 on the bioavailability of certain drugs was a result of its influence on P-gp, blocking the latter with a P-gp inhibitor would remove the effect of PEG 400 on the bioavailability of the P-gp substrates (ampicillin and ranitidine), but would have no influence on the absorption of the non-P-gp substrate (metformin). To test this hypothesis, male and female rats were pre-treated with the P-gp blocker cyclosporine A at 15min prior to administration of ampicillin, ranitidine or metformin in the absence or presence of PEG 400.

As expected, pre-treatment with cyclosporine A did not change the bioavailability of metformin in either male or female rats ($p>0.05$) (shown in Table 5 and Figure 5). In contrast, pre-treatment with cyclosporine A increased ampicillin bioavailability in both males and females ($p<0.05$), with the percentage increase in bioavailability being higher in males than in females (132% vs 42%). Addition of PEG 400 in cyclosporine A pre-treated rats had no significant effect on the drug bioavailability in either male or female rats, compared to the cyclosporine A pre-treated

rats ($p>0.05$) (Table 5 and Figure 6), i.e. once P-gp have been blocked by cyclosporine A, PEG 400 had no influence as its site of action was not available. The results are in a good agreement with our hypothesis that sex-specific influence of PEG 400 on drug bioavailability are mediated via the action of PEG 400 on the efflux transporter P-gp.

Similar results were observed for ranitidine (Table 5 and Figure 7). When the rats were pre-treated with cyclosporine A 15min before being given ranitidine, the ranitidine $AUC_{0\text{-}480}$ was increased by 113% in males and 42% in females, respectively, compared with the controls (which only received ranitidine). Meanwhile, in the cyclosporine A pre-treated rats, no effect of PEG 400 on ranitidine bioavailability was observed in either male or female rats.

3.4 Different Extents of P-gp in Males and Females

As seen in Figures 1-7 and Tables 3-5, the influence of PEG 400 and cyclosporine A on the bioavailability of P-gp substrates depends on the animals' sex, i.e. a greater response was observed in male rats compared to female ones, suggesting higher P-gp activity and/or expression in male rats than female ones.

Higher P-gp activity in males compared to females has previously been suggested in a study, where the effect of PSC833 (a P-gp inhibitor) on the intestinal transport of two P-gp-mediated drugs, ivermectin and Rho 123, was investigated using tissues from male and female rats (Ballent et al., 2012, Mariana et al., 2011). Greater ivermectin accumulation was observed in male intestines (by 141%) compared to female ones (by 14%). Similar results were observed for Rho 123, in which its efflux ratio significantly decreased from 4.33 to 1.51 in males ($p<0.05$) but not in females. While the reasons for greater P-gp activity in males compared to females have not been established, we speculate that the differential P-gp activity is modulated by P-gp modifying-mechanisms which may themselves be influenced by the sex of organism. For example, cyclosporine A alters the P-gp activity by inhibiting both the substrate stimulated- and the basal- P-gp ATPase (Watanabe et al., 1997), while PEG influences the function of P-gp by producing mitochondrial toxicity and depleting the amount of intracellular ATP (Johnson et al., 2002). Sex-related differences in the variations in P-gp ATPase or ATP level in cells could cause sex differences in the interaction between cyclosporine A/PEG and P-gp. Further work is

needed to clarify the mechanisms underlying this phenomenon.

In addition to P-gp activity, sex-related differences in P-gp expression could also explain our results. Sex difference in the expression of P-gp was first proposed in liver (Schuetz et al., 1995) and later reported in the intestine (Gerrard et al., 2004), where men were reported to have higher hepatic and enterocyte P-gp content than women. However, no sex-based difference in P-gp expression have been proposed in the upper duodenum (Paine et al., 2005). Meanwhile, no sex-related differences in the intestinal P-gp protein content and RNA expression were found (MacLean et al., 2008). The fact that rats were fed in this study (while fasted rats were used in our in vivo studies) shows that the possibility of sex-based differences arising in the intestines of fasted rats cannot be excluded, given that the fed/fasted states have been shown an impact on P-gp and other membrane transporters (Deferme and Augustijns, 2003, Deferme et al., 2003, Furumiya and Mizutani, 2008).

P-gp activity has been found to be largely similar in rats and humans (Li et al., 2015, Li et al., 2017). Based on this, the observed influence of PEG 400 (in this paper) on the bioavailability of P-gp substrates has implications for drug usage in humans. Indeed, we have already shown differential effects of PEG 400 on the oral bioavailability of ranitidine in men and women (Ashiru et al., 2008). It suggested that the bioavailability of a drug which is a P-gp substrate and which is formulated with PEG 400 as an excipient will be higher in men compared to women, with the possibility that the therapeutic level will not be reached in women. There may also be repercussions in polypharmacy. For example, if a medicine containing a non-P-gp substrate drug and PEG 400 as excipient is co-administered with another medicine which contains a P-gp substrate, the bioavailability of the latter could potentially be increased by the PEG 400, which could lead to toxic levels of the P-gp substrate in men.

4. CONCLUSION

The work reported in this paper further enhances our understanding of the influence of the supposedly inactive excipient PEG 400 on drug bioavailability, specifically its different action depending on the sex of the organism. We confirmed that PEG 400 had a greater drug

bioavailability-enhancing influence in males compared to females, such as ranitidine and ampicillin. Importantly, the sex-related influence of PEG 400 occurred only for drugs whose absorption is controlled by the efflux transporter P-gp. Thus, blocking of P-gp by cyclosporine A (a P-gp inhibitor) eliminated the effect of PEG 400 on the bioavailability of ampicillin and ranitidine. Cyclosporine A blocking of P-gp had its own sex-related effect on drug bioavailability. This differential sex-based influence on drug bioavailability could be due to greater P-gp activity and/or expression in males compared to females, as suggested by some existing literature. Given that many compounds are P-gp substrates and/or modulate P-gp activity, increasing attention to this topic is needed for an optimal usage of excipients.

Since the WHO Prequalification of Medicines Programme implemented biowaivers based on the Biopharmaceutics Classification System (BCS) in 2008, the European Medicines Agency (EMA) extended the BCS-based biowaiver to BCS Class III drugs (high solubility and poor permeability), such as ranitidine (Tsume and Amidon, 2010). However, the findings in our study have significant implications for the use of supposedly "inactive" excipients and also highlight the influence of an organism's sex. Excipients can affect the activity of transporters, thereby alter drug bioavailability, herapeutic efficacy and adverse side effects, which may be different in males and females.

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