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International Journal of Pharmaceutics 305 (2005) 105–111
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Porcine vaginal mucosa as an in vitro permeability model for human vaginal mucosa

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Received 3 June 2005; received in revised form 24 August 2005; accepted 3 September 2005
Available online 13 October 2005

Abstract

The availability of human tissue for experimental purposes is often problematical and use is thus made of animal tissue as models of the human tissue. In this study, porcine vaginal mucosa was used as an in vitro permeability model for human vaginal mucosa using tritium-labelled permeants (17β-estradiol, r-arecoline, vasopressin, oxytocin and water). Fresh porcine and human vaginal tissues were frozen in liquid nitrogen and stored at −85 °C. In vitro permeability studies were performed using a flow-through diffusion apparatus (24 h, 20 °C, 1.5 ml/h). The mean steady state flux values for water, r-arecoline and vasopressin were approximately 4, 12 and 5% lower, while those for 17β-estradiol and oxytocin were approximately 17 and 53% higher, through porcine vaginal mucosa as compared to human vaginal mucosa, respectively. Using a *F*-test (comparing whole curves), statistically significant differences in the diffusion of 17β-estradiol, r-arecoline and oxytocin were indicated when comparing human and porcine vaginal mucosa. Generally, porcine vaginal mucosa seems a good in vitro permeability model for human vaginal mucosa. However, permeability of these two mucosa towards all permeants tested does not always correspond closely. These differences must always be considered when using porcine tissue as an in vitro permeability model for human vaginal mucosa.

Keywords: Human; Mucosa; Permeability; Porcine; Vaginal

1. Introduction

The non-invasive vaginal route of drug administration has gained a lot of interest in recent years. The vaginal mucosa contains a dense network of blood vessels, making it an excellent route for delivering drugs in women for both local and systemic indications (Alexander et al., 2004; Justin-Temu et al., 2004; Hussain and Ahsan, 2005). The main advantages of this route of administration are ease of drug application, immediate availability to the systemic circulation because first-pass drug metabolism is bypassed and permeability towards peptides and proteins (Alexander et al., 2004; Justin-Temu et al., 2004; Hussain and Ahsan, 2005). Gastric acid- or digestive enzyme-mediated degradation within the gastrointestinal tract are also avoided. Furthermore, the presence of food in the stomach as well as the gastric emptying rate, do

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doi:10.1016/j.ijpharm.2005.09.002

not have any effect when this route of drug administration is used (Chen et al., 1999). There are, however, some drawbacks associated with the vaginal route, e.g. cultural sensitivity, personal hygiene, gender specificity, local irritation and influence of sexual intercourse (Hussain and Ahsan, 2005). Changes in the thickness of the vaginal epithelium also influence the rate and extent of absorption of intravaginally applied drugs (Hussain and Ahsan, 2005). The vagina and uterus have extensive vascular connections and a "first uterine pass effect" has been hypothesized when the vaginal route is used for hormone administration (Alexander et al., 2004).

Several drugs for intravaginal use have been approved for the treatment of local conditions, e.g. metronidazole, tioconazole, clotrimazole and spermicides (e.g. nonoxynol-9) (Lee and Chien, 1996; Alexander et al., 2004; Hussain and Ahsan, 2005). A number of vaginally absorbed drugs achieve serum levels high enough to produce systemic effects, e.g. misoprostol, oxytocin, dinaprostone, sildenafil, bromocriptine, indomethacin, contraceptives and hormone therapy preparations (Alexander et al., 2004; Hussain and Ahsan, 2005). The delivery systems used for the vaginal route include solutions (foams, douches), aerosols, semisolids (creams, ointments, gels), tampons, tablets, capsules, pessaries, suppositories, particulate systems, intravaginal rings, sponges and powders (Justin-Temu et al., 2004). The focus of current research on intravaginal drug delivery systems includes those being developed for preventing sexually transmitted diseases (STDs) and HIV infection. These systems are being developed to deliver topical intravaginal formulations of anti-HIV agents or microbicides in order to prevent mucosal and perinatal virus transmission (Alexander et al., 2004).

Studies have been done on the diffusion of toxic shock syndrome toxin-1 across various epithelial barriers, e.g. rat-, porcine- and rabbit vaginal mucosa, human intestinal epithelial cells and porcine endothelium (Davis et al., 2003; Peterson et al., 2005). Of these, only the porcine vaginal mucosa closely resembles the human vaginal mucosa. These two tissues share important similarities, i.e. both types of mucosa consist of stratified squamous epithelium that is supported by connective tissue lamina propria and also appear to have similar lipid compositions that are important for barrier function (van der Bijl et al., 1997; Kremer et al., 2001; Thompson et al., 2001). It is important to specifically use an animal model system that is comparable with human vaginal mucosa when performing in vitro permeability studies on drugs destined for human use.

In this study, in vitro permeability properties of human and porcine vaginal mucosa towards various permeants (water, 17β-estradiol, vasopressin, r-arecoline (reduced arecoline) and oxytocin), compounds of different lipophilicity and molecular weight, were compared. It was envisaged that the data would help to assess the predictive value of porcine vaginal mucosa as an in vitro permeability model for human vaginal mucosa.

2. Materials and methods

2.1. Porcine vaginal mucosa

Vaginal mucosal specimens were obtained from five freshly slaughtered pigs at the Maitland Abattoir, Maitland, Western Cape, South Africa.

2.2. Human vaginal mucosa

Specimens were obtained from excess tissue removed from seven postmenopausal patients, aged 40–70 years (mean age 61 ± 11 years S.D.), following vaginal hysterectomies at the Louis Leipoldt Hospital, Bellville, South Africa.

All the specimens were transferred to our laboratory within 1 h after being placed in a transport fluid, prepared as previously described (van der Bijl et al., 1997, 1998a,b, 2001; van Eyk and van der Bijl, 2004). After trimming away excess connective and adipose tissue, all specimens were snap-frozen in liquid nitrogen and stored at $-85\,^{\circ}C$. No specimens were obtained where there was clinical evidence of any disease that might have influenced the permeability characteristics of the mucosa. The study was approved by the Ethics Committee of Stellenbosch University and the Tygerberg Academic Hospital.

2.3. Permeability experiments

The diffusion kinetics of tritium-labelled water, 17β-estradiol, r-arecoline, oxytocin and vasopressin through human and porcine vaginal mucosa were determined. Prior to the experiment, frozen specimens were

thawed in PBS (pH 7.4) for 10 min. The thawed mucosal specimens were carefully cut, so as not to damage the epithelial surfaces, into 4 mm diameter sections. The mucosal sections were then mounted in flow-through diffusion cells (exposed areas 0.039 cm^2) as previously described and permeation studies performed on seven tissue replicates for each specimen (van der Bijl et al., 1997, 1998a,b, 2001; van Eyk and van der Bijl, 2004). Before each permeability experiment was started, tissue disks were equilibrated for 10 min in PBS (pH 7.4) at 20 °C in both the donor and acceptor compartments of the diffusion cells. The PBS was removed from the donor compartment and 1.0 ml of PBS added, containing either 1 μCi ^{3}H-water, 1.4 μCi ^{3}H-17β-estradiol, 0.5 μCi ^{3}H-reduced arecoline (r-arecoline), 0.15 μCi ^{3}H-oxytocin or 0.093 μCi ^{3}H-vasopressin. Tritium-labelled reduced arecoline was used in these studies because the labelled parent compound (arecoline) was not available commercially. Perkin-Elmer Life Sciences Inc. (Boston, MA) supplied the ^{3}H-oxytocin and all the other radioisotopes were obtained from Amersham Laboratories (Little Chalfont, Amersham, UK). Aliquots (100 μl) were removed within minutes from each of the seven donor compartments for the determination of donor cell concentration at time zero. Experiments were conducted at 20 °C and fractions collected by means of a fraction collector, at 2-h intervals for 24 h and a flow-rate of 1.5 ml/h. All permeability studies were performed under sink conditions, i.e. at the completion of each run the concentration of tritiated permeant in the acceptor chamber never reached 10% of that in the donor compartment. To each sample collected, 10 ml scintillation cocktail (PCS scintillation cocktail; Amersham Biosciences, SE-75184, Uppsala, Sweden) was added and the radioactivity determined using a liquid scintillation counter (Beckman LS 5000TD). The counting of the samples was continued until a 2-s value of 1% was reached. Quenching for each sample was automatically corrected for in the counter.

2.4. Calculation of flux values

The following relationship was used to calculate the flux values (J) across the membranes:

$$J = \frac{Q}{A} \times t \,(\mathrm{dpm\,cm^{-2}\,min^{-1}})$$

where Q is the quantity of substance crossing membrane (in dpm), A the membrane area exposed (in cm^2) and t is the time of exposure (in min).

2.5. Steady state kinetics

Steady state (equilibrium kinetics) was assumed for a particular mucosal specimen and tritiated permeant, when no statistically significant differences ($P < 0.05$, t-test) between flux values were obtained over at least two consecutive time intervals.

2.6. Statistical analysis

Non-linear regression analyses (third order polynomials) were performed using a GraphPad Prism, Version 4, 2003, computer programme. A F-test was used to compare entire curves (Motulsky, 1995). A significance level of 5% was used. Because 17β-estradiol, oxytocin (through human vaginal mucosa only) and vasopressin did not reach steady state during the 24 h time course of the experiments, estimated steady state flux values were used (mean of the flux values obtained at 20, 22 and 24 h).

3. Results

The mean flux values of water, 17β-estradiol, r-arecoline, vasopressin and oxytocin across human and

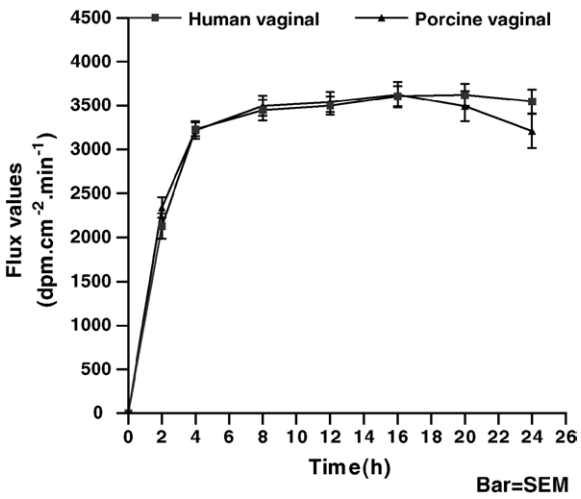

Fig. 1. Overall mean flux values of water across human vaginal and porcine vaginal mucosa.

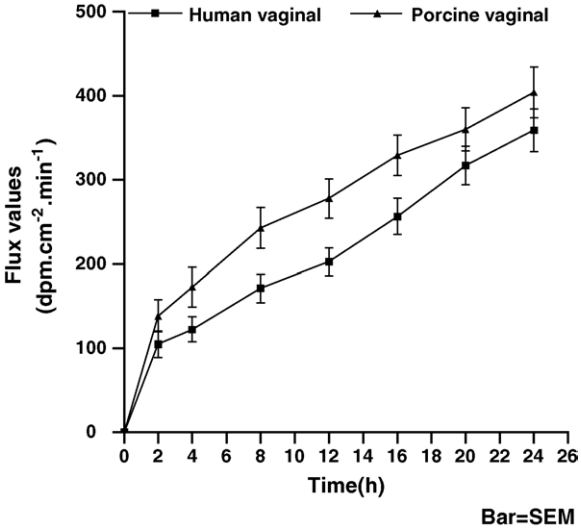

Fig. 2. Overall mean flux values of 17β-estradiol across human vaginal and porcine vaginal mucosa.

porcine vaginal mucosa versus time are shown in Figs. 1–5. Steady state for water and r-arecoline were obtained after approximately 8 and 20 h, respectively, across both human and porcine vaginal mucosa. Oxytocin steady state through porcine vaginal mucosa was reached at approximately 16 h, while steady state was not reached through human vaginal mucosa. Steady state was also not reached for 17β-estradiol and vasopressin across both types of mucosa. Estimated values for this parameter were then used (means of values obtained at 20, 22 and 24 h) in each case. The mean and mean estimated steady state flux values for water, r-arecoline and vasopressin were approximately 4, 12 and 5% lower, while those for 17β-estradiol and oxytocin were approximately 17 and 53% higher, through porcine vaginal mucosa compared to human vaginal mucosa, respectively (Fig. 6). Using a *F*-test and comparing whole curves, statistically significant differences between the flux values of 17β-estradiol ($P = 9.2 \times 10^{-4}$), r-arecoline ($P = 1.3 \times 10^{-2}$) and oxytocin ($P = 2.0 \times 10^{-21}$) were found for human and porcine vaginal mucosa. No

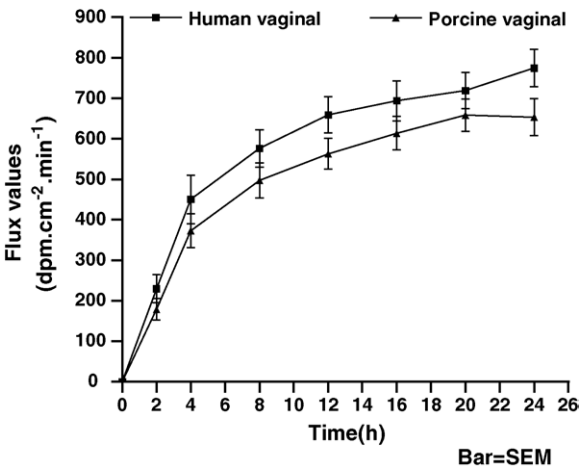

Fig. 3. Overall mean flux values of r-arecoline across human vaginal and porcine vaginal mucosa.

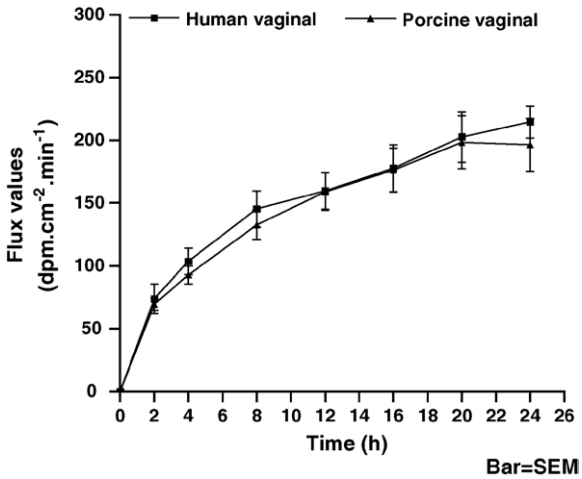

Fig. 4. Overall mean flux values of vasopressin across human vaginal and porcine vaginal mucosa.

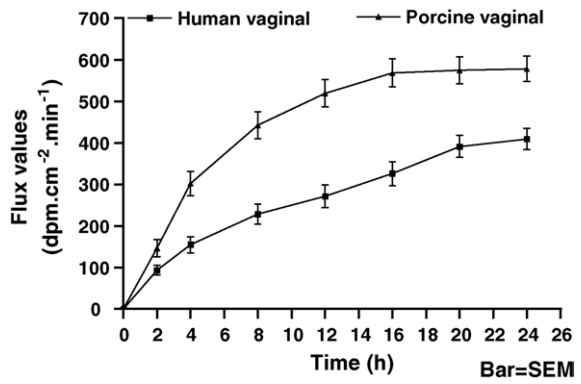

Fig. 5. Overall mean flux values of oxytocin across human vaginal and porcine vaginal mucosa.

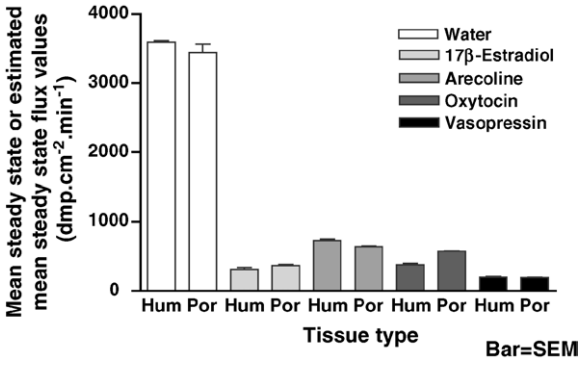

Fig. 6. Mean steady state and mean estimated steady state flux values of water, 17β-estradiol, r-arecoline, oxytocin and vasopressin through human and porcine vaginal mucosa. (Hum, human vaginal mucosa; Por, porcine vaginal mucosa.)

statistically significant differences could be shown for the diffusion of water ($P = 6.9 \times 10^{-1}$) and vasopressin ($P = 9.9 \times 10^{-1}$) through the two types of tissues.

4. Discussion

Administration of drugs other than via the oral route has attracted a great deal of attention over the last decade. Some of the alternatives investigated have included transmucosal buccal and vaginal delivery (Sandri et al., 2004). Diffusion of drugs through any mucosal is influenced by the physiochemical properties of the agent tested, i.e. molecular weight, solubility, dissolution rates, lipophilicity, ionisation characteristics and chemical stability as well as characteristics of the membrane (Williams, 2003; Justin-Temu et al., 2004; van der Bijl and van Eyk, 2004). Physiological factors also play a role in the diffusion process, e.g. cyclic changes in the thickness of the vaginal epithelium, fluid production volumes and composition as well as vaginal pH (Hussain and Ahsan, 2005). When the vaginal fluid volume is higher, absorption of poorly water-soluble drugs may be increased. Changes in the vaginal pH, might also have an effect on the release of pH sensitive drugs from vaginal delivery systems (Hussain and Ahsan, 2005). Lipophilic molecules will permeate faster than hydrophilic molecules and it has been suggested that, for the vaginal mucosa, the molecular weight cut-off points for permeation may be higher than that found for other mucosal surfaces (Williams, 2003; Justin-Temu et al., 2004). According to the pH partition hypothesis, ionisation will also affect the absorption of drugs and those in an unionised state will penetrate membranes more freely (Williams, 2003; Justin-Temu et al., 2004).

Chemical compounds cross the epithelial cell layers via two major pathways, i.e. the intercellular (paracellular) pathway where the compounds pass between cells and the intracellular (transcellular) pathway through the cells. For mucosal delivery of most drugs, the intercellular route of diffusion seems to predominate. Two types of pathways exist within the intercellular space. One of these is the hydrophobic pathway through the lipid bilayers and the other is a parallel hydrophilic pathway along the narrow aqueous regions that are associated with the polar head groups of the lipids. As the hydrophobicity of a permeant molecule increases, so the penetration of the compound through the epithelial barrier seems to increase (Wertz and Squier, 1991; Harris and Robinson, 1992; Williams, 2003).

Porcine vaginal mucosa was found to be very similar in many respects to human vaginal mucosa, i.e. their lipid compositions are comparable and these two types of mucosa also show histological similarities (Kremer et al., 2001; Thompson et al., 2001; Davis et al., 2003). In the present study, the in vitro permeability behaviour of these two types of mucosa towards various permeants of different molecular weight, lipophilicity and charge (water, 17β-estradiol, vasopressin, r-arecoline and oxytocin), demonstrated a number of similarities, however, major differences were also detected. For hydrophilic molecules, e.g. water (18 Da) and vasopressin (1083 Da), porcine vaginal mucosa as an in vitro permeability model of human tissue was accurate. However, for molecules that were more lipid-soluble, the permeation behaviour of some of these started to differ. Statistically significant differences in the flux values, comparing whole curves and using the *F*-test, of r-arecoline (160 Da), oxytocin (1007 Da) and 17β-estradiol (272 Da), were found (Figs. 2, 3 and 5). For oxytocin, substantial differences in the flux values through the two types of mucosa were observed. Mean steady state flux values of oxytocin through porcine vaginal mucosa were found to be 53% higher than the corresponding estimated values through human

vaginal mucosa (Figs. 5 and 6). The diffusion rates of vasopressin through both types of mucosa were also much lower than those observed for oxytocin (Figs. 4 and 5).

Water is a standard marker used in in vitro permeability studies. It is not the preferred permeant to be used for comparative studies on paracellular diffusion because it permeates the barrier not only by passive paracellular diffusion, but also by osmosis (Nielsen and Rassing, 2000). This would explain the near identical results obtained for water flux values through both tissues (Fig. 1). The hormone, 17β-estradiol, is rapidly degraded by first pass metabolism and was chosen specifically because in vivo vaginal absorption ensures direct entry into the blood. Steady state flux values for 17β-estradiol were not achieved through porcine nor human vaginal mucosa. This may be due to the wider distribution of 17β-estradiol into the lipophilic domains of the mucosal membranes (van der Bijl et al., 1998a). Mean estimated steady state flux values of 17β-estradiol through porcine vaginal mucosa were 17% higher ($P < 0.05$) than through human vaginal mucosa (Figs. 2 and 6). Arecoline is a potentially deleterious tertiary amine compound found in areca nut (betel nut) chewed by specific populations all over the world. It has cholinomimetic properties, but also crosses the blood brain barrier and has a psychoactive effect (Asthana et al., 1996). The chemical and physical properties of r-arecoline (160 Da) are similar to the parent compound (155 Da) and these two compounds will most certainly not differ much in diffusion through mucosa. At physiological pH, r-arecoline ($pK_a = 6.8$) will most probably contain a fairly high proportion of unionised species (lipophilic) (van der Bijl et al., 2001). Mean steady state values of r-arecoline through human vaginal mucosa were found to be 12% higher ($P < 0.05$) than through porcine vaginal mucosa (Figs. 3 and 6).

Vasopressin and oxytocin are small peptides (nine amino acids), easily degraded by gastrointestinal enzymes when they are administered via the oral route. These two peptides differ in only two amino acids, at positions 3 and 8. Oxytocin has an isoleucine at position 3 whereas vasopressin contains a phenylalanine at this position. The most significant difference occurs at position 8, where vasopressin contains a large arginine group and oxytocin a medium sized lipophilic group, i.e. leucine. At physiological pH the vasopressin molecule is more positively charged because of the protinated arginine amino acid at position 8. This renders the entire vasopressin molecule more hydrophilic. Furthermore, due to the larger arginine group, the molecular weight of vasopressin would be higher than that of oxytocin. Penetration through the lipophilic barrier of the mucosal membrane would therefore be more limited and this would explain the lower flux values found for vasopressin when compared to oxytocin in these studies (Foye et al., 1995). The porcine vaginal epithelial barrier allows greater permeation of more hydrophobic permeant molecules (oxytocin and 17β-estradiol) than that found for the human vaginal epithelial barrier. Slight differences in hydrophobicity, ionisation state at pH 7.4 and molecular size of the permeant molecules used in this study, indicate that there are differences in the epithelial barrier properties of these two species.

5. Conclusions

In conclusion it can be stated that porcine vaginal mucosa is generally a good in vitro permeability model for human vaginal mucosa. This appears to be particularly the case for hydrophilic compounds, but molecular size and charge may make substantial differences, as in the case of oxytocin. When the model is used to test lipophilic drugs, however, special care must be taken when interpreting and extrapolating results to human tissues, because large differences in permeability towards these compounds may occur. This being the case, it would be advisable to first investigate the permeation of any prospective drug through both mucosae if the in vitro porcine permeability model is going to be used for obtaining data on drugs destined for human use.

Acknowledgements

This material is based upon work supported by the National Research Foundation under Grant number 2053880. The authors would also like to thank the Stellenbosch University for their financial support. Special thanks are extended to the Maitland Abattoir, Maitland, Western Cape, South Africa, for the provision of porcine vaginal mucosa and Dr. C le Grange for supplying human vaginal mucosa.

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