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Permeability of human intestinal mucosa using a continuous flow-through perfusion system

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Abstract

Continued interest in in vitro methods for performing bioavailability/bioequivalence (BA/BE) studies for drug registration purposes, prompted us to investigate the suitability of a continuous flow-through perfusion system to determine diffusion of a wide variety of permeants, through human intestinal mucosa. Permeability of fresh and frozen intestinal mucosa towards water, 17β-estradiol, sumatriptan, arecoline and vasopressin was compared. Furthermore, diffusion studies of water, sumatriptan, arecoline, arecaidine, estradiol, cyclosporin and vasopressin across frozen/thawed intestinal mucosa specimens (− 85 °C) were performed. No statistically significant differences between the flux values of the five compounds tested across fresh and frozen intestinal tissue, were found. Furthermore, it was demonstrated that the flux rates of the various compounds across these tissues decreased with increasing molecular size. However, the flux rates across frozen intestinal mucosa for compounds with molecular weights > 300 Da, were low. Flux rates for the compounds studied across frozen/thawed human vaginal and buccal mucosa were 36–160% higher than those across frozen intestinal mucosa. We concluded that the continuous flow-through perfusion system used shows promise as an in vitro method for permeability determination through intestinal mucosa. However, other human mucosa e.g. vaginal mucosa, may have to be considered as alternatives to intestinal mucosa if therapeutic agents with molecular weights > 500 Da are to be compared for in vitro BA/BE purposes, and further studies in this respect are warranted.

Keywords: Human intestinal mucosa; Permeability studies; Various compounds

1. Introduction

During recent years there has been ongoing international discussion regarding in vivo/in vitro bioavailability/bioequivalence (BA/BE) studies in the regulatory process for therapeutic agents. Two guidelines with new regulations were issued recently, the CPMP Note, for Guidance on the Investigation of Bioavailability and Bioequivalence (CPMP Note, 1998) in Europe and, the FDA Guidance for Industry 'Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System' in

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the USA (FDA Guidance, 2000). In the latter guidelines, one of the methods suggested for determining the permeability of a drug substance from the gastrointestinal tract includes in vitro permeation studies using excised intestinal tissues from human or animal origin.

Previous in vitro permeability studies have enabled us to thoroughly evaluate a continuous flow-through mucosal perfusion system to determine the diffusion characteristics of a wide variety of therapeutic agents and other chemical compounds through fresh and frozen human vaginal and buccal mucosa, skin and cornea (Van der Bijl et al., 1997, 1998a,b,d, 2000b,c, 2001a,b; Van der Bijl and Van Eyk, 2001). The main advantages of this system include the use of small tissue samples (4 mm ∅) and the maintenance of a continuous high gradient of permeant across the biological barrier membrane to be studied. We could find no information on the in vitro diffusion kinetics of a wide range of compounds, with varying molecular weights, across human intestinal mucosa using a continuous flow-through mucosal perfusion system.

Since in vitro diffusion experiments have potential applications in the proposed guidelines for BA/BE studies, the objective of the present study was to determine the suitability of the above mucosal perfusion system for studying the diffusion kinetics of a wide range of therapeutic agents and other compounds, differing in chemical nature, molecular size, conformation, hydrophilicity and degree of ionization, across freshly harvested and frozen/thawed human intestinal mucosa.

2. Materials and methods

2.1. Intestinal mucosa

Small intestine specimens (mean distance from duodenojejunal flexure: 92 ± 57 cm SD; range 20–150 cm) were obtained from excess tissue removed from eight patients (four females, mean age 62 ± 12 SD (range: 47–72) year and four males, mean age 57 ± 11 SD (range: 43–67) year), following various surgical procedures, from the Department of Surgery, Tygerberg Hospital. Surgical specimens were immediately placed in a transport fluid, prepared as previously described (Van der Bijl et al., 1997, 1998a,b,d, 2000b,c, 2001b; Van der Bijl and Van Eyk, 2001) and transferred to our laboratory within 1 h. On arrival in the laboratory, one half of the specimens was used immediately and the other half snap frozen in liquid nitrogen and stored at -85 °C for no longer than 1 month before use.

No specimens were included in the study where there was clinical evidence of any disease that might have influenced the permeability characteristics of the intestinal mucosa.

The study was approved by the Ethics Committee of the University of Stellenbosch and the Tygerberg Hospital.

2.2. Permeability experiments

Prior to each permeability experiment tissue specimens were either freshly used or thawed at room temperature in phosphate buffered saline (PBS, pH 7.9). Initially, the diffusion kinetics of tritium labelled water, 17β-estradiol, sumatriptan, arecoline and vasopressin through fresh and frozen intestinal mucosa were determined for comparitive purposes. Hereafter, frozen/thawed intestinal specimens were used for all the other permeability experiments with the various labelled compounds. After equilibration of the intestinal mucosal specimens in PBS, they were carefully cut, so as not to damage the epithelial surfaces, into sections (4 mm ∅) and mounted in flow-through diffusion cells (exposed areas 0.039 cm^2) as previously described (Van der Bijl et al., 1997, 1998a,b,d, 2000b,c, 2001a,b; Van der Bijl and Van Eyk, 2001) and permeation studies performed on seven tissue replicates for each patient. Prior to commencing each permeability experiment, tissue disks were equilibrated for 10 min with PBS (pH 7.9) at 20 °C in both the donor and acceptor compartments of the diffusion cells. Following equilibration, the PBS was removed from the donor compartment and replaced with 1.0 ml of PBS containing either 1 μCi ^{3}H-water, 1.4 μCi ^{3}H-17β-estradiol, 1 μCi ^{3}H-sumatriptan, 0.093 μCi ^{3}H-vasopressin, 0.83 μCi (mebmt-β-^{3}H cyclosporin), 0.5 μCi ^{3}H-reduced arecoline or 0.5

μCi ^{3}H-reduced arecaidine. The ^{3}H-17β-estradiol was obtained from Sigma Chemical Company, St. Louis, MO, all 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 determination of donor cell concentration at time zero. PBS at 20 °C was pumped through the acceptor chambers at a rate of 1.5 ml h^{-1} and collected, by means of a fraction collector, at 2-h intervals for 24 h. The permeability study was 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. Scintillation cocktail (15 ml) (Ready Protein + ™; Beckman Instruments, Fullerton, CA, USA) was added to each sample collected and counted in a liquid scintillation counter (Beckman LS 5000TD) until a 2-s value of 1% was reached. Quenching for each sample was automatically corrected in the counter.

2.3. Calculation of flux values

Flux (J) values across membranes were calculated by means of the relationship,

$$J = Q/A \times t \text{ (cpm cm}^{-2} \text{ min}^{-1}\text{)},$$

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

2.4. Steady-state kinetics

When no statistically significant differences ($P < 0.05$) (ANOVA and Duncan's multiple range test) between flux values were obtained over at least two consecutive time intervals, a steady-state (equilibrium kinetics) was assumed to have been reached for a particular specimen and tritiated permeant.

2.5. Statistical analysis

An unpaired t-test with Welch's correction was used to investigate possible differences between flux means of corneal tissues at 2-h intervals. A significance level of 5% was used for all tests and comparisons.

3. Results

Overall mean flux values for water, 17β-estradiol, sumatriptan, arecoline and vasopressin through fresh and frozen/thawed intestinal mucosa versus time are shown in Fig. 1. While steady-state flux conditions were reached for water, sumatriptan and arecoline after approximately 15 h across intestinal mucosa, this was not the case for estradiol and vasopressin. No significant differences, at the 5% level, between the flux rates of all five permeants across fresh and frozen tissues could be demonstrated by the t-test at any of the 2-h time points. Overall means of the flux rates for the various permeants (water, sumatriptan, arecoline, arecaidine, cyclosporin, estradiol and vasopressin) over time across frozen/thawed intestinal mucosa are shown in Fig. 2. Steady-state conditions were again not reached for estradiol, vasopressin and cyclosporin during the 24-h time course over which the experiment was conducted. Overall mean flux rates of the various permeants studied versus time across intestinal and vaginal mucosa are shown in Fig. 3. Flux rates of the various permeants across vaginal mucosa were obtained from previous studies (Van der Bijl et al., 1997, 1998a,b, 2000b, 2001b; Van der Bijl and Van Eyk, 2001). Mean ($\pm$sem) steady-state flux values (16–24 h) for water, sumatriptan, arecoline and arecaidine across vaginal and intestinal mucosa were 2176 ± 8, 797 ± 18, 761 ± 12, 512 ± 5 and 1283 ± 18, 343 ± 9, 488 ± 6 and 364 ± 6 cpm cm^{-2} min^{-1}, respectively. For estradiol, vasopressin and cyclosporin 'steady-state' flux values were estimated by averaging flux values between 20 and 24 h. For estradiol, vasopressin and cyclosporin, these estimated 'steady-state' flux values were 300, 200 and 70 across vaginal and 120, 75 and 51 cpm cm^{-2} min^{-1} across intestinal mucosa, respectively. Mean steady-state and estimated 'steady-state' flux values for the various permeants versus molecular weight (Da) across intestinal mucosa are shown in Fig. 4.

4. Discussion

While preference should be given to using fresh, viable tissues for in vitro permeability studies, this is not always possible. Although it has been demonstrated that human buccal and vaginal mucosa specimens can be frozen and banked without their permeability properties to water being changed, this cannot be assumed to be the case for all types of tissues and different permeants (Van der Bijl et al., 1998c). Because no data on the effects on the in vitro permeability characteristics of frozen, banked human intestinal tissue could be found in the literature, this aspect was also investigated in the present study. Statistical comparison of the flux values across fresh and frozen/thawed human intestinal mucosa of water, 17β-estradiol, sumatriptan, arecoline and vasopressin obtained in the present study at various times, revealed no significant differences at the 5% level (Fig. 1). This finding demonstrated that human intestinal mucosa may be frozen and banked, and after thawing, used for in vitro permeability experiments whenever it is not practical to use the tissue within several hours following removal from donors. The temperature (20 °C) at which the diffusion experiments in the present study were conducted was chosen as a matter of convenience, its exact value not really being of importance for comparative studies. We have previously demonstrated that flux rates increase linearly with rises in temperature (Van der Bijl et al., 1998e, 2000a), probably because of increases in barrier lipid fluidity. Therefore, comparative flux rates obtained at 20 °C will be equally comparable, albeit higher, at 37 °C.

Steady-state flux rates were reached for water, sumatriptan, arecoline and arecaidine after approximately 15 h of the experiment. However, this was not the case for estradiol, vasopressin and cyclosporin, the flux rates of all three compounds gradually increasing during the course of the ex-

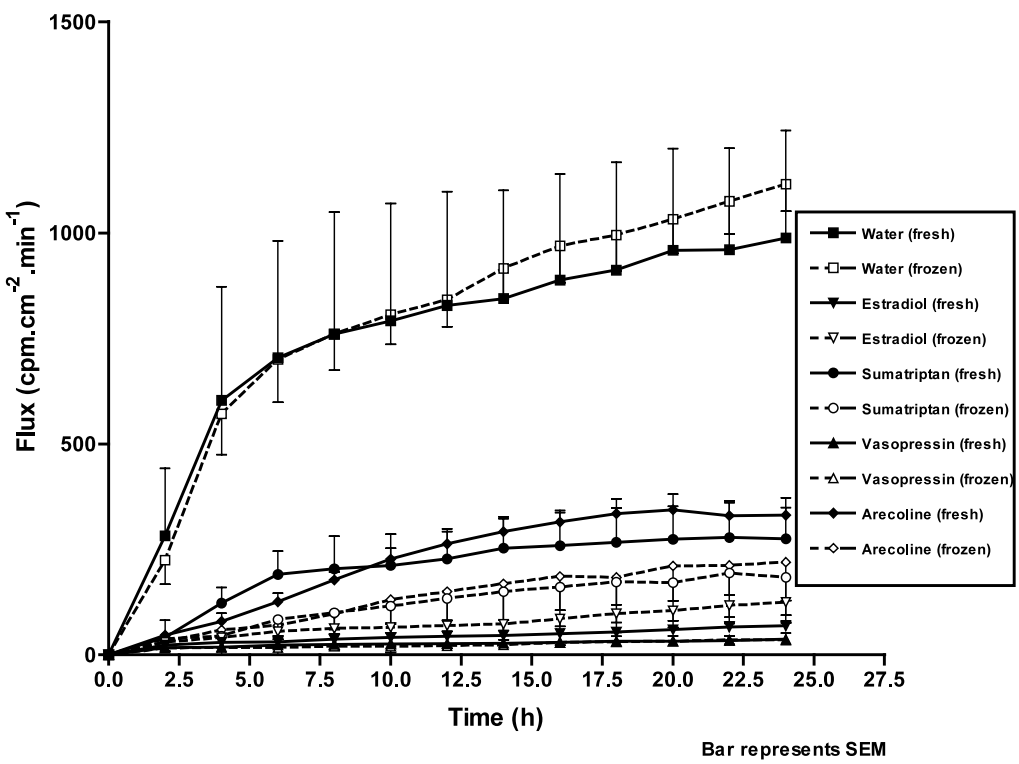

Fig. 1. Overall mean flux values for various permeants across fresh and frozen/thawed human intestinal mucosa.

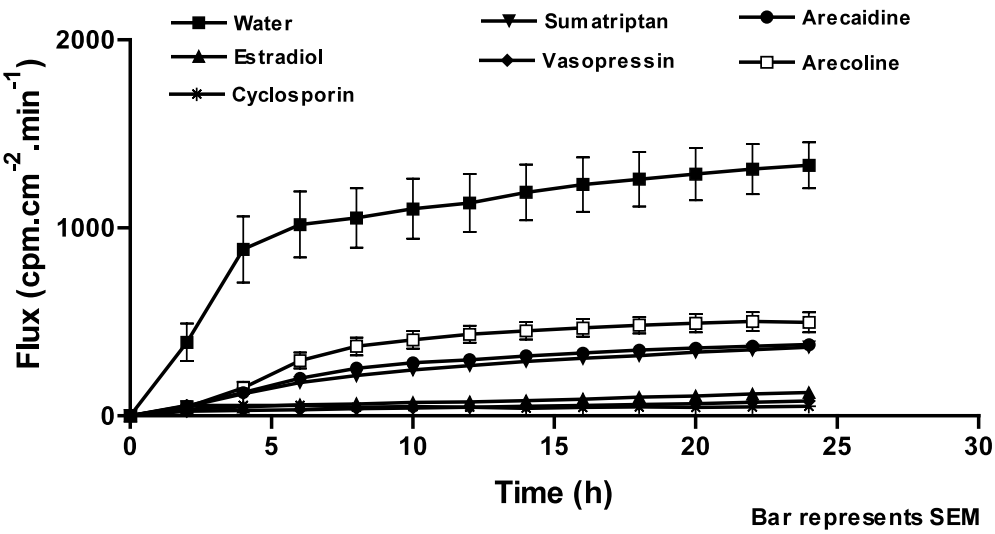

Fig. 2. Overall mean flux values for various permeants across frozen/thawed human intestinal mucosa.

periment (Fig. 2). These results concur with those found in previous studies using human buccal and vaginal mucosa, two tissues which are comparable, and have been attributed to the relatively large size of these molecules and the wider distribution of the hydrophobic estradiol into the lipophilic domains of the tissues (Van der Bijl et al., 1997, 1998a,b, 2000b, 2001b; Van der Bijl and Van Eyk, 2001). This may also be the case for the lipophilic molecule, cyclosporin. In general, as would be expected, the magnitude of the flux rates of the various permeants across intestinal mucosa were related to molecular size, the larger compounds with molecular weights >300 Da, having low flux rates (Fig. 4). This is in accordance with results obtained in previous studies performed on human jejunum using hydrophilic monodisperse polyethyleneglycols (PEG) with molecular weights ranging between 194 and 502 Da (Artursson et al., 1993). The results from these studies have demonstrated that human intestinal epithelium has a limited permeability to PEG with a molecular weight of 502 Da. This is in contrast to human buccal and vaginal mucosa, which are relatively freely permeable to dextrans of 4.4 kDa, i.e. having molecular weights approximately 10 times higher than those molecules which diffuse across intestinal mucosa with relative ease (Van der Bijl et al., 1998d). Only for compounds with molecular weights >12 kDa, do the flux rates across buccal and vaginal mucosa become low. It is also interesting to note that the flux rates of arecoline (M_w 160 Da), the methyl ester of arecaidine, are somewhat higher than those of arecaidine itself (M_w 146 Da). This concurs with our previous observations for the diffusion of these two alkaloids across human buccal and vaginal mucosa (Van der Bijl and Van Eyk, 2001; Van der Bijl et al., 2001b). It is also in agreement with the general observation that although molecular size is an important determinant for permeability, the pH-dependent degree of ionization also plays a role as is the case for the weak organic acid, arecaidine. The combined effect of these factors, which is not always precisely predictable on theoretical grounds, constitutes the final diffusion characteristics of the compound across biological barriers.

It is well known that the human small intestine contains a multitude of luminal, cellular and epithelial cell membrane brush border peptidases (Woodley, 1994). However, the degradative effects of these enzymes on the nonapeptide vasopressin were considered to be insignificant in the present study. Apart from the obvious absence of luminal peptidases, the in vitro experimental conditions were unfavourable to efficient tissue enzyme activ-

ity and a relatively large quantity of vasopressin was used relative to the area of tissue exposed (0.039 cm^2). Furthermore, the low and gradually increasing flux rates of vasopressin across the intestinal tissue concur with a negligible enzymic degradation rate of the peptide. In the presence of a significant degree of hydrolysis, detectably higher increases in flux rates associated with smaller radio-labelled cleavage fragments, would have been anticipated.

It is also well known that several active transport systems are associated with the absorption of therapeutic agents from the human gastrointestinal tract. However, the conditions under which the present in vitro diffusion experiments were conducted, are not suitable for evaluating these transport systems.

It is clear that for all permeants studied, the flux rates across vaginal mucosa were 36–160% higher than those across intestinal mucosa (Fig. 3). In this respect the structural and functional differences between these two types of mucosa probably played a role. The intestinal mucosa consists of a single layer of three types of columnar epithelium (enterocytes, goblet and entero-endocrine cells) and differs from the stratified squamous epithelium of the buccal and vaginal mucosa (Schumacher and Schumacher, 1999). Although, both types of mucosa also protect against the ingress of a range of deleterious substances, the barrier characteristics of the two types of mucosa appear to differ. In the intestinal epithelium the barrier is believed to primarily be comprised of the mucosal membrane in conjunction with the tight junctions, while in the buccal and vaginal epithelium the main diffusion barrier is thought to reside in the lipoidal layer located in the upper third of the epithelium (Wertz et al., 1993). However, some authors consider the effect of tight junctions, at least in guinea pig vagina, to be of importance in regulating permeability (Winterhager and Kuhnel, 1985).

In conclusion, we have shown that freshly harvested specimens of human intestinal mucosa can be snap frozen in liquid nitrogen and stored at −85 °C for periods of at least 1 month and used for in vitro experiments, with no significant changes in permeability to a variety of permeants.

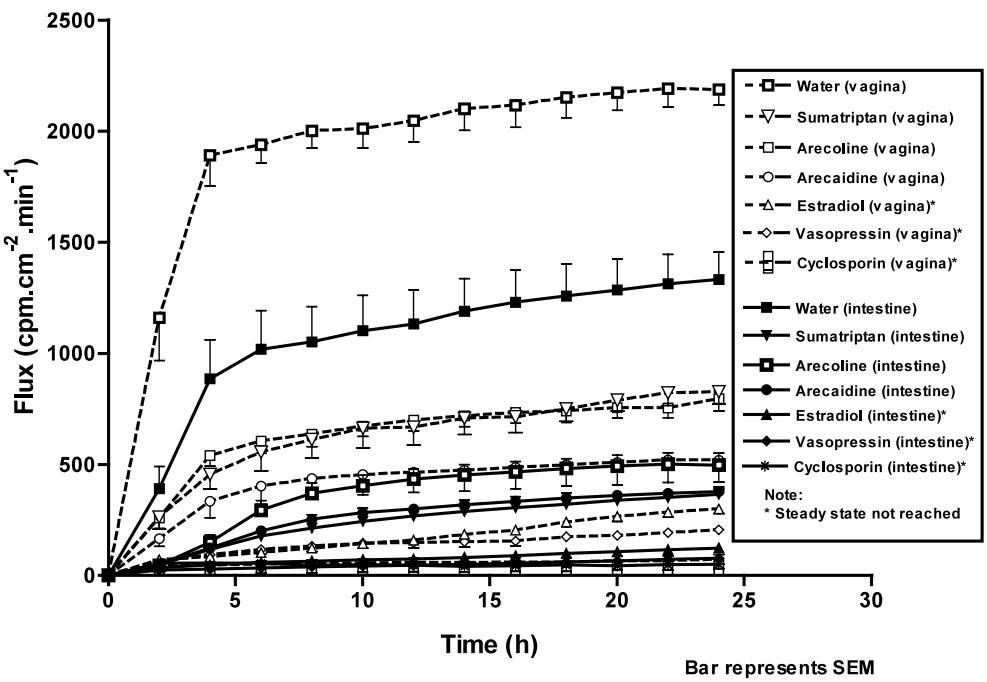

Fig. 3. Overall mean flux values for various permeants across frozen/thawed human intestinal and vaginal mucosa.

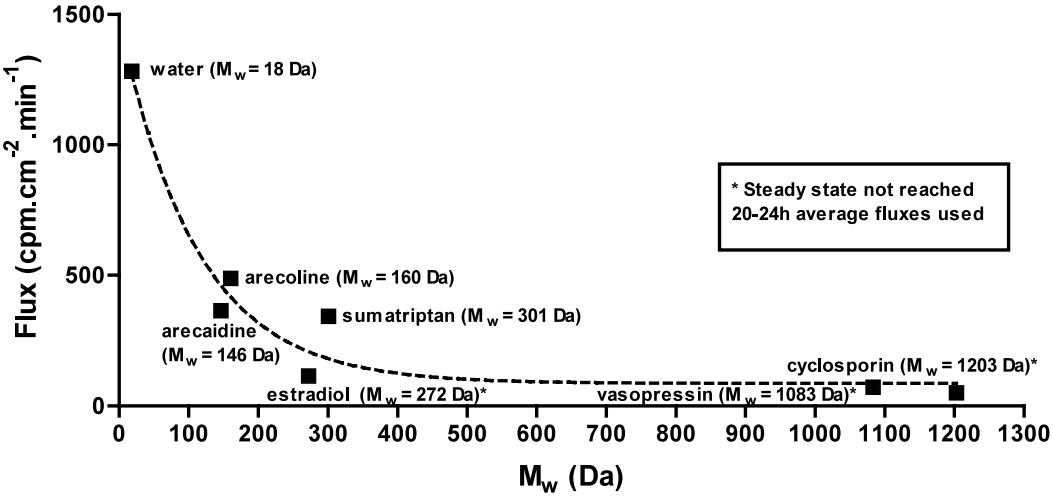

Fig. 4. Mean flux steady-state (18–24 h) flux values for various permeants across frozen/thawed human intestinal mucosa versus molecular weight (Da).

We have also determined the diffusion kinetics of a variety of therapeutic agents and other compounds of varying molecular size across small specimens of human intestinal mucosa. The results from this study have demonstrated that the continuous flow-through mucosal perfusion system used in conjunction with human intestinal mucosa, shows promise as an in vitro method for determining the permeability of a drug substance from the gastrointestinal tract for drug registration purposes. However, due to the fact that intestinal mucosa used in the above in vitro system is not very permeable to therapeutic agents with molecular weights > 500 Da, other mucosae e.g. vaginal mucosa, may have to be considered as alternatives to intestinal mucosa if therapeutic agents with molecular weights > 500 Da are to be compared for in vitro BA/BE purposes, and further studies in this respect are warranted.

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