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International Journal of Pharmaceutics 133 (1996) 47–58

international journal of pharmaceutics

In vitro measurement of drug transport using a new diffusion chamber compatible with Millicell® culture supports: performance with caco-2 monolayers

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Received 21 June 1995; revised 31 October 1995; accepted 29 November 1995

Abstract

A diffusion chamber compatible with Millicell® culture supports has been developed for the measurement of permeability coefficients across caco-2 cell monolayers, these monolayers themselves having been demonstrated to be a valuable in vitro model of intestinal drug absorption. Attributes of the chamber include its unibody design of clear polycarbonate (Lexan®), temperature control by water bath submersion and fluid agitation by magnetically driven stir bars. Characterization of the apparatus with respect to control of unstirred water layer (UWL) thickness was accomplished by studying, as a function of stirring rate, both the dissolution rate of *p*-aminobenzoic acid and testosterone flux across caco-2 cell monolayers. These studies indicated that the design was capable of reducing UWL thickness by as much as 90% in the case of testosterone flux and converting the rate limiting step in flux of this steroid from the UWL to the caco-2 barrier. Importantly, based on the consistency in the flux of mannitol across the range of stirring rates applied, as well as the low permeability of this leakage marker across the caco-2 monolayers compared to testosterone ($< 0.5\%$ of testosterone permeability), stirring did not adversely affect the barrier integrity of the monolayers.

Keywords: Millicell® cell culture supports; Caco-2; Intestinal permeability models; Unstirred water layer; Drug absorption

1. Introduction

A comprehensive approach to rational drug design includes an assessment of the biopharmaceutical properties of drug candidates. For several therapeutic targets, in which oral dosing is the preferred route of administration, consideration of the physical-chemical properties of drug candidates influencing their intestinal absorption is important. It is also prudent to use experimental approaches for measuring independently the potential for intestinal transport and first pass metabolism of a series of drug candidates. With regard to the former, the development of the caco-2 cell line as a model of the intestinal epithe-

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SSDI 0378-5173(95)04415-7

lium (Hidalgo et al., 1989) has generated much attention as a rapid and convenient in vitro based approach isolating the intestinal drug absorption process. The capability of this cell line to transport drugs and nutrients by carrier-mediated mechanisms (Hu and Borchardt, 1990, 1992; Chandler et al., 1993; Thwaites et al., 1993; Dantzig et al., 1994; Zheng et al., 1994; Ma et al., 1994) and, thus, to mechanistically probe drug transport, has further solidified its position as a valid experimental tool in intestinal drug transport work.

A complication in measuring intestinal transport using any experimental approach is the formation of a large and non-physiological unstirred water layer (UWL) through which a compound must diffuse prior to reaching the absorptive surface (Barry and Diamond, 1984; Winne, 1984). For hydrophobic drug molecules that are transported passively, flux measured in the absence of fluid agitation will be determined primarily by their rate of diffusion across this UWL (Komiya et al., 1980; Anderson et al., 1988). The UWL has also been shown to bias the transport parameters (K_m and J_{max}) of compounds that are transported by a carrier mediated process (Dietschy and Westergaard, 1975) and can even lead to a false conclusion regarding the mechanism of drug transport (Hu et al., 1988). Accordingly, the design of well-stirred systems that are compatible with cell culture systems employing microporous membranes has received attention from several laboratories (Shah et al., 1990; Hidalgo et al., 1991; Karlsson and Artursson, 1991; Leuenberger et al., 1991; Karlsson and Artursson, 1992). The goal of the present report is to communicate the design of a novel diffusion chamber that accepts Millicell® culture inserts. The unique design of the Millicell® insert relative to other commercially available products encouraged the design of a diffusion chamber with some unique features. These included the methods of temperature control and stirring, the type of plastic used in its construction and the method used for mounting an insert into the diffusion chamber without damaging the barrier integrity of the caco-2 cell monolayer. A supplementary goal of the report is to communicate the performance of the design in minimizing UWL effects in the transport of testosterone, a hydrophobic passively-transported steroid, while simultaneously preserving the barrier integrity of the caco-2 cell monolayer. The effects of stirring and insertion of Millicell® wells into the chambers on monolayer integrity were assessed using mannitol as a membrane impermeable leakage marker.

2. Materials and methods

2.1. Materials

Caco-2 cells were obtained from the Memorial Sloan-Kettering Cancer Center by permission. D-[1-^{14}C]Mannitol (57 mCi/mmol) and [1,2,6,7-^{3}H]testosterone (90 Ci/mmol) were obtained from Amersham Life Science, Arlington Heights, IL. Unlabelled mannitol was purchased from J.T. Baker Chemical Co., Philipsburg, NJ and testosterone from Aldrich Chemical Co. Inc., Milwaukee, WI. *Para*-aminobenzoic acid (PABA) was purchased from Sigma Chemical Co., St. Louis, MO. Millicell®-PCF inserts, 30 mm polycarbonate Isopore membrane, 0.4 μm pore size, were purchased from Millipore Corporation, Bedford, MA. Growth media consisted of Dulbecco's Modified Eagle's Medium (DMEM)/F12 3:1 (GIBCO, Grand Island, NY) supplemented with 5% fetal bovine serum (Hyclone Corp., Logan, UT), 20 mM *N*-[2-hydroxyethyl]-piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) (US Biochemical, Cleveland, OH) and 50 μg/ml Tobramycin (Eli Lilly and Co., Indianapolis, IN). Transport buffer was a modified Kreb's-Ringer solution, buffered to pH 6.0 on the apical (donor) side using 25 mM 2-[*N*-morpholino]-ethanesulfonic acid (MES, Sigma Chemical Co.) and pH 7.4 on the basolateral (receiver side) using 25 mM HEPES. Other transport buffer components were of analytical reagent grade and were present at a final concentration of 125 mM NaCl, 5.2 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$ and either 10 mM mannitol (donor side) or 10 mM glucose (receiver side).

Diffusion chambers and water bath were fabricated by personnel in the machine shop at Eli Lilly and Company. These were fabricated from

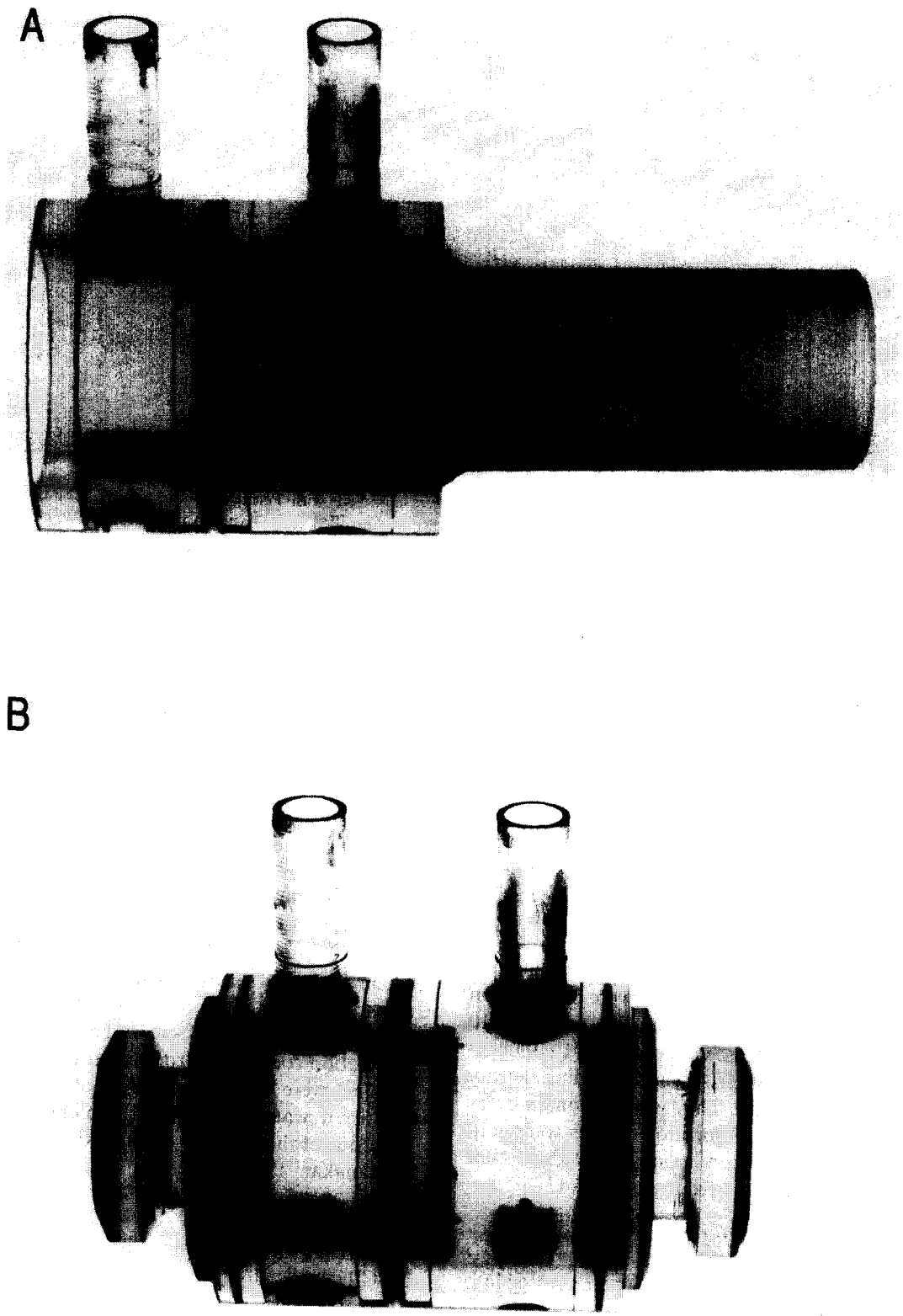

Fig. 1. Side view of the novel diffusion chamber shown with a Millcell® well just prior to insertion (A) and in final position (B). An O-ring between the side wall of the Millicell® well and the Lexan® wall of the chamber forms a water tight seal that defines the donor and receiver compartments. O-rings are also used to form watertight seals at the two ends of the chamber.

Fig. 2. Front view of an array of six assembled diffusion chambers complete with PermeGear stir motors. The chambers are immersed in a covered water trough that is connected to a temperature controlled recirculating water bath.

polycarbonate (Lexan® brand) rods (chambers) or sheets (water bath); and were purchased from Auburn Plastics and Rubber, Indianapolis, IN. Polyisoprene O-rings were purchased from Seal Products, Indianapolis, IN and teflon coated stir bars purchased from Curtin Matheson Scientific Inc., Houston, TX. Electric stir motors (PermeGear) were purchased from AMIE Systems (Riegelsville, PA), and were slightly modified by making the plastic disks covering the rotating magnets flush with the top of the stainless steel console housing the stir motors.

2.2. Cell culture

Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air and were passaged on a weekly basis at ~90% confluency using a 1:10 split ratio. Cells were seeded on Type 1 collagen-coated cell culture inserts using a seeding density of 1.1×10^5 cells/cm^2. They were fed on an every other day basis. All absorption studies were conducted using monolayers from 20–30 days after seeding. The cells were from passages 47–49.

2.3. Dissolution of PABA

Between 225–230 mg of PABA were pressed into a 13 mm disk of 1.35 mm thickness using 10 tons of pressure. A disk was loaded into a nylon insert that was subsequently placed into a diffusion chamber. The rate of appearance of PABA on each side of the insert was monitored with respect to time up to 20 min by UV absorbance at 266 nm. These experiments were conducted using pH 7.4 transport buffer on both sides of the disk. The dissolution rate (dM/dt) at a given stirring speed was determined by linear regression analysis of the amount dissolved vs. time plot and the dissolution rate constant (k, cm/s) determined from Equation 1 (Banakar, 1992a):

$$k = (dM/dt)/C_s \times A \quad (1)$$

where C_s is the saturation solubility of PABA in transport buffer (75.6 mM) and A is the surface area of the disk (1.33 cm^2). The square of the correlation coefficients from linear regression analysis (r^2) were 0.99 or greater at all stirring rates. Unstirred water layer (UWL) thickness (h_{aq}) on each side of the disk was calculated using Equation 2 (Banakar, 1992b):

$$h_{aq} = D_{paba}/k \quad (2)$$

where D_{paba} is the diffusion coefficient of PABA at 37°C (1.1×10^{-5} cm^2/s).

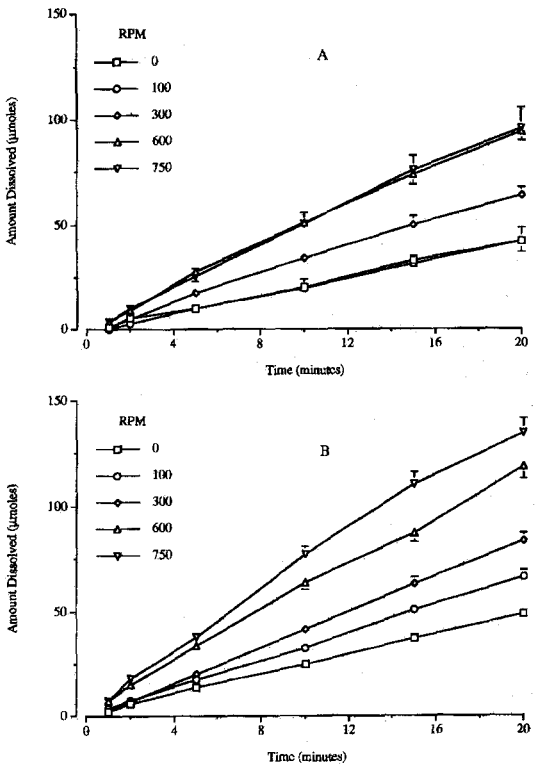

Fig. 3. PABA dissolution profiles as a function of stirring rate (RPM) in the donor (A) and receiver (B) compartments. Each point represents the average of 3 determinations. For clarity, only positive or negative error bars are shown; these represent one standard deviation.

2.4. Transport studies

Mannitol and testosterone fluxes across caco-2 monolayers were determined simultaneously at a given stirring rate by monitoring their rate of appearance on the receiver (basolateral) side. This was accomplished by removing 200 μl at several time points and detecting both C-14 (mannitol) and H-3 (testosterone) using standard liquid scintillation techniques with external standards. Fresh transport buffer, pH 7.4, was added back to the receiver side immediately after each sampling in order to maintain a constant volume. Experiments were conducted at 37°C and were initiated by the addition of 0.5 ml of a stock mannitol/testosterone solution to the donor (apical) side of a chamber (14.0 ml final volume). The concentration of mannitol in these experiments was 1.0 mM (2.0 μCi per chamber) and testosterone concentration was 0.1 mM (10.0 μCi per chamber). Selection of the concentration of testosterone in an experiment was based on previous work showing that at this concentration testosterone metabolism by caco-2 cells was sufficiently small and saturated (Buur and Mørk, 1992).

Apparent permeability coefficients (P_{app}) for mannitol and testosterone were calculated according to Equation 3:

$$P_{app} = (dQ/dt)/(A \times C_0) \quad (3)$$

where dQ/dt is the steady state flux (mol/s), A is the surface area of the monolayer (4.1 cm^2) and C_0 is the initial concentration of either mannitol or testosterone (mol/cm^3). All experiments were carried out under sink conditions (that is, less than 10% transported).

2.5. Statistics

Statistical differences between two means were assessed using an unpaired, two-sided Student's t-test. Differences among more than two means were evaluated using a one-way analysis of variance followed by the Tukey-Kramer HSD test for multiple comparisons (Kramer, 1956). In all cases, statistical significance was defined as $P \leq 0.05$.

3. Results

3.1. Design characteristics of the diffusion chamber

The diffusion chambers are made of polycarbonate (Lexan®) in order to maximize their durability and minimize adsorption of protein and lipophilic compounds. With regard to minimizing the adsorption of lipophilic compounds, the recovery of testosterone, based on an average of 22 trials, was 95 ± 4.9%. As shown in Fig. 1, a

Table 1
PABA dissolution rate, dissolution rate constant and unstirred layer thickness measured in donor and receiver compartments at various stirring rates

Stirring rate (rev./min)	Donor			Receiver			Total Thickness of UWL (μm)
	Dis. rate[a] (nmol/s/cm^2)	Dis. rate constant[b] ($\times 10^{+4}$, cm/s)	Thickness of UWL[c] (μm)	Dis. rate (nmol/s/cm^2)	Dis. rate constant ($\times 10^{+4}$, cm/s)	Thickness of UWL (μm)	
0	26.8 ± 2.0	3.5 ± 0.5	320 ± 25	30.6 ± 1.5	4.0 ± 0.2	279 ± 14	599 ± 21
100	27.5 ± 4.0	3.6 ± 0.6	315 ± 49	41.6 ± 6.3	5.4 ± 0.9	209 ± 34	524 ± 40
300	41.7 ± 2.4	5.5 ± 0.3	205 ± 12	54.6 ± 6.9	7.2 ± 0.9	158 ± 19	363 ± 17
600	59.6 ± 3.3	7.9 ± 0.4	144 ± 8	72.2 ± 1.1	10.0 ± 0.1	118 ± 2	262 ± 7
750	60.8 ± 6.0	8.0 ± 0.8	141 ± 14	85.5 ± 10.0	11.2 ± 1.3	101 ± 12	242 ± 12

[a]Dissolution rate was calculated from the slope obtained from linear regression of the amount dissolved vs. time and normalizing this result to surface area exposed to the dissolution medium, $A = 1.33$ cm^2. Results are reported as mean ± S.D.; $n = 3$. For the condition 750 rev./min on the receiver side, r^2 was 0.997 ± 0.0012.
[b]Dissolution rate constant was calculated from Equation 1. $C_s = 75.6$ μmol/ml.
[c]Thickness of the unstirred water layer was calculated from Equation 2.$D_{paba} = 1.1 \times 10^{-5}$ cm^2/s.

water tight seal is attained by passing a Millicell® well through a rubber O-ring that is recessed in the center of the cylinder. This insertion process is accomplished in one step using a plastic tool that does not contact the filter, thus preventing monolayer damage. Once a Millicell® well has been inserted, the volume of each half chamber is 14 ml on the monolayer side of the filter support and 13 ml on the back side of the filter. As shown in Fig. 2, temperature control is achieved by submersion of individual chambers in a water bath that rests directly on several stirrers arranged in series. The water bath is made of 1/4 inch thickness polycarbonate sheets, thus providing excellent visibility of the contents of each chamber as well as proximity

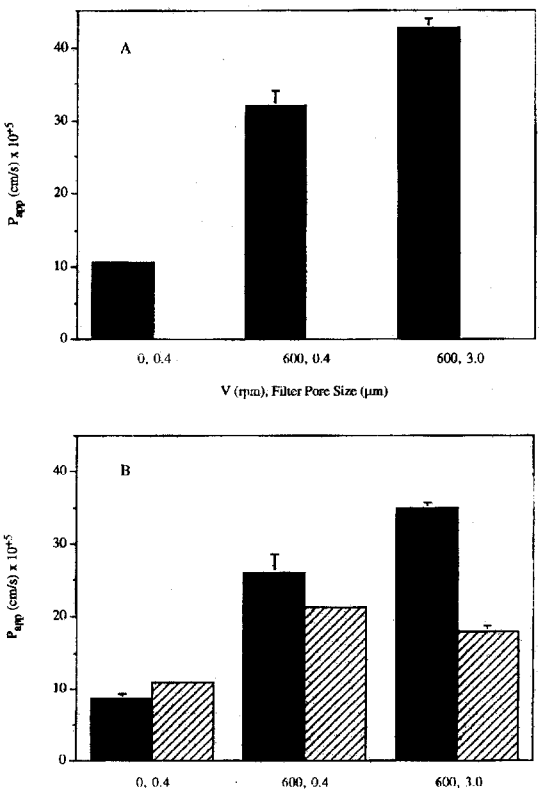

Fig. 4. Measured permeability coefficients (P_{app}) for mannitol (A) and testosterone (B) in either the absence (solid bars) or presence (hatched bars) of caco-2 cells. The two compounds were applied as a mixture. Each point represents the average of 3 or 4 determinations. For clarity, only positive error bars are shown; these represent one standard deviation. Refer to Table 2 for actual values.

of the stir bars to the stir motors. In the majority of experiments, the specific model of stir motor used provides a fixed stirring speed of 600 rev./min; however, a variable speed model is also available from Amie Systems.

3.2. Dissolution of PABA

Dissolution profiles of PABA in both the donor and receiver compartments as a function of stirring rate are shown in Fig. 3. The amount of PABA dissolved in 20 min ranged from 2.5 ± 0.34% (0 rev./min, donor) to 8.1 ± 0.79% (750 rev./min, receiver) of the total amount present. The average concentration of PABA in the receiver compartment at this time and at 750 rev./min stirring was 13.1% of saturation solubility (75.6 mM). These observations supported the application of zero order and sink conditions in the calculation of the dissolution rates and rate constants shown in Table 1. In the donor compartment, a reduction in UWL thickness to 44% of the estimated thickness obtained in the unstirred condition was observed as a result of applying a stirring rate of 750 rev./min. The effect of stirring was more pronounced in the receiver compartment in that a reduction to 36% of the estimated thickness obtained in the unstirred condition was observed at 750 rev./min. In fact, with the exception of the unstirred condition, UWL thickness in the receiver compartment was significantly less than in the donor compartment at the various stirring rates ($P < 0.05$). This result was not unexpected given the greater distance from stir bar to dissolving or absorbing surface, as the case may be, in the donor compartment and the greater depth of the stir bar in the stirring well of the donor compartment. The combined effect of stirring on both sides was to reduce the total UWL thickness to about 40% of the non-stirred condition.

3.3. Mannitol and testosterone permeability across cell-free and caco-2 filters

In the absence of cells, mannitol and testosterone diffusion were sensitive to stirring rate and filter porosity (Fig. 4). A 3-fold increase in both

Table 2
Apparent permeability coefficients for mannitol and testosterone transport across control (cell free) and caco-2 cell monolayers formed on Millicell® polycarbonate filter supports at 0 and 600 rev./min

V (rev./min)/filter pore size (μm)	Apparent permeability[a] ($\times 10^{+5}$ cm/s)			
	Mannitol		Testosterone	
	Control	Cells	Control	Cells
0/0.4	10.7 ± 0.14	0.06 ± 0.008	8.7 ± 0.59	10.9 ± 0.46
600/0.4	32.0 ± 2.02	0.07 ± 0.014	26.0 ± 2.50	21.4 ± 0.43
600/3.0	42.7 ± 1.21	0.08 ± 0.004	35.0 ± 0.78	18.0 ± 0.76

[a]Values are mean ± S.D.; $n = 3$ or 4.

mannitol and testosterone permeability was observed in going from the unstirred condition to a stirring rate of 600 rev./min with the 0.4 μm pore size filter (Table 2). The greater permeability of both compounds observed at 600 rev./min when using the 3.0 μm filter instead of the 0.4 μm filter indicated a departure from the UWL as the rate determining step in the transport process as a consequence of stirring.

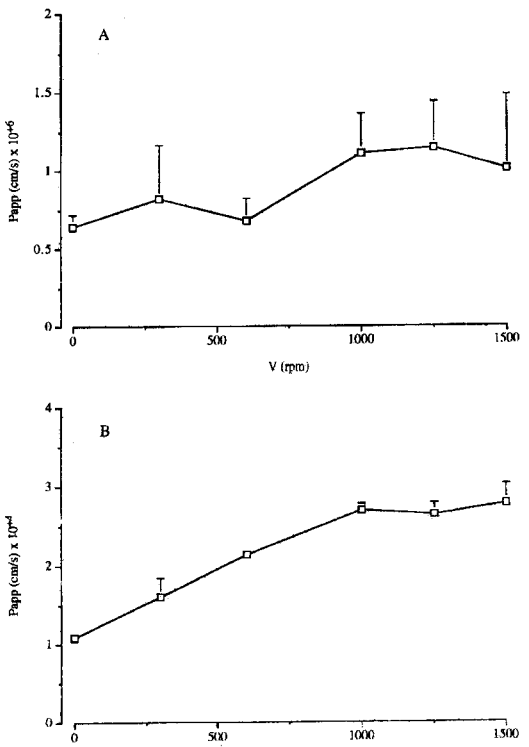

Fig. 5. Measured permeability coefficients (P_{app}) for mannitol (A) and testosterone (B) across monolayers at different stirring rates (V). Each point represents the average of 3 or 4 determinations. For clarity, only positive error bars are shown; these represent one standard deviation. Error bars for testosterone are within the plot symbols.

Permeability of mannitol in the presence of a cell barrier at both 0 and 600 rev./min was reduced to $< 1\%$ of the permeability observed in the absence of cells (Table 2). Importantly, as shown in Fig. 4, mannitol permeability was unaffected by stirring, thus indicating stability of the cell barrier in the presence of stirring and over the time course of the experiment (120 min). Filter pore size also did not affect the integrity of the cell barrier.

In the presence of the caco-2 barrier, testosterone permeability was approximately doubled as a result of stirring (Fig. 4). Further, compared to cell free filters, the caco-2 barrier resulted in a significant ($P < 0.05$) reduction in testosterone permeability across either 0.4 μm or 3.0 μm pore size membranes. Such a reduction was not observed in the absence of stirring. Combined with results obtained in the absence of cells, these findings indicate that, as a result of stirring, the UWL was no longer the rate determining step in testosterone transport.

3.4. Measurement of UWL thickness in relation to testosterone transport across caco-2 monolayers

The relationship between stirring rate and the measured permeability coefficient of testosterone

Table 3
Effect of stirring rate on UWL thickness relative to 0.1 mM testosterone flux across caco-2 cells grown on Millicell® 0.4 μm polycarbonate filter supports

V (rev./min)	P^{a}_{app} ($\times 10^{+5}$, cm/s)	$1/P^{b}_{uwl}$ ($\times 10^{-3}$, s/cm)	h^{c}_{uwl} (μm)
0	10.9 ± 0.46	6.3 ± 0.38	492 ± 30.1
300	16.1 ± 2.37	3.4 ± 0.99	265 ± 77.6
600	21.4 ± 0.43	1.7 ± 0.10	136 ± 7.8
1000	26.9 ± 0.90	0.8 ± 0.12	61 ± 9.7
1250	26.4 ± 1.60	0.9 ± 0.24	67 ± 18.9
1500	27.8 ± 2.50	0.7 ± 0.35	53 ± 27.4

[a]Values are means ± S.D.; $n = 3$ or 4.
[b]$1/P_{uwl} = 1/P_{app} - (1/P_{caco\text{-}2} + 1/P_{filter})$.
[c]$h_{uwl} = (1/P_{uwl}) \times D_{testosterone}$, 37°C. $D_{testosterone}$, 37°C = 7.84 x 10^{-6} cm^2/s.

is shown in Fig. 5. Using the approach developed by Karlsson and Artursson (Karlsson and Artursson, 1991), the relationship between stirring rate and measured testosterone permeability (P_{app}), was transformed to a linear relationship in order to define the contribution of the various components of resistance (filter, UWL and cells) to the total (measured) resistance ($1/P_{app}$). Linear regression analysis of the transformed data yielded a slope of 2.94 ± 0.163 × 10^3 s/cm; $r^2 = 0.951$. Based on this analysis, the caco-2 specific permeability coefficient ($P_{caco\text{-}2}$) for testosterone was 52.1 ± 2.89 × 10^{-5} cm/s. Unstirred water layer resistance and associated thickness at the various stirring rates are summarized in Table 3. The effect of stirring was to reduce the estimated thickness of the UWL to as little as 11% of the non-stirred condition. Overall, UWL thickness ranged between 50 and 500 μm. As shown in Fig. 6, stirring resulted in a shift in the principal rate controlling step of testosterone transport from the UWL to the caco-2 barrier. Importantly, mannitol permeability measured in the same experiments was independent of stirring rate (Fig. 5).

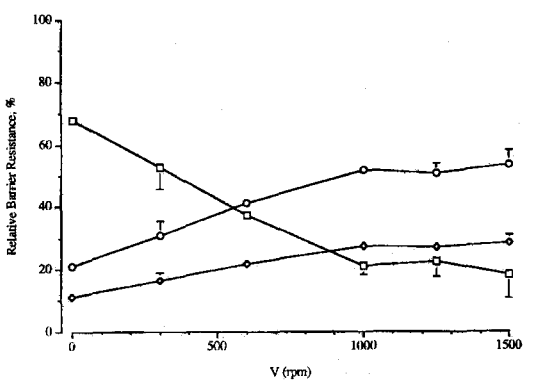

Fig. 6. Relative contributions of the monolayer (circles), UWL (squares) and polycarbonate filter (diamonds) to the total resistance to testosterone transport at various stirring rates (V). Each point represents the average of 3 or 4 determinations. For clarity, only positive or negative error bars are shown; these represent one standard deviation.

4. Discussion

Based upon our experience and that reported by others (Hidalgo et al., 1991), it is important to minimize the manipulation of caco-2 monolayers prior to using them in an experiment so as to avoid diminishing their barrier integrity. To address this sensitivity, we designed a diffusion chamber in which Millicell® inserts are loaded in one step without touching the filter or its plastic housing. Specifically, insertion is accomplished by using a lexan rod that contacts only the 3 'feet' of the insert that are used to suspend the filter during cell culture. The greater than 100-fold reduction in mannitol permeability in the presence of cells vs. their absence indicates that the integrity of the monolayer was not disrupted by this insertion procedure (Table 2). Our mannitol permeability results, including precision, are also comparable to those reported in the literature (Cogburn et al., 1991; Artursson et al., 1993; Adson et al., 1994).

Improving the usefulness of caco-2 cells as a model of intestinal drug absorption by minimizing UWL effects on measured drug transport rates has been the impetus in recent years for the development of several diffusion chambers incorporating some type of fluid agitation (Shah et al., 1990; Hidalgo et al., 1991; Karlsson and Artursson, 1991; Leuenberger et al., 1991; Karlsson and Artursson, 1992). Given our familiarity with the magnetically driven stirring apparatus commercially available from AMIE Systems, as well as our familiarity and preference for Millicell® inserts, we decided to construct a Millicell® compatible diffusion chamber around the AMIE Systems apparatus. Achieving compatibility between these two designs necessitated placement of the stir bar on the cell free side of the filter surface (receiver compartment) closer to this surface than the stir bar on the cell containing side of the filter (donor compartment). According to PABA dissolution rates, this asymmetry resulted in about a 20% smaller UWL thickness on the receiver side vs. the donor side (Table 1, 600 rev./min).

The total UWL thicknesses calculated from the PABA dissolution experiments (Table 1) were consistently larger by ~ 100 μm than those determined at corresponding stirring rates by applying the Karlsson and Artursson approach (Karlsson and Artursson, 1991) to testosterone transport (Table 3). The difference most likely represents dual control of the PABA dissolution rate by diffusion and the interfacial reaction process of the PABA transition from the solid to the dissolved state (Banakar, 1992a); whereas, in the case of testosterone, only diffusion applied. In addition, the lack of close agreement is not surprising considering the fact that these were two different measurement approaches and that UWL thickness in the presence of cells is highly compound dependent (Winne, 1984).

As demonstrated by others (Karlsson and Artursson, 1992), both the pattern and intensity of fluid agitation influence the barrier integrity of the caco-2 monolayer to drug transport. The consistency of mannitol permeability from 0–1500 rev./min indicated that our design did not adversely affect monolayer integrity (Fig. 5). In addition, the relationship between testosterone permeability and stirring rate from 0–1500 rev./min indicated that minimization of the UWL for this highly permeable drug was attained between 600 and 1000 rev./min, and resulted in a shift in the principal rate controlling barrier from the UWL to the cell monolayer (Fig. 6). Results of testosterone transport in the absence vs. the presence of cells also support this conclusion (Fig. 4). Thus, the chamber design fulfilled our 2-fold objective of (1) preserving the barrier integrity of the cells and (2) simultaneously providing sufficient fluid agitation to transfer the rate controlling step in the transport of highly permeable molecules to this barrier. Fulfilment of this objective will consequently enable superior resolution of measured caco-2 permeability coefficients of candidate drug molecules in an SAR. In addition, the diffusion chambers were recently used to study the kinetics of transport of loracarbef, a beta-lactam antibiotic, across caco-2 cells (Hu et al., 1994a). In this study, it was possible to differentiate the carrier-mediated and passive components of transport, suggesting that sufficient minimization of the UWL to dissect the two transport pathways had been attained.

The minimum UWL thickness obtained from the testosterone experiments (61 ± 10 μm, Table 3) was similar to two recent estimates obtained using the Transwell® cell culture insert design and either an ELISA shaker plate or a modification of the Grass-Sweetana diffusion apparatus (128 ± 10, Karlsson and Artursson, 1991 and 52 ± 4, Karlsson and Artursson, 1992, respectively). Thus, our design provides a similar reduction of UWL thickness and generates a thickness that is comparable to a recent estimate obtained in humans (Strocchi and Levitt, 1991).

From a pragmatic standpoint, in addition to minimizing drug adsorption, the lexan construction has proven extremely durable, as the original chambers have been in continuous use for over 4 years. The chamber design is also easily modified to accept 12 mm Millicell® inserts, although it is more practical to use the larger surface area 30 mm inserts from the standpoint of studying drug metabolism. In this regard, the chambers were recently used to study the metabolism of a dipeptide, Phe-Pro, by caco-2 cells (Hu et al., 1994b). We have also developed a means of supplying air

to the transport buffer on both sides of the monolayer through use of the sampling ports, which themselves are sufficiently small in order to reduce evaporation of transport buffer during the course of an experiment. However, as the presently reported results demonstrate maintenance of an intact barrier throughout a 2-h experiment, we have thus far found such auxiliary supply to be unnecessary.

Acknowledgements

The authors are grateful to Darrell Barlow for his expertise in manufacturing the chambers, and to Tony Gardner and Don McClure for supplying caco-2 cells.

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