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A method for the determination of cellular permeability coefficients and aqueous boundary layer thickness in monolayers of intestinal epithelial (Caco-2) cells grown in permeable filter chambers

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Summary

A linear relationship between apparent permeability coefficients (P_{app}) and agitation rate was used to determine cell permeability coefficients (P_c) and the thickness of the aqueous boundary layer (h_{aq}) in monolayers of Caco-2 cells. Drugs with different physical/chemical properties were studied. P_{app} values for 'highly permeable drugs' (testosterone, corticosterone, propranolol, metoprolol, warfarin) were dependent on the agitation rate, i.e. on the h_{aq}. The permeation of testosterone was studied in detail. In the absence of agitation, the measured P_{app} value of testosterone was $35.7 \pm 3.3 \times 10^{-6}$ cm/s while the P_c value was $137.4 \pm 8.1 \times 10^{-6}$ cm/s, i.e. 3.8 times higher. P_{app} values for 'less permeable drugs' (hydrocortisone, salicylic acid, sulphasalazine, mannitol) were not dependent on the agitation conditions. In the absence of agitation, h_{aq} for testosterone was 1544 ± 142 μm and the contribution of the resistance of the aqueous boundary layer to the total diffusional resistance was 70%. h_{aq} was reduced to 128 ± 10 μm when the highest agitation rate was used. Under these conditions, the contributions of the resistances of the aqueous boundary layer and the Caco-2 cell monolayers were 16 and 73%, respectively. Similar results were obtained for the other highly permeable drugs. The results indicate that the P_{app} values for highly permeable drugs are controlled not only by the aqueous boundary layer but also by the Caco-2 cell monolayers.

Introduction

In absorption experiments, a liquid layer adjacent to the surface of the cell membrane, the so called aqueous boundary layer (ABL), is a significant barrier to the absorption of drugs and nutrients (Barry and Diamond, 1984; Winne, 1984). If the ABL is not taken into account, absorption parameters such as permeability coefficients may be underestimated. The absorption of highly permeable drugs such as testosterone and warfarin has been shown to depend on the luminal stirring conditions, i.e. on the thickness of the ABL (e.g., Komiya et al., 1980; Anderson et al., 1988). Trans-

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port parameters of actively transported compounds such as glucose are also biased by the ABL (Wilson and Dietschy, 1974; Lherminier and Alvarado, 1981).

Various methods have been introduced to correct for the ABL (reviewed by Winnie, 1984). In in situ experiments, these corrections can be avoided by the use of the laminar flow model (Amidon et al., 1980; Elliott et al., 1980; Levitt et al., 1988). However, many drug absorption studies are performed in Ussing chambers or in cell culture chambers, i.e. under conditions where the laminar flow model is not applicable.

Recently, a number of cell culture models for drug absorption studies have been developed (reviewed by Borchardt et al., 1991). In these models, epithelial or endothelial monolayers are cultivated in permeable cell culture inserts. The single-use inserts are placed in the wells of conventional cell culture plates where drug absorption studies can be performed without further manipulation. The influence of the ABL on apparent permeability coefficients (P_{app}) in these systems has been recognized (Hidalgo et al., 1989), but so far no quantitative determinations of unbiased cell epithelial permeability coefficients (P_c) and of the thickness of the ABL (h_{aq}) have been presented. In this paper, a method for the determination P_c and h_{aq} in monolayers of intestinal epithelial (Caco-2) cells grown in conventional single-use Transwell cell culture inserts is presented.

Materials and Methods

Drugs and radiolabeled markers

[^{3}H]Metoprolol (0.4 mCi/mmol) and unlabeled metoprolol were obtained from Dr Kurt-Jörgen Hoffman, Hässle AB, Göteborg, Sweden. [^{14}C]Sulfasalazine (77.5 mCi/mmol) and unlabeled sulfasalazine were obtained from Dr Peter Edman, Pharmacia AB, Uppsala, Sweden. [^{14}C]Warfarin (56 mCi/mmol) was purchased from Amersham, Arlington Heights, IL, U.S.A. [^{3}H]Corticosterone (101.6 Ci/mmol), [^{3}H]hydrocortisone (80.4 Ci/mmol), [^{3}H]mannitol (19.1 Ci/mmol), [^{3}H]propranolol (26.6 Ci/mmol), [^{14}C]salicylic acid (58.2 Ci/mmol) and [^{3}H]testosterone (180.0 Ci/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. The radiolabeled compounds had a radiochemical purity of 97–99%. All other drugs were purchased from Sigma, St. Louis, MO, U.S.A.

Cell culture

Caco-2 cells, originating from a human colorectal carcinoma (Fogh et al., 1977) were obtained from American Tissue Culture Collection, Rockville, MD. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% heat-inactivated fetal calf serum (FCS), 1% non-essential amino acids, benzylpenicillin (100 U/ml) and streptomycin (10 μg/ml), in an atmosphere of 90% air and 10% CO_2 as described elsewere (Artursson, 1990). All tissue culture media were obtained from Gibco through Laboratorie Design AB, Lidingö, Sweden. The cells were grown in uncoated polycarbonate filter chamber inserts (Transwell, Costar, Badhoevedorp, The Netherlands; pore size 0.4 μm; diameter 24.5 mm). 2×10^6 cells were added to each insert and cells of passage number 85–99 were used. The cells were fed every second day and were allowed to grow and differentiate for up to 30 days before the monolayers were used in drug absorption experiments.

Drug transport studies

The drugs and the corresponding radiolabeled markers were dissolved in air buffered DMEM (pH 7.4) containing 0.1% human serum albumin, 1% penicillin/streptomycin (PEST) and 25 mM Hepes buffer or in Hanks' Balanced Salt Solution (HBSS) containing 1% PEST and 25 mM Hepes buffer to give a final concentrations of 1×10^{-4} M. 2.0 ml of the radiolabeled drug solutions was added to the apical side of the cells and an equal volume (2.0 ml) of the corresponding medium without drug was added to the basolateral side. The cells were incubated in air at 37°C and a relative humidity of 95%. At regular time intervals, 100 μl samples were withdrawn from the basolateral chambers. The samples were corrected for dilution. The initial drug concentration (C_0) in the apical chamber was determined from a 100 μl sample of the radiolabeled drug solutions. The integrity of the cell monolayers was checked at the

end of each experiment by measurement of the transepithelial electrical resistance, as described elsewere (Artursson, 1990). In additon, the paracellular marker mannitol was used as an integrity marker (Dawson, 1977). 10 ml of scintillant was added to the samples and the radioactive samples were counted in a liquid scintillation spectrometer (Tricarb 1900 CA, Packard Instruments).

Determination of permeability coefficients

Apparent permeability coefficients (P_{app}). All rate constants were obtained under 'sink' conditions (i.e. before > 10% of the drug had diffused across the cell monolayers) from the linear drug appearance curves in the basolateral chambers and were expressed as mol/min.

The apparent permeability coefficient (P_{app}) was determined according to the following equation:

$$P_{app} = \frac{\Delta Q}{\Delta t}\frac{1}{AC_0}\ (cm/s) \qquad (1)$$

where $\Delta Q/\Delta t$ is the permeability rate (steady-state flux, mol/s), C_0 is the initial concentration in the apical chamber (mol/ml) and A is the surface area of the membrane (cm^2).

The reciprocal of P_{app} ($1/P_{app}$) denotes the measured resistance (R_{app}) to drug absorption and is equal to the sum of the different diffusional resistances in the Caco-2 model (Flynn et al., 1974; Komiya et al., 1980; Barry and Diamond, 1984; Winne, 1984). Thus, the inverse of P_{app} can be related to the sum of the inverse permeability of the the aqueous boundary layers (ABL) adjacent to the apical cell surface and the basolateral surface of the polycarbonate filter (P_{aq}), the cell monolayer (P_c) and the supporting polycarbonate filter (P_f) by:

$$R_{app} = \frac{1}{P_{app}} = \frac{1}{P_{aq}} + \frac{1}{P_c} + \frac{1}{P_f} \qquad (2)$$

Filter permeability coefficients (P_f). The permeability for a drug in the polycarbonate filter can be calculated according to Eqn. 3 (King, 1988):

$$\frac{1}{P_f} = \frac{h_f}{n\pi r_p^2 D_{aq}} \qquad (3)$$

where h_f is the thickness, i.e. the pore length (10 μm), n is the number of pores per unit area (1×10^8 pores/cm^2) and r_p is the pore radius (0.2 μm) of the polycarbonate filter (data given by the manufacturer). The aqueous diffusion coefficients (D_{aq}) for the drugs were estimated with the Stoke-Einstein equation and the D_{aq} of mannitol. D_{aq} for mannitol is 6.1×10^{-6} cm^2/s at 25°C and a water viscosity of 0.8904 cP (Neast and Astle, 1981–1982). The corresponding D_{aq} at 37°C and a water viscosity of 0.6915 cP is 9.14×10^{-6} cm^2/s.

Cellular permeability coefficients (P_c). The permeability of a drug across the ABL in the Caco-2 model can be related to the agitation of the cell monolayers by:

$$P_{aq} = KV \qquad (4)$$

where K is a constant incorporating the aqueous diffusivity of the drug in the ABL, kinematic viscosity and geometrical factors of the Caco-2 model and V is the agitation rate (rpm). The cell monolayers were agitated with a calibrated plate shaker (Titertec, Flow Laboratories Ltd, U.K.). The agitation rates were determined with a digital tachometer (Shimpo DT-201, Shimpo Industrial Co, Kyoto, Japan).

Substituting for P_{aq} from Eqn 4 into Eqn 2 yields an expression where a double reciprocal plot of $1/P_{app}$ as a function of $1/V$ permits the determination of P_c from the intercept ($1/P_c + 1/P_f$) on the y-axis (Komiya et al., 1980):

$$\frac{1}{P_{app}} = \left(\frac{1}{P_c} + \frac{1}{P_f}\right) + \frac{1}{KV} \qquad (5)$$

In order to avoid possible errors in the extrapolating to the y-axis, both sides in Eqn 5 were multiplied by V to give Eqn 6. The extrapolation can thus be avoided. The ($1/P_c + 1/P_f$) value is obtained from the slope of the curve which increases

the accuracy of the determination (Cornish-Bowden and Wharton, 1988):

$$\frac{V}{P_{app}} = \frac{1}{K} + \left(\frac{1}{P_c} + \frac{1}{P_f}\right)V \qquad (6)$$

Diffusional resistance (R_{aq}) and the thickness of the ABL (h_{aq}). The diffusional resistance for a drug in the ABL (R_{aq}) at a given agiation rate can be calculated according to Eqn 2, when P_c and P_f are known, and the thickness of the ABL (h_{aq}) is given by:

$$R_{aq} = \frac{1}{P_{aq}} = \frac{h_{aq}}{D_{aq}} \qquad (7)$$

Statistics

P_c was obtained by linear regression analysis of the slope in Eqn 6. The confidence limits of P_c are given by: $P_c \pm t \times$ S.D., where S.D. is obtained from the standard deviation of the slope and the t value is taken at the desired confidence level and $(n-2)$ degrees of freedom (Table 3; Miller and Miller, 1988). Unpaired two-tailed Student's t-test was used to test the significance of the difference between the means of P_{app} at 135 and 1090 rpm. $P < 0.05$ was considered as significant (n.s = not significant; Table 4).

Results

Permeability of testosterone

Initially, the effects of increasing agitation rates (V) on the apparent permeability coefficients (P_{app}) were studied with testosterone as a model drug. Testosterone is a highly lipophilic drug (log $D = 3.31$) that is rapidly absorbed from the intestine. Its absorption is therefore considered to be controlled by the ABL (Komiya et al. 1980). The permeability of testosterone increased with increasing agitation rate (Fig. 1). When the agitation rate was increased from 0 to 1090 rpm, the P_{app} values increased from $35.7 \pm 3.3 \times 10^{-6}$ to $100.8 \pm 7.9 \times 10^{-6}$ cm/s.

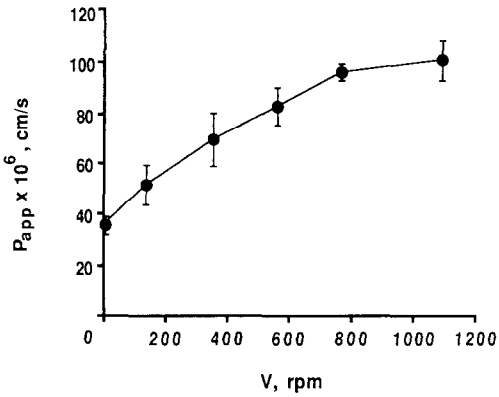

Fig. 1. Apparent permeability (P_{app}) of testosterone at different agitation rates (V). Values are means ± S.D.; $n = 3$.

The P_{app} values for testosterone between 135 and 1090 rpm were inserted into Eqn 6 and the corresponding graph is shown in Fig. 2. Linear regression analysis of the curve gave a slope of $8.29 \pm 0.49 \times 10^3$ s/cm. This value is the sum of the cell monolayer ($1/P_c = R_c$) and the polycarbonate filter ($1/P_f = R_f$) resistances. An R_f value of 1.02×10^3 s/cm and an R_c of $7.28 \times 10^3 \pm 0.43 \times 10^3$ s/cm was calculated. This corresponds to a P_c of $137.4 \pm 8.1 \times 10^{-6}$ cm/s which should be compared to a P_{app} value of $35.7 \pm 3.3 \times 10^{-6}$ cm/s in the absence of agitation (Table 1). Thus, in the absence of agitation the P_{app} value for testosterone is significantly influenced by the ABL.

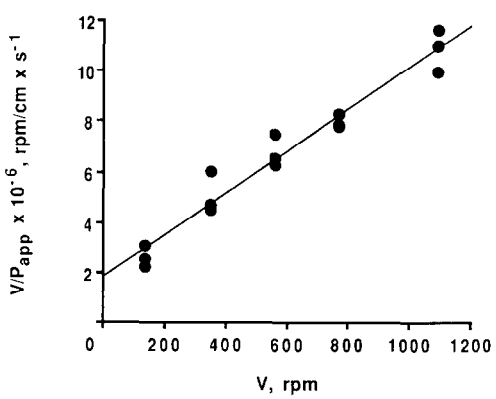

Fig. 2. The cellular permeability (P_c) for testosterone was determined from the slope of the linear relationship between agitation rate (V)/P_{app} and agitation rate (V). The data points represent individual experiments.

TABLE 1

Apparent permeability coefficients, resistances and thickness of the aqueous boundary layers for testosterone, at different agitation rates

V (rpm)	P_{app} [a] ($\times 10^6$) (cm/s)	R_{aq} [b] ($\times 10^{-3}$) (s/cm)	h_{aq} [c] (μm)
0	35.7 ± 3.3	19.70 ± 1.81	1544 ± 142
135	51.8 ± 7.9	11.01 ± 1.67	863 ± 131
350	69.9 ± 10.5	6.01 ± 0.90	471 ± 70
559	83.1 ± 7.5	3.74 ± 0.34	293 ± 27
767	96.3 ± 3.1	2.09 ± 0.07	164 ± 5
1090	100.8 ± 7.9	1.63 ± 0.13	128 ± 10

[a] Values are means ± S.D.; $n = 3$ ($n = 6$ at 0 rpm).
[b] R_{aq} was calculated from Eqns 6 and 2.
[c] h_{aq} was calculated from Eqn 7 and a D_{aq} of 7.84×10^{-6} cm^2/s.

The diffusional resistance of the ABL (R_{aq}) and the thickness of the ABL (h_{aq}) at different agitation rates were also determined. In the absence of agitation, a h_{aq} value of 1544 ± 142 μm was obtained (Fig. 3, Table 1). This value decreased to 471 ± 70 μm at an agitation rate of 350 rpm. A further decrease to 128 ± 10 μm was observed at the highest agitation rate. Thus, by varying the agitation rate, a thickness of the ABL between approx. 1500 and 100 μm could be obtained.

The relative contributions of R_{aq}, R_c and R_f to the total diffusional resistance (R_{app}) to testosterone, under given agitation conditions, are shown in Fig. 4. In the unstirred system, the contribution of R_{aq}, R_c and R_f to the total diffusional resistance of the system was 70.4 ± 6.5, 26.0 ± 2.4 and 3.6 ± 0.3%, respectively. At the highest agitation rate, the corresponding values were 16.4 ± 1.3, 73.4 ± 5.8 and 10.2 ± 0.8%. Thus, the resistance of the ABL dominates in the unstirred system but its influence on the total diffusional resistance is reduced in the agitated system. Instead, the contribution from the cell monolayer becomes significant.

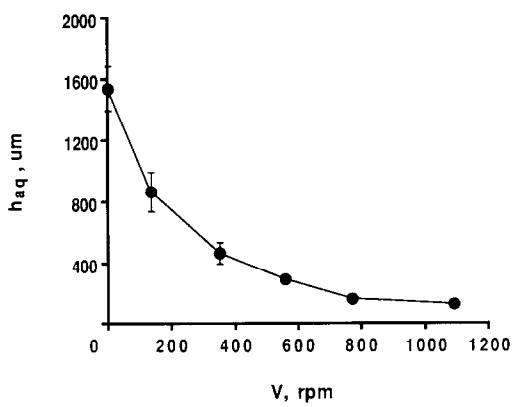

Fig. 3. The thickness of the aqueous boundary layer (h_{aq}) for testosterone decreases when the agitation rate (V) is increased. Values are means ± S.D.; $n = 3$.

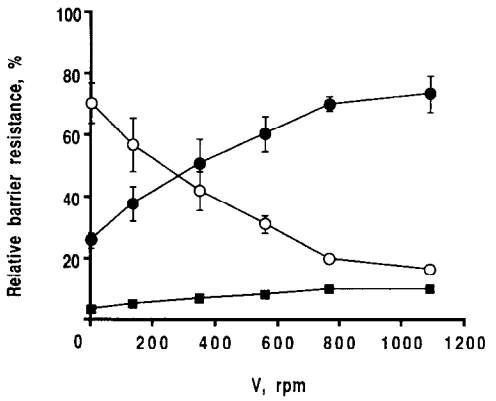

Fig. 4. Relative contribution of the resistances of the cell monolayer; R_c (●), the aqueous boundary layer; R_{aq} (○) and the polycarbonate filter; R_f (■) to the total diffusional resistance to testosterone at different agitation rates (V). Values are means ± S.D.; $n = 3$.

Permeability of warfarin

The experiments were repeated with warfarin — a drug that is less lipophilic (log $D = 0.12$) and has a lower P_{app} than testosterone (Artursson et al., 1990). Like testosterone, the absorption of warfarin has been reported to be controlled by the ABL (Levitt et al., 1988). When the permeability of warfarin was studied at different agitation rates, a similar linear relationship to that presented for testosterone was obtained. The influence of agitation rate on the P_{app} value was not as pronounced for warfarin as for testosterone (Table 2). Whereas an increase in agitation rate from 105 to 1090 rpm increased the P_{app} value for testosterone 2.8 times, the corresponding increase for warfarin was 1.8 times. These results indicate that our method for

TABLE 2

Apparent permeability coefficients, resistances and thickness of the aqueous boundary layers for warfarin, at different agitation rates

V (rpm)	P_{app} [a] ($\times 10^6$) (cm/s)	R_{aq} [b] ($\times 10^{-3}$) (s/cm)	h_{aq} [c] (μm)
0	31.6 ± 1.6	14.68 ± 0.13	1125 ± 58
135	39.5 ± 1.9	8.63 ± 0.76	641 ± 31
350	44.8 ± 1.0	5.36 ± 0.12	411 ± 9
559	48.4 ± 2.9	3.69 ± 0.22	283 ± 17
767	50.7 ± 0.5	2.77 ± 0.03	213 ± 2
1090	55.7 ± 3.8	0.99 ± 0.07	76 ± 5

[a] Values are means ± S.D.; $n = 3$ ($n = 6$ at 0 rpm).
[b] R_{aq} was calculated from Eqns 6 and 2.
[c] h_{aq} was calculated from Eqn 7 and a D_{aq} of 7.67×10^{-6} cm^2/s.

determinating P_c is applicable to drugs with different physical/chemical properties. However, the method is time consuming since five determinations of P_{app} are required in order to establish a linear relationship similar to that shown in Fig. 2.

TABLE 3

Determination of cellular permeability coefficients from the slopes of agitation rate/P_{app} vs agitation rate

Drug	V (rpm)	n	P_c [a] ($\times 10^6$) (cm/s)	r [b]
Testosterone	135–1090	15	137.4 ± 17.5	0.978
	135/1090	6	131.9 ± 24.0	0.991
Warfarin	135–1090	15	62.8 ± 4.8	0.992
	135/1090	6	62.8 ± 8.5	0.995

[a] P_c was determined from five (135–1090) and from two (135/1090) agitation rates. The P_c values are given with the 95% confidence limits.
[b] Correlation coefficient of V/P_{app} vs V.

It would be advantageous if reliable P_c values could be established from fewer P_{app} determinations.

Therefore, P_c values for testosterone and warfarin were calculated from the results obtained at the lowest (135 rpm) and the highest (1090 rpm) agitation rate. The P_c values (Table 3) obtained

TABLE 4

Phycical/chemical properties, apparent permeability coefficients at agitation rates of 135 and 1090 rpm and cellular permeability coefficients

Drug	MW	D_{aq} [a] ($\times 10^6$) (cm^2/s)	log D [b]	P_{app} [c] ($\times 10^6$) (cm/s)		P [d]	P_c [e] ($\times 10^6$) (cm/s)
				135 rpm	1090 rpm		
Testosterone	288	7.84	3.31 [f]	51.8 ± 7.9	100.8 ± 7.9	< 0.01	131.9 ± 8.7
Corticosterone	346	7.37	1.89 [f]	64.3 ± 2.5	98.9 ± 6.0	< 0.01	120.6 ± 5.3
Propranolol	259	8.12	1.54 [g]	58.8 ± 1.7	90.1 ± 5.9	< 0.01	107.3 ± 5.2
Metoprolol	267	8.04	0.068 [g]	51.4 ± 1.3	63.3 ± 2.4	< 0.01	69.9 ± 1.9
Warfarin	308	7.67	0.12 [h]	39.5 ± 1.9	55.7 ± 3.8	< 0.01	62.8 ± 3.1
Hydrocortisone	362	7.26	1.53 [f]	17.1 ± 1.3	16.8 ± 0.50	n.s.	17.3 ± 0.91
Salicylic acid	138	10.02	−2.14 [h]	7.65 ± 0.58	7.11 ± 0.44	n.s.	7.45 ± 0.51
Mannitol	182	9.14	−3.10 [i]	0.220 ± 0.079	0.264 ± 0.108	n.s.	0.228 ± 0.095
Sulfasalazine	398	7.04	−0.13 [j]	0.130 ± 0.012	0.134 ± 0.020	n.s.	0.132 ± 0.015

[a] Aqueous diffusion coefficient calculated as described in Materials and Methods.
[b] Literature values of distribution coefficients for octanol/water or octanol/phosphate buffer at pH 7.4. [f] Komiya et al., 1980; [g] Tavaloki-Saberi and Audus, 1988; [h] Hansch and Elkins, 1971; [i] Grass and Sweetana, 1988; [j] Dr Peter Edman, Pharmacia AB, Uppsala, Sweden.
[c] Values are means ± S.D.; $n = 3$ ($n = 8$ for mannitol).
[d] Significance test of the differences between the means of P_{app} at 135 and 1090 rpm. Two-tailed Student's t-test. $p < 0.05$ was considered as significant (n.s. = not significant).
[e] Values are means ± S.D.; $n = 6$ ($n = 16$ for mannitol). P_c of the upper five drugs were calculated from the slope obtained from two agitation intensities (Eqns 6 and 2). P_c of the other drugs were calculated from $1/P_c = 1/P_{app} - 1/P_f$.

by this procedure were not significantly different from those obtained from five agitation rates. This indicates that it is possible to use only two (one low and one high) agitation rates to calculate P_c values for testosterone and warfarin.

Permeability of other drugs

P_{app} values obtained at 135 and 1090 rpm were used to calculate P_c values for a number of drugs with different physical/chemical properties (Table 4). Drugs with higher P_{app} values than warfarin (referred to as 'highly permeable drugs') were significantly influenced by the ABL and their absorption rates were approx. 1.2–1.9 times faster at 1090 rpm than at 135 rpm. Thus, at 135 rpm the P_{app} values of the highly permeable drugs were dependent on the ABL. The contribution of R_{aq} to the total diffusional resistance for these drugs was 21.5–57.0% at 135 rpm.

The absorption of drugs with lower permeability coefficients than warfarin (referred to 'as less permeable drugs') was not influenced by the agitation rate. This indicates that the permeability of these drugs is completely controlled by the cell monolayers (P_c) and that the integrity of the monolayers is maintained under the applied agitation conditions. A possible exception was the paracellular marker mannitol (Table 4). The P_{app} value for mannitol increased from $0.220 \pm 0.079 \times 10^{-6}$ cm/s to $0.264 \pm 0.108 \times 10^{-6}$ cm/s. However, this increase was not significant at $P = 0.05$. Further evidence for the integrity of the monolayers was derived from the transepithelial electrical resistance values, which were not affected by the agitation conditions (data not shown).

It should be noted that both lipophilic (hydrocortisone) and hydrophilic drugs were represented among the less permeable drugs (Table 4). This indicates that other factors than lipophilicity determine the permeability of drugs across the cell monolayer.

The effect of the agitation rate on the P_{app} values is summarized in Fig. 5. At 135 rpm, the influence of the ABL on P_{app} was significant and became rate limiting for drugs with P_c values greater than approx. 60×10^{-6} cm/s. The ABL had no effect on the P_{app} values for the drugs with lower P_c values than approx. 20×10^{-6} cm/s. At

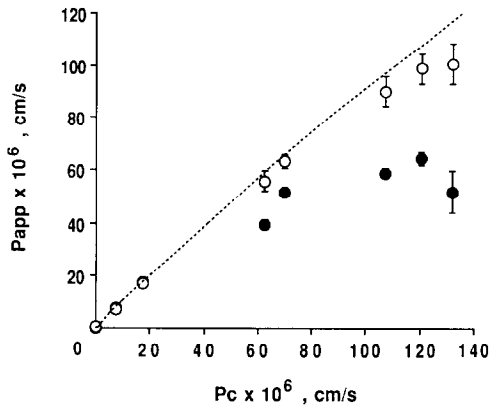

Fig. 5. Apparent permeability coefficients (P_{app}) as a function of cellular permeability coefficients (P_c) at different agitation rates. (●) 135 rpm; (○) 1090 rpm. (The P_{app} and P_c values were taken from Table 4.) Error bars represent ± S.D. of P_{app}. The dotted line represents the relation between P_{app} and P_c when $h_{aq} = 0$, i.e. when $1/P_c = 1/P_{app} - 1/P_f$.

1090 rpm, the influence of the ABL was less. These results are in agreement with theoretically calculated curves for the relationship between P_{app} at different h_{aq} and the actual membrane permeability (P_m; Barry and Diamond, 1984).

Discussion

A problem associated with the use of cell culture inserts in drug absorption experiments is that the hydrodynamic conditions in these small circular chambers are undefined. In conventional Ussing chambers, a gas lift is usually used to stirr the liquids in the apical and lateral chambers (Grass and Sweetana, 1988) but this method is not easily adapted to the small cell culture inserts. A special diffusion cell similar to those used in whole tissue experiments has recently been developed for cell culture studies (Borchardt et al., 1991). In this diffusion cell, the stirring flow rates can be controlled by a gas lift. A larger, magnetically stirred diffusion cell for cell monolayers has also been developed (Leuenberger et al., 1991). However, with these methods, the cell culture inserts have to be removed from the culture wells and mounted in the diffusion cells.

A conventional shaker for ELISA plates offers a simpler and less time consuming method for agitating the single-use cell culture inserts in the culture wells in a controlled manner. The shaker rotates and vibrates simultaneously in order to create adequate agitation conditions. The agitation rate was chosen so that h_{aq} for testosterone could be varied from approx. 100 to 1500 μm. Literature data indicate that h_{aq} in the intestine varies between 200 and 800 μm, depending on the experimental conditions (Anderson et al., 1988). Recently, h_{aq} values of approx. 100 μm have been reported in non-anesthetized rats (Anderson et al., 1988). Thus, h_{aq} values similar to those found in vivo could be selected in the agitated cell culture model.

Initially we determined h_{aq} in permeable cell culture inserts in the absence of cell monolayers (Cooper et al., 1987; Artursson and Karlsson, 1991). With this method, all drugs with similar D_{aq} will diffuse at the same rate across the permeable membranes and consequently give the same calculated h_{aq}. Other physical/chemical properties of the drugs, such as lipophilicity, are not taken into account in this procedure. However, by definition, h_{aq} is not only dependent on the permeability of the drug in the aqueous boundary layer (P_{aq}) but also on the permeability in the cell monolayer (P_c; Winne, 1984). h_{aq} was therefore determined in the presence of the Caco-2 cell monolayer.

The use of cell monolayers allowed the determination of P_c for a number of drugs. In contrast to h_{aq}, P_c is a constant. The P_c values in this report can therefore be used to calculate h_{aq} from experimental P_{app} values obtained in other cell culture systems based on Caco-2 cells. Moreover, the Caco-2 cell line develops spontaneously to well differentiated intestinal epithelial cells in culture (reviewed by Neutra and Louvard, 1989). The cells form tight polarized monolayers that are morphologicaly similar to normal intestinal epithelium. Thus, the P_c values given in this report can presumably be used for calculations on normal intestinal epithelium.

Our findings indicate that the resistance of the cell monolayer contributes significantly to the total diffusional resistance of both highly permeable and less permeable drugs. Under well stirred conditions, R_c became rate limiting for all of the investigated drugs. This is in contrast to the opinion that the absorption of lipophilic drugs such as testosterone and warfarin is totally controlled by the ABL even under well stirred conditions (Komiya et al., 1980; Levitt et al., 1988).

Calculations of h_{aq} based on the assumption that the absorption of testosterone is completely controlled by the ABL will lead to an overestimation of h_{aq}. These calculations give an h_{aq} for testosterone of 778 ± 61 μm at an agitation intensity of 1090 rpm as compared to the 128 ± 10 μm obtained in this study (when the resistance of the cell monolayer and the polycarbonate filter were included in the calculation of h_{aq}).

The importance of the ABL as a rate limiting barrier to drug and nutrient absorption has been convincingly shown in several in vivo and in situ studies (reviewed by Winne, 1984). These studies are normally performed in the presence of the intestinal mucus layer since it is complicated to separate the ABL from the mucus layer in situ. Thus, the two layers are treated as a joint barrier to drug absorption (Smithson et al., 1981). The relative importance of the ABL and the mucus layer as barriers to drug absorption is therefore unclear although some reports suggest that the mucus layer may be a significant barrier to the absorption of some drugs (Nimmerfall and Rosenthaler, 1980; Smithson, 1981; Grass et al., 1990).

Caco-2 cells do not produce a mucus layer (Wikman, A., unpublished observation). Thus, the barrier properties of the ABL and the epithelial cell monolayer may be compared directly. Our findings indicate that the barrier function of the (mucus-free) ABL is significant in the unstirred Caco-2 model when highly permeable compounds are studied. However, the importance of the ABL as a barrier decreases when the monolayers are adequately agitated. Instead, the cell monolayer barrier dominates when h_{aq} values similar to those found in vivo are chosen. This indicates that the mucus layer contributes significantly to the resistance of the ABL in vivo. Further studies on the role of the mucus layer as a barrier to drug absorption are indicated.

Conclusions

A linear relationship between apparent permeability coefficients and agitation rate was used to determine cellular permeability coefficients (P_c) for drugs in Caco-2 monolayers. The reported P_c values are constants and can be used to determine the thickness of the ABL (h_{aq}) in other absorption models where Caco-2 cells are used. h_{aq} can be determined for any agitation rate. By selecting appropriate agitation rates on the plate shaker, h_{aq} values similar to those found in different in situ or in vivo models can be obtained in the Caco-2 model. The absorption of highly permeable drugs such as testosterone is considered to be controlled by the ABL. However, our findings show that Caco-2 monolayers (which do not have a mucus layer) contribute significantly to the total barrier resistance to these drugs. The absorption of highly permeable drugs is therefore also controlled by the cell monolayer in the Caco-2 model.

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