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A new diffusion chamber system for the determination of drug permeability coefficients across the human intestinal epithelium that are independent of the unstirred water layer

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A new method for determining permeability coefficients, that are independent of the unstirred water layer (UWL), has been developed. The method was used to determine the cellular permeability coefficient of the rapidly absorbed drug testosterone in monolayers of the human intestinal epithelial cell line, Caco-2. Using a new diffusion cell with an effective stirring system based on a gas lift, the cellular permeability coefficient for testosterone was $(1.98 \pm 0.13) \cdot 10^{-4}$ cm/s which is 3.5-times higher than the permeability coefficient obtained in the unstirred system. The thickness of the UWL obtained with the well stirred diffusion cell was 52 ± 4 μm. This value is much lower than those previously reported in various well stirred in vitro models. The calculated cellular permeability of testosterone was 13–23-times lower than that for an UWL of the same thickness as the epithelial cell (17–30 μm). We conclude that the permeability of the epithelial monolayer must be included in calculations of the thickness of the UWL.

Introduction

Several drug absorption models based on epithelial or endothelial cell cultures have recently been developed (reviewed in Ref. 1). In these models, the cell layers are cultivated in permeable cell culture inserts placed in conventional cell culture plates (reviewed in Ref. 2). Thus, absorption studies can be performed directly in the cell culture inserts. However, as in all in vitro models, adequate stirring conditions are needed in order to reduce the effects of the unstirred water layer (UWL; the aqueous boundary layer) on the drug absorption rates [3].

Adequate stirring can be achieved by placing the cell culture plates, containing the cell culture inserts, on a plate shaker designed for agitation of microtiter plates. Using this technique, we were able to determine passive drug absorption coefficients in human intestinal epithelial cell monolayers that are independent of the unstirred water layer [4]. Although useful in routine studies on drug absorption, we recently found that our method has some limitations when applied to more precise studies on the mechanisms of drug absorption. Firstly, the rather vigorous stirring generated by the plate shaker limits the duration of the experiments. When longer stirring times are applied, the integrity of the monolayers is affected. Secondly, the plate shaker reduces the thickness of the UWL to a minimum of approx. 100 μm. Although this value is lower than published values obtained in other in vitro models, recent studies in dogs and humans suggest that the UWL has a maximal apparent thickness of only 40 μm in vivo [5]. It would therefore be advantageous if stirring conditions that reduce the thickness of the UWL to a value comparable to that found in humans could be used.

A new diffusion chamber system, specially designed for cell culture inserts with an effective stirring system based on a gas lift, has recently been described [6]. Several rapidly absorbed drugs (whose absorption rates are reduced by the UWL) were absorbed at higher rates in this diffusion chamber system as compared to in our model [4,6]. However, since a simplified determination of the UWL was used, which does not take the absorption barrier of the cell layer and the cell culture insert into account, we decided to evaluate the diffusion chambers further by applying our new determination of the UWL and cell permeability coefficients. By using the same cell culture model, monolay-

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ers of human intestinal epithelial Caco-2 cells, and the same drug, testosterone, as in our previous study, we could also evaluate our determination of cellular permeability coefficients. The passively absorbed drug testosterone was chosen as a model drug since its absorption is determined to a large extent by the UWL [4,6,7]. The results suggest that our new determination of cellular permeability coefficients is generally applicable since it gives similar results in two completely different drug absorption models. Further this method establishes that the intestinal epithelium is a significant barrier to the absorption not only of slowly, but also of rapidly absorbed compounds. In addition, the results show that the diffusion chamber has an effective stirring system that does not compromise the integrity of the Caco-2 cell monolayers.

Materials and Methods

Materials

The Caco-2 cell line, isolated by Fogh et al. [8], was obtained from American Type Culture Collection Rockville, MD, USA. Dulbecco's modified Eagle medium (DMEM), foetal calf serum (FCS), non-essential amino acids (NEAA), penicillin-streptomycin solution (10000 U/ml and 10 mg/ml, respectively) and Hanks' balanced salt solution (HBSS) were obtained from Gibco Laboratories through Laboratorie design AB, Lidingö, Sweden. 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes), testosterone and raffinose were from Sigma, St. Louis, MO, USA. Transwell™ and Snapwell™ inserts, polycarbonate membrane, 12 mm diameter and 0.4 μm pore size, were obtained from Costar, Bedford, MA, USA. The side-by-side diffusion chambers, gas manifold, and block heater were from Precision Instrument Design, Los Altos, CA, USA. [1,2,6,7,16,17(n)-^{3}H]Testosterone (spec. act. 141.1 Ci/mmol) and [G-^{3}H]raffinose (spec. act. 7.0 Ci/mmol) were from New England Nuclear, Boston, MA, USA.

Cell culture

The Caco-2 cells were maintained as described previously [9]. The cells were seeded on uncoated cell culture inserts at a density of $4.2 \cdot 10^5$ cells/cm^2. The cells were grown in culture medium consisting of DMEM supplemented with 10% (v/v) heat inactivated FCS, 1% (v/v) NEAA and 1% (v/v) penicillin-streptomycin. They were incubated at 37°C in a humidified atmosphere of 10% CO_2 in air. The medium was changed every second day. Cells of passage numbers 90 to 100 were used throughout. All absorption studies were performed with 20–30 days old monolayers, since the permeabilty of the monolayers to passively absorbed compounds is constant in this interval (Ref. 9, and unpublished results Karlsson and Artursson).

Absorption studies

Diffusion chamber system. The design of the diffusion chamber system was similar to one described previously with the exception that cell monolayers of larger surface area (1.13 cm^2) were used [6]. The diffusion chambers were stirred by the gas lift system, which produces a homogeneous circulation of the bathing fluids. The valves on the 12 port air manifold were adjusted to give a uniform delivery of the gas mixture (5% CO_2/air) to each half chamber. A gap flowmeter (Platon Flowbits Ltd., Basingstoke, UK) was connected in series with the air manifold to control the flow rate (60–600 ml/min). The inlet pressure to the flowmeter was set at 1 bar. With this arrangement it was possible to achieve reproducible stirring flow rates in the diffusion chambers.

When the gas lift was on, the absorption studies were performed in HBSS containing 15.0 mM $NaHCO_3$ and 1% (v/v) penicillin-streptomycin (pH = 7.4 at 37°C). In absorption studies where the gas lift was off, HBSS with 25 mM Hepes and a lower concentration of $NaHCO_3$ (4.1 mM) was used.

The cell monolayers were inserted into the diffusion chambers and 5.00 ml of HBSS was added to the donor and receiver reservoirs. The diffusion chambers were placed in the block heater, which was connected to a thermostatically controlled recirculating water bath to maintain a constant temperature of 37.0°C. The cell monolayers were allowed to equilibriate in the diffusion chambers before the start of the experiment (15–30 min). The transport experiments were started by adding 1.00 ml of drug-free HBSS to the receiver chamber and 1.00 ml of HBSS containing [^{3}H]testosterone or [^{3}H]raffinose to the donor chamber. The applied initial concentration in the donor chamber (including unlabelled substance) was 0.10 mM for testosterone and 1.0 μM for raffinose. After one minute, samples were taken both from the donor (50 μl) and the receiver (300 μl) side to determine the initial donor concentration. Subsequently, a 300 μl sample was collected from the receiver chamber at regular intervals. The samples were replaced with an equal volume of fresh HBSS. 10.0 ml of scintillation cocktail was added to each sample and the radioactivity was analyzed on a liquid scintillation spectrometer (Tricarb 1900 CA, Packard Instrument, Downers Grove, IL, USA).

Transwell system. The transport experiments were performed in air at 95% relative humidity and 37.0°C in HBSS containing 1% (v/v) penicillin-streptomycin and 25 mM Hepes. 0.4 ml of HBSS containing [^{3}H]testosterone (0.10 mM) or [^{3}H]raffinose (1.0 μM) was added to the apical side of the cells and 1.5 ml of marker-free HBSS was added to the basolateral chamber. At regular intervals, the cell culture inserts were transferred rapidly to a new basolateral chamber. The initial donor concentration in the apical chamber, C_0,

was determined from the mean value of at least three samples (50 μl) taken from the radiolabelled drug solutions. The radioactivity was analyzed in the liquid scintillation spectrometer and the accumulated radioactivity was calculated for each time point.

The Transwell system was stirred by agitating the culture plates on a calibrated plate shaker (Titertec, Flow Laboratories, UK) as described previously [4]. The agitation rates were determined with a digital tachometer (Shimpo DT-201, Shimpo Industrial, Kyoto, Japan).

Transepithelial electrical resistance measurements

The transepithelial electrical resistance (TEER) of the cell monolayers was measured at the end of the absorption experiments, using the Millicell-ERS device (Millipore, Västra Frölunda, Sweden). The measured TEER values were corrected for the resistance of cell culture inserts without cells.

Calculations

Apparent permeability coefficients (P_{app}). The apparent permeability coefficient (P_{app}; cm/s) was calculated using the following equation:

$$P_{app} = \frac{dC}{dt} \cdot \frac{V_R}{A \cdot C_0} \quad (1)$$

where dC/dt is the steady-state rate of change in the radiochemical concentration in the receiver chamber (dpm ml^{-1} s^{-1}), V_R the volume of the receiver chamber (ml), A the surface area of the cell monolayer (i.e., 1.13 cm^2) and C_0 the initial concentration in the donor chamber (dpm ml^{-1}). All experiments were carried out under 'sink' conditions.

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 [3,4,7,10,11]. Thus, the inverse of P_{app} is given by the sum of the inverse permeabilities of the the UWLs 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):

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

Filter permeability coefficients (P_f). The permeability of a drug in the polycarbonate filter can be calculated according to Eqn. 3 [12]:

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

where h_f is the thickness, i.e., the pore length (10 μm), n is the number of pores per unit area ($1 \cdot 10^8$ pores/cm^2), r_p is the pore radius (0.2 μm) of the polycarbonate filter (data given by the manufacturer) and D_{aq} is the aqueous diffusion coefficient. D_{aq} for testosterone at 37°C, $7.84 \cdot 10^{-6}$ cm^2/s, was calculated from the expression $D_{aq} \cdot M_r^{1/3}$ = constant, using mannitol (M_r = 182; $D_{aq} = 9.14 \cdot 10^{-6}$ cm^2/s) as the reference solute [13].

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

$$P_{aq} = K \cdot V^{\gamma} \quad (4)$$

where K is a constant incorporating the aqueous diffusivity of the drug in the UWL, kinematic viscosity and geometrical factors of the diffusion system and V is the stirring rate (rpm or ml/min).

Substituting for P_{aq} (Eqn. 4) in Eqn. 2 yields an expression where a double reciprocal plot of $1/P_{app}$ as a function of $1/V^{\gamma}$ permits the determination of P_c from the intercept ($1/P_c + 1/P_f$) on the y-axis [7]:

$$\frac{1}{P_{app}} = \left(\frac{1}{P_c} + \frac{1}{P_f}\right) + \frac{1}{K \cdot V^{\gamma}} \quad (5)$$

In order to avoid possible errors in the extrapolation, both sides of Eqn. 5 were multiplied by V^{γ} 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 [14]:

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

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

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

Results and Discussion

The integrity of the cell monolayers under different stirring conditions was investigated by two methods, transepithelial electrical resistance measurements and permeability to the passively absorbed and metabolically inert hydrophilic marker [^{3}H]raffinose [15,16].

TABLE I

Effect of stirring flow rate in the diffusion chamber system on the apparent permeability (P_{app}) of raffinose and the transepithelial electrical resistance (TEER) of the Caco-2 cell monolayers

The apparent permeability coefficients were calculated as described in Materials and Methods. Each flow rate was studied for 225 min. Results are means ± S.D. of four monolayers. The stirring did not increase the permeability of raffinose and TEER significantly (results analyzed by one way ANOVA).

Flow rate (ml/min)	P_{app} (cm/s) ($\times 10^7$)	TEER ($\Omega \times cm^2$)
0	2.27 ± 0.79	211 ± 6
100	1.27 ± 0.19	224 ± 32
200	1.45 ± 0.18	227 ± 10
400	0.99 ± 0.05	205 ± 9
600	2.19 ± 0.30	205 ± 12

Both of these methods have been used previously to follow changes in the epithelial integrity of the intestinal mucosa (e.g., Refs. 17–19). Maximal stirring for 60 min on the plate shaker was detrimental to the integrity of the cell monolayers, while stirring flow rates of up to 600 ml/min could be used for up to 225 min in the diffusion chambers without affecting the integrity of the Caco-2 monolayers (Table I, Figs. 1–2). Thus, the stirring conditions in the diffusion chamber system were superior to the those generated by the plate shaker.

When a compound is rapidly absorbed, its concentration in the fluid adjacent to the cell layer decreases relative to the concentration in the bulk of the lumen. By definition, the thickness of the UWL is determined by this concentration difference [3]. Thus for a slowly absorbed compund, no measurable concentration difference (and consequently no UWL) will be formed since the diffusion of the compound from the bulk to the surface of the cell layer will be faster than its disappearance by absorption. As can be seen in Table

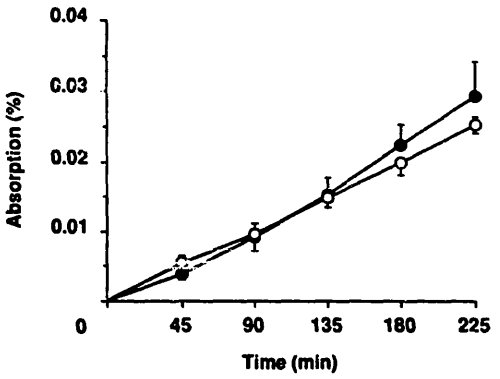

Fig. 2. Transport of raffinose (1.0 μM) across the Caco-2 cell monolayers at stirring flow rates of 100 ml/min (●) and 400 ml/min (○) in the diffusion chamber system. Values are means ± S.D; $n = 4$.

I, raffinose does not have a significant UWL. The absorption of raffinose is very slow and no significant UWL is formed. Therefore, the stirring conditions have no influence on its permeability.

With rapidly absorbed compounds such as testosterone, a significant concentration difference between the liquid layer adjacent to the epithelial surface and the bulk is produced. As a result, the absorption rate varies with the stirring conditions and the thickness of the UWL can be calculated. Such calculations are often based on the assumption that the UWL is the only significant barrier to absorption and no consideration is given to the possible contribution of the epithelial cell layer barrier [5]. However, we have recently shown that this assumption is erroneous and that the cell monolayer contributes significantly to the total resistance to absorption of rapidly absorbed drugs [4]. Therefore, in the present study, the contributions of all barriers to the absorption of testosterone were incorporated in the calculations of the thickness of the UWL.

The effects of stirring conditions on the absorption rate of testosterone in the diffusion chambers and on

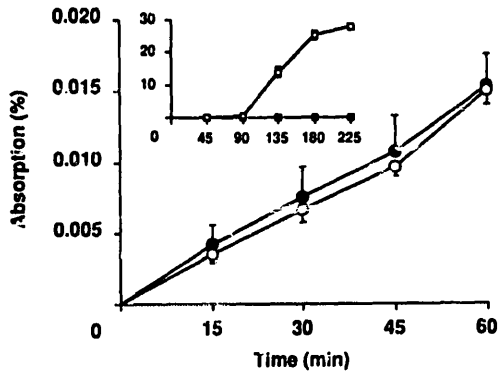

Fig. 1. Transport of raffinose (1.0 μM) across the Caco-2 cell monolayers during 60 min of low; 135 rpm (●) and high; 767 rpm (○) agitation rate on the plate shaker. The inset shows the transport during 225 min of low; 135 rpm (■) and high; 767 rpm (□) agitation rate on the plate shaker. Values are means ± S.D; $n = 4$.

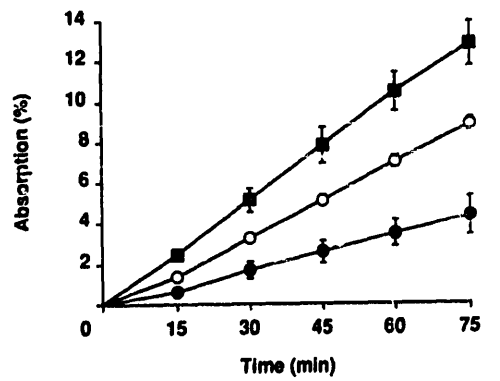

Fig. 3. Transport of testosterone (0.10 mM) across the Caco-2 cell monolayers at different stirring flow rates in the diffusion chamber system:0 ml/min (▲), 100 ml/min (○) and 400 ml/min (■). Values are means ± S.D.; $n = 4$.

TABLE II

Apparent permeability (P_{app}) of testosterone across monolayers of Caco-2 cells under different stirring conditions

Testosterone was added to the apical side of the cells at a concentration of 0.10 mM. The transport of testosterone across the monolayers under different stirring conditions was studied for 75 min in the diffusion chamber system and for 20–30 min on the plate shaker. The apparent permeability coefficients were calculated as described in Materials and Methods. Values represent the means ± S.D. for the number of determinations indicated within parentheses.

Stirring system (a) Diffusion chamber (b) Plate shaker	P_{app} (cm/s) ($\times 10^5$)
(a) Flow rate (ml/min)	
0	5.6 ± 1.1 (6)
100	10.0 + 0.94 (12)
200	12.0 + 1.3 (8)
400	14.9 + 1.2 (8)
600	14.5 ± 0.95 (4)
(b) Agitation rate (rpm)	
0	5.1 ± 0.75 (4)
135	6.1 ± 0.66 (4)
767	10.7 + 0.34 (4)

the plate shaker are shown in Fig. 3 and Table II. Increasing the stirring rates resulted in marked increases in the absorption rates in both models. However, the P_{app} value obtained at the highest flow rate in the diffusion chamber system was approx. 35% higher than that obtained at the maximal shaking rate on the plate shaker, indicating that the gas lift produced the most effective stirring conditions.

With the new methods presented here, we can now determine the cellular permeability (P_c) to passively absorbed solutes [4] (Fig. 4 and Table III). The P_c value of testosterone obtained on the plate shaker was approx. 1.4-times lower than that obtained in the diffusion chamber system. The reason for the discrepancy is not known, but may be related to the more effective stirring obtained in the diffusion chambers. However, the difference between the P_c values is small and we conclude that our method can be applied to different

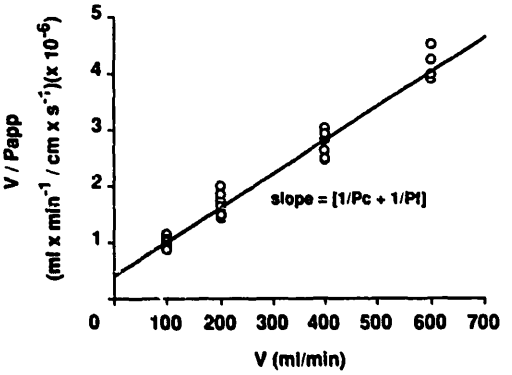

Fig. 4. The cellular permeability (P_c) for testosterone was determined from the slope of the linear relationship between apparent permeability (P_{app}) and stirring flow rate (V). The data points represent single determinations.

diffusion models for in vitro studies on, e.g., drug absorption. The results in Table III corroborate our previous finding that P_c values can be determined using only two stirring rates [4]. Thus, no significant difference between the P_c values was obtained when two (100 and 600 ml/min) or five stirring rates were used.

Our results clearly indicate that the cellular diffusion barrier to rapidly absorbed compounds is significantly greater than an aqueous diffusion barrier of similar thickness: The epithelial resistance to the absorption of testosterone ($1/P_c = (5.05 \pm 0.33) \cdot 10^3$ s/cm; Table III) equals an aqueous diffusion barrier with a thickness of 396 ± 26 μm. Filter-grown Caco-2 cells are generally 17–30 μm thick [20,21] and the true epithelial resistance is therefore 13–23-times higher than that of an UWL of the same thickness. Thus, if the epithelial resistance is assumed to be negligible, the calculated thickness of the UWL will be overestimated, Fig. 5 [5]. Further, the relative contribution of the epithelial resistance to the total resistance increases when the thickness of the UWL decreases. This means that the epithelial resistance is more important in the well stirred situation, i.e., under conditions when it is generally assumed to be negligible.

TABLE III

Comparison of cellular permeability coefficients (P_c) of testosterone obtained in the diffusion chamber system and on the plate shaker

The cellular permeability coefficient for testosterone was determined from the linear relationship between apparent permeability and stirring rate as described in Materials and Methods. The combined resistance of the cell monolayer and the filter support, $[1/P_c + 1/P_f]$, was obtained from the slope of stirring rate/P_{app} vs. stirring rate (see Fig. 4). The slope values were determined from four (100–600) and from two stirring rates (100 and 400; 135 and 767). P_c was calculated after subtracting $1/P_f$ ($= 1.02 \cdot 10^3$ s/cm) from the slope value. The results are expressed as means ± 95% confidence limits.

Stirring method	Stirring rates	No. of data points	$[1/P_c + 1/P_f]$ (s/cm) ($\times 10^{-3}$)	P_c (cm/s) ($\times 10^5$)
Gas lift chamber	100–600 (ml/min)	38	6.07 ± 0.40	19.8 ± 1.3
Gas lift chamber	100 and 600 (ml/min)	20	5.67 + 0.[illegible]	21.[illegible] ± 1.9
Plate shaker	135 and 767 (rpm)	8	7.84 ± 0.63	14.6 ± 1.2

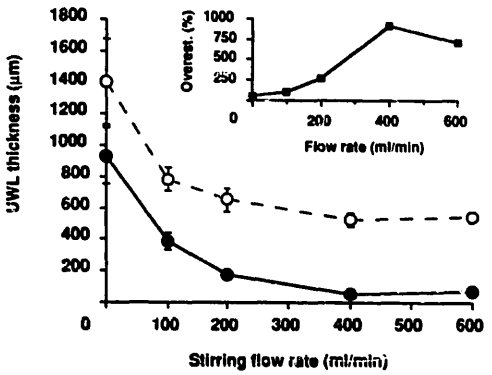

Fig. 5. The thickness of the unstirred water layer (h_{aq}) for testosterone as a function of stirring flow rate in the diffusion chamber system. The h_{aq} values were calculated using two methods: ●, h_{aq} determined using the method in the present paper, i.e., where the resistance of the cell monolayer and the cell culture insert are included in the calculations ($h_{aq} = R_{aq} \cdot D_{aq}$); ○, h_{aq} determined using the standard procedure, i.e., the resistance of the cell monolayer and the cell culture insert are neglected (h_{aq} [app] = $R_{app} \cdot D_{aq}$). Values are means ± S.D.; n = 4–12. The inset shows the overestimation of h_{aq} (in %) when the standard procedure is used.

Calculations of the thickness of the UWL at different stirring rates showed that the gas lift reduced the thickness to 52 ± 4 μm (Table IV). To the best of our knowledge, this value is the lowest reported value of the thickness of the UWL in vitro and should be compared with a recently calculated maximal UWL thickness in dogs and humans of 40 μm [5]. Thus, the gas lift system creates stirring conditions that reduce the thickness of the UWL to values similar to those obtained in humans. However, it should be stressed that the thickness of the UWL is not a constant but a value that is determined by the absorption rate of each compound [3,4]. Only compounds that are absorbed at the same rate have an UWL of the same thickness.

The validity of testosterone as a model compound in drug absorption studies has recently been questioned [22]. Buur et al. showed that testosterone is partly metabolized to androstenedione by monolayers of Caco-2 cells. However, a significant metabolism was observed only when the testosterone concentration was lower than the concentration used in the present study. Thus, at the present testosterone concentration (0.10 mM) only approx. 1% was converted to androstenedione during a 40-min absorption experiment [22]. This indicates that the results presented in this study were not affected by testosterone metabolism.

In conclusion, the results in the present study suggest that our method for determining cell permeability coefficients is generally applicable. Further, the application of this method clearly shows that the intestinal epithelium is a significant barrier to the absorption not only of slowly, but also of rapidly absorbed compounds. In addition, the results show that the stirring flow produced by the gas lift in the diffusion chamber system is superior to that obtained with the plate shaker.

TABLE IV

Effect of varying the stirring conditions on the thickness of the unstirred water layer (UWL)

The thickness of the unstirred water layer at the indicated stirring conditons was calculated according to Eqns. 2 and 7 using the values of $[1/P_c + 1/P_f]$ and P_{app} in Tables II and III. The results are expressed as in Table II.

Stirring system (a) Diffusion chamber (b) Plate shaker	Thickness of the UWL (μm)
(a) Flow rate (ml/min)	
0	934 ± 181 (6)
100	386 ± 51 (12)
200	179 ± 20 (8)
400	52 ± 4 (8)
600	67 ± 4 (4)
(b) Agitation rate (rpm)	
0	911 ± 133 (4)
135	671 ± 73 (4)
767	119 ± 4 (4)

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