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International Journal of Pharmaceutics 222 (2001) 77–89

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Permeability of lipophilic compounds in drug discovery using in-vitro human absorption model, Caco-2☆

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Received 4 August 2000; received in revised form 11 April 2001; accepted 12 April 2001

Abstract

Highly lipophilic compounds are often encountered in the early stages of drug discovery. The apparent permeability (Papp) of these compounds in Caco-2 cell could be underestimated because of considerable retention by the Caco-2 monolayer and non-specific binding to transwell surface. We have utilized a general approach for the determination of permeability of these compounds, which includes the addition of 1–5% DMSO in the apical (AP) and 4% bovine serum albumin (BSA) in the basolateral (BA) side. Two highly lipophilic and highly protein bound Schering compounds, SCH-A and SCH-B, exhibited poor recovery and low Papp in the conventional Caco-2 system that included 1% DMSO in the AP and BA sides. In contrast, both compounds were well absorbed in cynomolgus monkeys. Inclusion of BSA (up to 4%) in the BA side provided necessary absorptive driving force similar to in vivo sink conditions improving both recovery and Papp of these compounds as well as progesterone, a model highly lipophilic and highly protein bound compound. Whereas, the recovery and Papp of mannitol (high recovery, low permeability) and propranolol (high recovery, high permeability) remained unaffected. The presence of 4% BSA increased Papp of SCH-A, SCH-B, and progesterone by five-, four-, and three-fold, respectively. We also compared this approach with a second, based on the disappearance of the compound from the AP side, which resulted in a reasonable estimate of the permeability (23.3×10^{-6} cm/s) for SCH-A. The results demonstrated that the reliable estimates of permeability of highly lipophilic compounds that are subjected to considerable retention by the cell monolayer and exhibit non-specific binding are obtained by the addition of BSA to the BA side. © 2001 Published by Elsevier Science B.V.

Keywords: Caco-2; Bovine serum albumin; Non-specific binding; Permeability; Oral absorption; Lipophilic compounds

Abbreviations: AP, apical or donor side; BA, basolateral or receiver side; dpm, disintegration per minute; HTS, high-throughput screening; mol. wt., molecular weight; NME's, new molecular entities; Papp, apparent permeability; PEG, polyethylene glycol; p-gp, P-glycoprotein; SD, sample standard deviations; TEER, trans-epithelial electrical resistance; TM, transport media.

☆ A part of this work was presented as an abstract and poster at First AAPS Frontier Symposium, 'From Good Ligands to Good Drugs: Optimizing Pharmaceutical Properties by Accelerated Screening', February 19–21, 1998, Bethesda, MA.

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0378-5173/01/$ - see front matter © 2001 Published by Elsevier Science B.V.

PII: S0378-5173(01)00698-6

1. Introduction

The majority of new molecular entities (NME's) generated in drug discovery in the pharmaceutical industry are intended for oral administration because of convenience, safety, and better patient compliance. Thus, oral absorption potential, if not assessed early in drug discovery, could seriously jeopardize the development of a lead compound mainly due to pharmacokinetic deficiencies (Barr et al., 1996). Among various in vitro systems to predict oral absorption in humans, Caco-2 monolayers seem to be the best available model in terms of throughput and reliability. Accordingly, the permeability evaluation in Caco-2 monolayers is becoming the most widely used screen for oral absorption potential in a drug discovery setting in the pharmaceutical industry (Yee, 1996). This system is amenable to higher throughput, which can meet the challenge of the vast increase in the number of NME's emanating as a result of combinatorial chemistry and high throughput screening (HTS).

Studies have shown a good correlation between permeability in the Caco-2 monolayers and the oral absorption in humans (Bailey et al., 1996; Artursson et al., 1996; Rubas et al., 1993). However, these correlations, for the most part, were made with compounds that were well characterized. Unfortunately, at a very early stage of drug discovery, it is not possible to fully characterize compounds in terms of physicochemical properties. Moreover, the in vitro pharmacological screens such as receptor binding assays tend to be selective toward more lipophilic and poorly water-soluble compounds. These compounds pose a major challenge in terms of estimating permeability in the Caco-2 monolayers because of their poor solubility and high lipophilicity, which may result in considerable retention of compounds by the cell monolayer as well as non-specific binding or adsorption to in vitro systems.

Systematic studies addressing permeability as it is affected by the recovery of the compound are lacking. The pre- or post- experimental approaches reported earlier do not address the problems associated with considerable retention of compounds in the Caco-2 monolayers. Additionally, approaches such as post-experimental incubation with organic solvents, (e.g. dimethylsulfoxide and methanol) are tedious and not suitable for higher-throughput (Augustinjns et al., 1993; Hosoya et al., 1996). Other approaches such as the pre-treatment with human serum albumin (HSA) may not work for some compounds because of the extensive adsorption of compounds to the system despite the pre-treatment with HSA (Chan et al., 1996) or considerable uptake by the cell monolayers (Wils et al., 1994). The use of surfactants such as Tween 80, or cremophore EL to minimize non-specific binding seems promising. However, surfactants may interfere with p-glycoprotein (p-gp) which may confound the results (Nerurkar et al., 1996).

Therefore, a universal rather than a compound specific approach is needed to improve recovery so that a reasonable estimate of permeability could be attained. This has an added importance in drug discovery, since the quantities of compounds are very limited, precluding reiterative studies. In this report, we have systematically investigated the approach that might be useful in determining permeability of compounds that could otherwise be underestimated because of significant retention by cell monolayers and non-specific binding to in vitro set-up. Next, this approach was compared with another that is based on the disappearance of the compound from the apical side.

2. Experimental

2.1. Materials

^{3}H-propranolol, and ^{3}H-progesterone were purchased from Sigma Chemical Co (St. Louis, MO). ^{14}C-mannitol was from Moravek Biochemicals (Brea, CA). Corresponding unlabeled chemicals were from Sigma Chemical Co. (St. Louis, MO). SCH-A, SCH-B, and SCH-E were from the Chemical Research Division, Schering-Plough Research Institute, Kenilworth, NJ. The radiochemical purity of the compounds used was $>95\%$.

2.2. Cell and Transwell culture

Caco-2 cells were obtained from American Type Culture Collection (ATCC) at passage # 17. The cells from a passage number between 30 and 50 were used in the experiments. The cells were grown as earlier described (Rubas et al., 1993; Yee, 1996). The cells from a passage number between 30 and 50 were used in the experiments. Briefly, cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Biowhittaker) with high glucose (4.5 g/l) that was supplemented with 10% fetal bovine serum (Sigma), 1% nonessential amino acids (Biowhittaker, Walkersville, MD), 1% penicillin streptomycin solution (PEST; Biowhittaker), and 2% L-glutamine (Biowhittaker). The cells were seeded at a cell density of approximately 10^5 cells per cm^2 on Transwell-Clear polyester membrane filter inserts (0.45 μ pore size, 4.71 cm^2; Corning Costar, Cambridge, MA) in a 6-well transwell plate format. The apical (AP) and basolateral (BA) compartments received 1.6 and 2.5 ml of the culture media. The monolayer integrity was assessed by the inverted light microscopy and the measurement of trans-epithelial electrical resistance (TEER) on a regular basis.

2.3. Caco-2 permeability studies

Caco-2 monolayers exhibiting a TEER value of more than 160 Ω cm^2 were used within 30 days post-seeding. The culture media was removed from both AP and BA sides and the monolayers were washed twice with cold phosphate buffered saline with calcium and magnesium (PBS; Biowhittaker). The monolayers were pre-incubated at 37°C for 30 min in a CO_2 incubator with pre-warmed transport media (TM). The TM consisted of Hanks Balanced Salt solution with calcium and magnesium (HBSS, Biowhittaker), 10 mM HEPES buffer (*N*-2-hydroxyethyl piperazine-*N*'-2-ethanesulfonic acid, Biowhittaker), 25 mM D-glucose, and the pH was adjusted to 7.4.

At the end of the pre-incubation period, the TM was removed and the appropriate volume of labeled and unlabeled compound solution in TM (1–50 μM for SCH-A, -B and -E, and 10–100 μM for all other compounds; total radioactivity ~0.5 μCi/well) was added to the AP side. Lower concentrations were used for SCH-A, -B, and -E because of the lower solubility compared with other compounds (aqueous solubility in the low μg/ml range). All compounds were dissolved in methanol or dimethylsulfoxide (DMSO), and reconstituted with TM to achieve a final organic solvent concentration of up to 5%. The TM for the BA side had either 1% organic solvent or up to 4% bovine serum albumin (BSA, Sigma). Wherever possible, radioactive mannitol (^3H or ^{14}C) was included as a marker for leak and to compare recovery values.

About 10 μl AP samples were taken immediately after the addition of compound to the apical side (zero time) and at the end of experiments (180 min). About 1 ml samples were taken from the BA side at 30, 60, and 180 min. These time points were used since this scheme provided similar estimate of the permeability compared to that from preliminary studies with extensive time points. The BA sample volumes removed were replenished with equal volumes of the fresh TM for the BA side containing BSA. The TEER was measured both in the beginning and at the end of experiment.

The samples were mixed with Flow-Scint scintillation cocktail (Packard, Meridan, CT) and counted in a dual count mode with appropriate correction for spillover using a PACKARD TRI-CARB 2250 CA scintillation counter. The permeability was calculated as follows:

$$\text{Papp} = \frac{\mathrm{d}M/\mathrm{d}t}{A*C}$$

where Papp is apparent permeability in cm/s, $\mathrm{d}M/\mathrm{d}t$ is cumulative radioactivity per unit time (disintegration per minute (dpm) dpm/s) in the basolateral side, A is the surface area (4.71 cm^2), and C is the initial concentration (dpm/ml) in the AP side. All experiments were done in triplicates. Wherever appropriate, statistical comparisons were made using Student's t-test. The recovery was calculated from the AP and BA samples.

2.4. Recovery

At the end of the experiment, the remaining sample was removed from the AP and BA sides. Both sides were washed twice with cold PBS. The filter membrane with monolayers was cut off from the filter insert and counted for adsorbed radioactivity. The AP and BA sides of each well were incubated separately with 5 ml methanol for 30 min to release adsorbed compound. The methanol washes were counted for radioactivity. The recovered radioactivity was determined for AP and BA samples, methanol wash from AP and BA sides, and Caco-2 monolyers with filter. In certain control experiments, Caco-2 monolayers were separated from filter at the end of experiment to determine the amount of radioactivity retained by Caco-2 monolayers and filter separately. In all cases, the filter was found to retain less than 15% of the added radioactivity. All recovery values were expressed as a percent of the amount added at zero time.

2.5. Mathematical approach to determine permeability

A second approach to correct for adsorption to the transwell was evaluated. This approach was based on the disappearance of the compound from the AP side with a delineation of the adsorption component (Chan et al., 1996). For this approach, experiments with SCH-A and progesterone were done with 10 μM compound in the AP side with the Caco-2 monolayer grown on the filter insert. In another experiment to evaluate the adsorption to the transwell system alone, the compound solution was placed both in AP and BA sides of transwell with filter in the absence of Caco-2 monolayers. The apical samples were taken at the same time points used above in Section 2.3 (0, 30, 60, and 180 min). The disappearance rate constants were determined from the linear regression of the log fraction remaining in the apical side (expressed as a fraction of the amount added at zero time) versus time curve in the presence and absence of Caco-2 monolayers. The permeability was calculated as follows:

$$\text{Papp} = \frac{V(K_{\text{total}} - K_{\text{ads}})}{A}$$

where, K_{total} is the disappearance rate for the Caco-2 and filter and K_{ads} is the disappearance rate for adsorption of compound to the transwell system with filter alone, V is initial volume in the apical side (1.6 ml), and A is the effective surface area available for transport (4.71 cm^2).

2.6. Physciochemical properties

The ACD/log P suite from Advance Chemistry Development, Inc (Toronto, Canada) was used to estimate Log octanol/water partition coefficient values (log P). All Schering compounds were basic compounds with pKa around 2.5. The structures of the compounds (SCH-A and SCH-B) are very similar to those reported in a recent publication (Taveras et al., 1999). The generic structures of SCH-A and SCH-B are shown in Fig. 1.

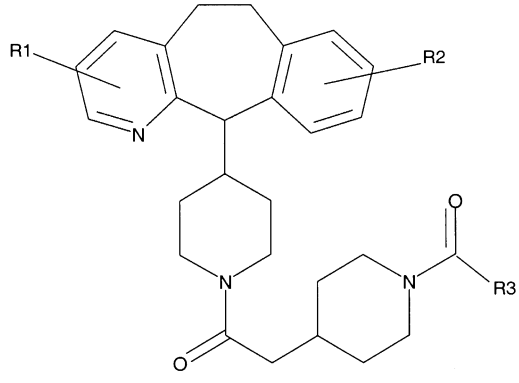
3. Results

The recovery and permeability of propranolol, progesterone, mannitol, and three NME's (SCH-A, SCH-B, and SCH-E) that were structurally unrelated except SCH-A and SCH-B were first evaluated. The recovery values without the methanol wash were about 80 and 100% for propranolol and mannitol, respectively. The recovery was $< 70\%$ for a relatively more lipophilic compound, progesterone.

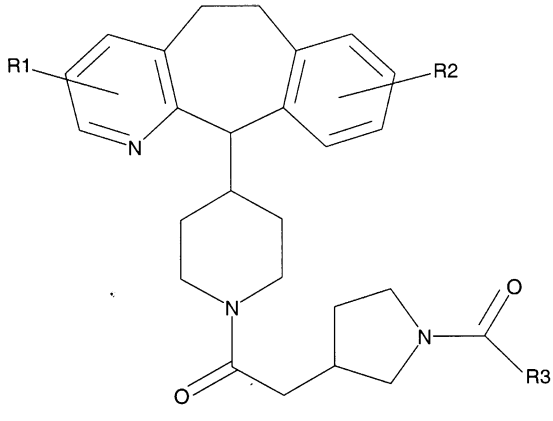
SCH-A and progesterone, lipophilic and consequently high protein bound (> 99 and 96–99%, respectively,) compound with passive transcellular diffusion, were selected as prototype compounds. Fig. 2 shows the permeability of SCH-A in the presence of 1% of either CH_3OH or DMSO in both AP and BA sides. The results showed that the permeability was low ($\sim 3–4 \times 10^{-6}$ cm/s) and was similar in the presence of either co-solvent. However, the total recovery was only about 30%. Therefore, it was postulated that the low permeability of SCH-A was due to the its low recovery. An experiment was conducted to localize the missing radioactivity in which CH_3OH was used to wash the radioactivity associated with

both AP and BA sides as well as Caco-2 cells. The results showed that the majority of missing radioactivity was recovered from the Caco-2 cells (Table 1), since the radioactivity retained in the filter, that was determined in a separate control experiment, was less than 15%.

The recovery and permeability values for SCH-A at 50 μM were similar (Fig. 2) to those found at 10 μM. Again, the majority of the radioactivity ($\sim 54\%$) was found to be associated with the Caco-2 monolayers (data not shown). The recovery for mannitol was $\sim 100\%$, which was present in the same well with SCH-A. Mannitol was not retained by the Caco-2 cells as would be expected for a paracellularly transported hydrophilic compound. The recovery and permeability values for SCH-A at 1 μM were also similar to those found at 10 and 50 μM (data not shown).



SCH-A



SCH-B

Fig. 1. Generic structures for SCH-A and SCH-B. Rs are either halogens or other functional groups.

Experiments were also carried out to reduce the cell associated compound by adding BSA to the BA side. Fig. 3 shows that the permeability in the presence of 1% DMSO in the AP side and in the absence of 1% DMSO or BSA in the BA side ($3.8 \pm 0.33 \times 10^{-6}$ cm/s) is identical to permeability in the presence of 1% DMSO in both AP and BA side ($3.8 \pm 0.52 \times 10^{-6}$ cm/s; Fig. 2) suggesting that 1% DMSO in the BA side does not enhance permeability. However, permeability increased considerably with increasing concentration of BSA in the BA side. This increase in the permeability directly correlated with a decrease in the amount retained by the cells (Fig. 4). In the presence of 4% BSA in the BA side, the Caco-2 monolayer together with filter accounted for only $\sim 26\%$ of the added amount for SCH-A. The AP and BA sides without methanol wash accounted for 75% of the added amount, which was much higher than that with 1% DMSO in both AP and BA side (34%). Interestingly enough, SCH-A amount associated with the apical surface (from the AP methanol wash) also decreased considerably from 8.5 to 0.2% (~ 43-fold) even though the apical TM was the same (1% DMSO) for both treatments. This suggested that BSA provides sink conditions similar to in vivo situation and minimizes the adsorption not only to BA side where it is present but also to the AP side where it is not present perhaps by reducing the residence time of the compound in the AP side. The direct result of the considerable reduction in the amount associated with Caco-2 monolayer and the amount adsorbed to the AP side was reflected in the Papp of SCH-A, which increased by about five-fold (from 3.8 to 18.8×10^{-6} cm/s).

A similar trend was also seen with SCH-B (Fig. 5), another lipophilic and highly protein bound ($> 99\%$) compound with passive transcellular diffusion. The Papp was increased by about four-fold (from 2.2 to 9.4×10^{-6} cm/s), when 4% BSA was used in the BA side. Again, this increase in the Papp was primarily due to a decrease in the

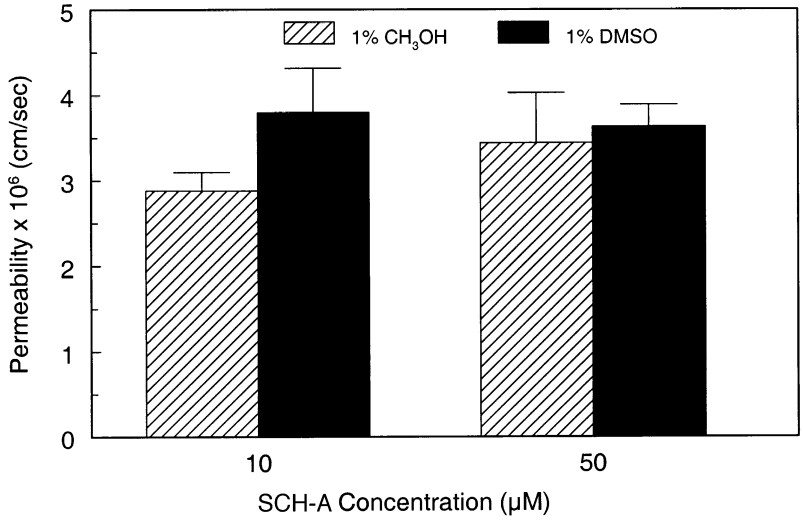


Fig. 2. Permeability of SCH-A in Caco-2 monolayers at different initial apical SCH-A concentration in the presence of 1% CH_3OH (solid bar), and 1% DMSO (hatched bar) in the AP and BA sides. Values are mean of triplicate with the error bar representing one standard deviation from the mean. Differences in the mean permeability between two co-solvents were not statistically significant at 95% confidence interval.

Table 1
Recovery for SCH-A and mannitol

Recovery	BA (1%DMSO)		BA (4% BSA)	
	Mannitol	SCH-A	Mannitol	SCH-A
AP (sample)	99.9 (2)	19.8 (19)	102.2 (3)	15.4 (17)
AP (methanol wash)	0.3 (36)	8.5 (17)	0.4 (38)	0.2 (21)
BA (sample)	4.4 (9)	11.6 (7)	4.3 (16)	59.7 (6)
BA (methanol wash)	0.1 (32)	1 (21)	0.1 (77)	0.4 (10)
AP+BA (total)	104.7	40.9	107.0	75.7
Caco-2+Filter	0	54.2 (20)	0	26.2 (14)
Total recovery	104.7	95.1	107	101.9

Mannitol was present in the same well as a leak marker with SCH-A. Apical (AP) side transport media (TM) had 1% DMSO and basolateral (BA) side TM had either 1% DMSO or 4% BSA. Values are expressed as mean (%CV) of triplicate; coefficient of variation; CV is shown for actual measurements, e.g. AP (sample) and AP (methanol wash), but not for AP+BA (total), which was obtained by adding AP (sample), AP (methanol wash), BA (sample), and BA (methanol wash) amounts.

amount retained by the Caco-2 monolayer in the presence of 4% BSA. The recovery values for SCH-B are presented in Table 2. The recovery values for yet another relatively less lipophilic Schering candidate, SCH-E, are also presented in Table 2. The recovery values for this compound were similar in the presence or absence of 4% BSA in the BA transport media. As would be expected, the permeability remained unaffected by the inclusion of 4% BSA in the AP side Fig. 5.

Also shown in Fig. 5 are the permeability values for various compounds in the presence of 1% DMSO or 4% BSA. The inclusion of BSA in the BA side did not affect the permeability of structurally diverse model compounds, mannitol (passive paracellular transport) and propranolol (passive transcellular transport). However, for a relatively more lipophilic model compound,

progesterone, the permeability increased by about three-fold (from 8.2 to 22.6 $\times 10^{-6}$ cm/s) with 4% BSA compared with 1% DMSO in the BA side.

The semilog plot of fraction remaining as a function of time is shown in Fig. 6. The calculated Papp (in cm/s $\times 10^{-6}$) for SCH-A based on the disappearance from the AP side alone and appearance in the BA side were 23.3 and 3.3, respec-

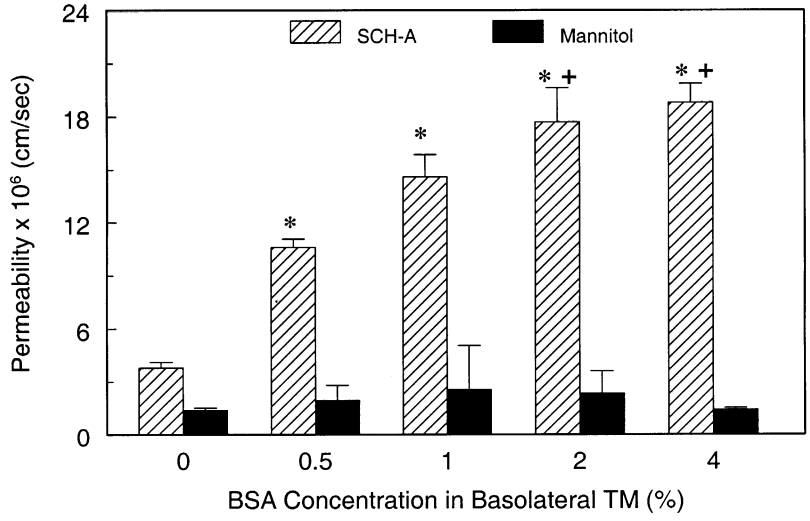


Fig. 3. Effect of BSA concentration in the BA side on the permeability of SCH-A (10 μM; hatched bar) and mannitol (solid bar) in Caco-2 monolayers. The AP side had TM with 1% DMSO. Values are of triplicate with the error bar representing one standard deviation from the mean. (*) Statistically, the mean permeability values with BSA (from 0.5 to 4%) were significantly different from the mean permeability values without BSA in the BA side; $P < 0.05$ using the Student's t-test. (+) Also, the mean permeability values with both 2 and 4% BSA were significantly different from the mean permeability values with 0.5, and 1% BSA; $P < 0.05$ using the Student's t-test.

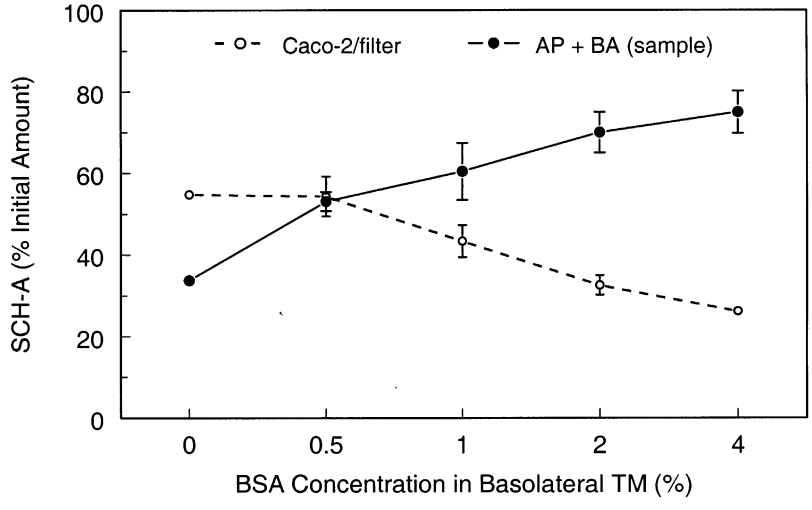


Fig. 4. Relationship SCH-A recovery (expressed as the percentage of SCH-A amount added initially) with increasing BSA concentration in BA side. Data are mean of triplicate with the error bar representing one standard deviation from the mean. Error bars, if smaller, could have been hidden by symbols.

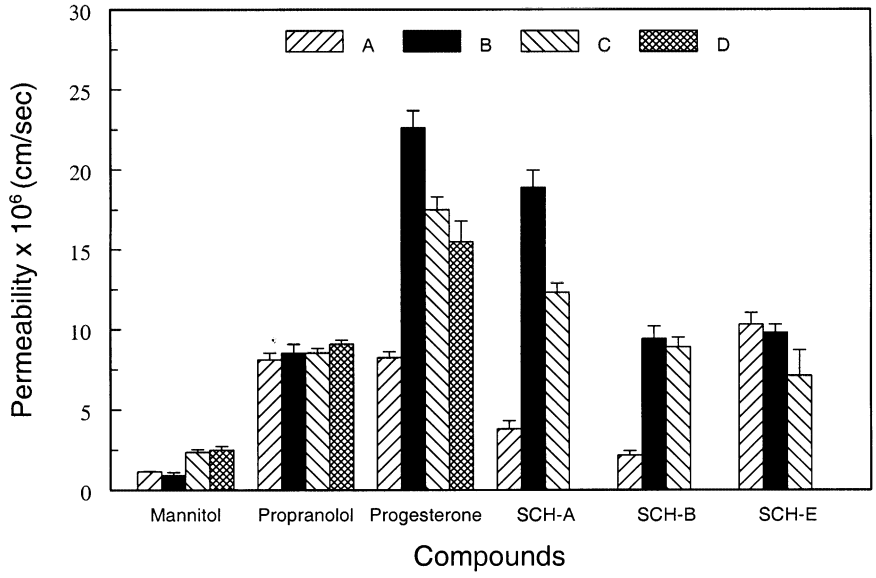


Fig. 5. Permeability of compounds across Caco-2 monolayers. A, 1% DMSO in the AP and BA sides; B, 1% DMSO in the AP and 4% BSA in the BA sides; C, 5% DMSO in the AP side and 4% BSA in the BA side; D, 5% DMSO in the AP side and 5% DMSO + 4% BSA in the BA side. Values are mean of triplicate with the error bar representing one standard deviation from the mean. Error bars, if smaller, could have been hidden by symbols.

Table 2
Recovery for SCH-B and SCH-E. Apical (AP) side TM contained 1% DMSO, and basolateral (BA) side TM had either 1% DMSO or 4% BSA

Recovery	SCH-B (BA side)		SCH-E (BA side)	
	1% DMSO	4% BSA	1% DMSO	4% BSA
AP (sample)	30.6 (66)	26.4 (21)	51.4 (3)	57 (3)
AP (methanol wash)	12.6 (8)	4.6 (29)	1.2 (26)	1.2 (26)
BA (sample)	6.8 (43)	29.8 (9)	32.6 (7)	31.2 (5)
BA (methanol wash)	1.1 (21)	0.1 (43)	0.1 (14)	0.1 (50)
AP+BA (total)	51.1	60.9	85.3	89.5
Caco-2+filter	50.2 (6)	36.5 (2)	15.8 (5)	11.4 (3)
Total recovery	101.3	97.4	101.1	100.9

Values are expressed as mean (%CV) of triplicate; coefficient of variation; CV is shown for actual measurements, e.g. AP (sample) and AP (methanol wash), but not for AP+BA (total), which was obtained by adding AP (sample), AP (methanol wash), BA (sample), and BA (methanol wash) amounts.

tively, which is illustrated in Fig. 7. The Papp for SCH-A based on the appearance in the BA side in the presence of 4% BSA was 19.5. Similarly, the calculated Papp for progesterone based on the disappearance from the AP side alone and appearance in the BA side was 8.2 and 14.4, respectively. The Papp for progesterone based on the appearance in the BA side in the presence of 4% BSA was 22.6, which was higher than that from the disappearance (mathematical) approach.

4. Discussion

SCH-A, SCH-B, and SCH-E are all moderate to well absorbed compounds in cynomolgus monkeys (unpublished data); however, SCH-A and SCH-B exhibited low permeability in conventional Caco-2 system with 1% DMSO or 1% CH_3OH as a co-solvent in the AP and BA sides. The log *P* values for SCH-A and SCH-B, were higher than that of progesterone, with a rank order of SCH-A > SCH-B (Table 3). The log *P* value for SCH-E was lower than that of propra-

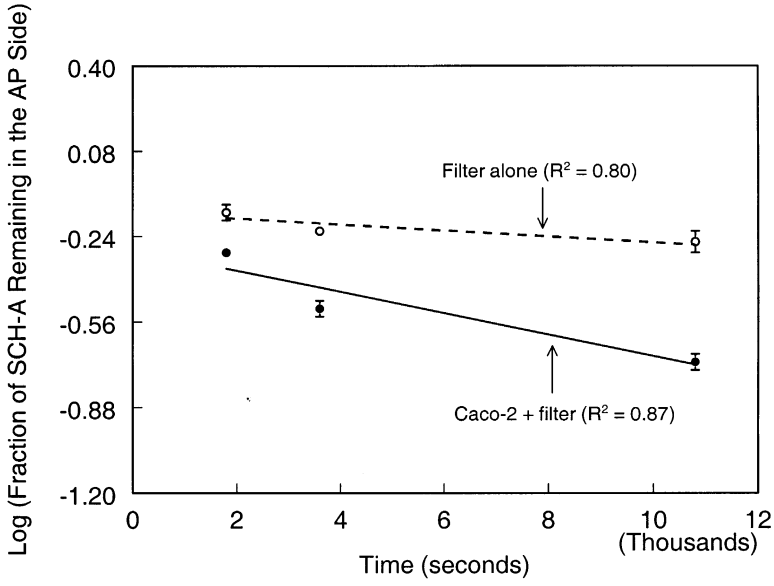


Fig. 6. SCH-A disappearance from the AP side in the presence and absence of Caco-2 monolayers. The lines are regression lines for all the data points from triplicate experiments. Data points are mean of triplicate with the error bar representing one standard deviation from the mean. Error bars. If smaller, could have been hidden by symbols.

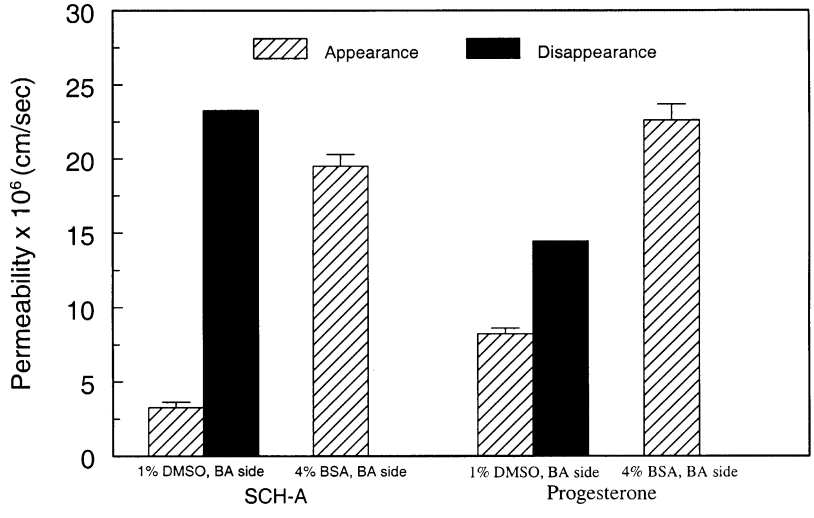


Fig. 7. Permeability calculated from both appearance in the BA side and disappearance from the AP side for SCH-A and progesterone. The AP side contained 1% DMSO and BA side had either 1% DMSO or 4% BSA.

Table 3
Molecular weight and log *P* for compounds used in these studies

Compound number	Compound	Mol.ecular weight	Log *P*
1	Mannitol	182.2	−2.50
2	SCH-E	563.3	2.18
3	Propranolol	259.3	2.53
4	Progesterone	314.5	3.87
5	SCH-B	624.8	5.89
6	SCH-A	638.8	6.32

nolol (Table 3). Consistent with high lipophilicity, protein binding for SCH-A, SCH-B, and progesterone is also high.

It appears that Caco-2 permeability values of SCH-A, SCH-B, and progesterone, were underestimated because of the poor recovery from AP and BA sides. In contrast, the permeability was not underestimated for SCH-E, propranolol, and mannitol that were found to have good recovery values ($> 70\%$) in the presence as well as absence of 4% BSA in the BA side. In the presence of 4% BSA in the BA side, however, both recovery and permeability values were increased considerably for SCH-A, SCH-B, and progesterone. This is illustrated in Fig. 8. The permeability values for other structurally diverse model compounds, PEG-4000 (passive transport; very low Papp), cimetidine (passive paracellular/transcellular transport; low Papp), L-glucose (passive transport; low Papp), and D-glucose (active transport; high Papp) were not affected by the inclusion of 1% DMSO in the AP and 4% BSA in the BA sides, since the recovery values in the absence of BSA were more than 70%.

This study clearly demonstrates that inclusion of BSA provides the necessary absorptive driving force reflecting in vivo sink conditions; thereby resulting in a dramatic reduction of cell associated as well as non-specifically bound compound to the AP side, which ultimately enhanced the transport of SCH-A, SCH-B, and progesterone. The presence of 4% BSA in the BA side appeared to better mimic in vivo sink conditions where serosal side (tantamount to the BA side in an in vitro setting) is perfused with the blood that has about 4% albumin. The albumin, that can bind lipophilic compounds, provides necessary driving force.

The material of the permeable support (filter) that is used to grow monolayer has been shown to affect the transport of compounds across Caco-2 monolayers (Nicklin et al., 1992). Therefore, to

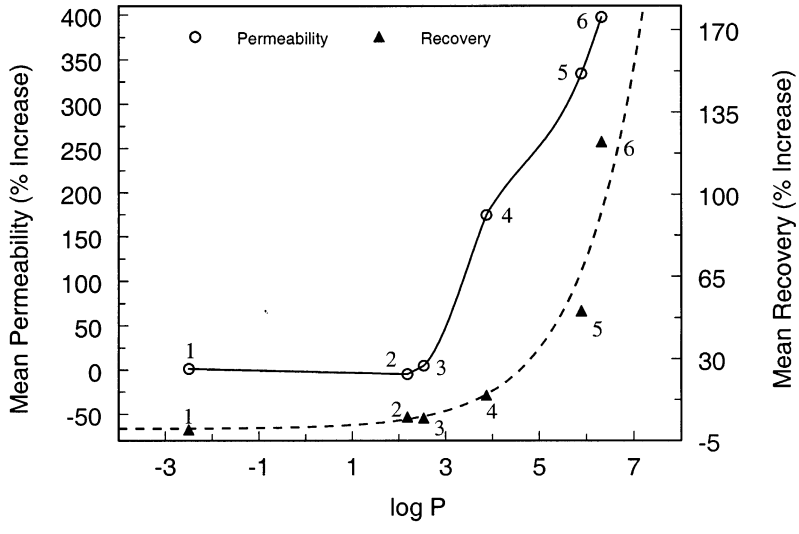


Fig. 8. Relationship of the log *P* with percentage increase in the mean permeability values (open circle) and percentage increase in the mean recovery values (solid triangles), when 4% BSA as opposed to 1% DMSO was used in the BA side. The TM in AP side was with 1% DMSO. The numbers correspond to compound numbers in Table 3.

preclude this possibility, the permeability in the filter alone was determined separately for compounds and it was found not to be rate-limiting. The involvement of the p-glycoprotein (p-gp) in the transport of SCH-A through the Caco-2 monolayer grown in our laboratory was ruled out by evaluating unidirectional fluxes in forward (AP to BA) and backward (BA to AP) directions which were found to be similar (Papp ratio AP to BA and BA to AP ~ 1). In addition, the AP to BA permeability in our study did not change appreciably with the inclusion of 100 μM verapamil, a known inhibitor (Mintenig et al., 1993) for the p-glycoprotein pump (Hunter et al., 1993). Moreover, western blot analysis revealed that the expression level of p-gp in Caco-2 cells grown in our laboratory was very low (Krishna et al., 1999), confirming that our results are not confounded by the effect on p-gp. The post-experimental wash of the BA side with methanol did not improve permeability values for SCH-A, SCH-B and progesterone precluding the utility of this approach (Augustinjns et al., 1993; Hosoya et al., 1996) to evaluate permeability of highly lipophilic compounds in drug discovery. Also, the pre-treatment of AP and BA sides with 10% fetal bovine serum for more than 3 weeks before initiating experiments did not improve permeability (Chan et al., 1996).

In the presence of Caco-2 monolayers, the disappearance of the compound from the AP side was due to both adsorption (if any) and transport. In the absence of Caco-2 monolayers with the compound solution in both sides to prevent any transport, the disappearance was due to adsorption alone. The permeability estimated from the disappearance approach curve was reasonable for SCH-A. The permeability estimate for progesterone, however, was lower than that utilizing 4% BSA in the BA side. We recognize that since limited number of samples were taken to characterize the disappearance kinetics, the observed differences may not be significant. Nevertheless, the disadvantage of mathematical approach is that it involves additional control experiments without the Caco-2 monolayers and as a result requires more compounds and generates twice as many samples in comparison to the method involving the inclusion of BSA in the BA side. Therefore, although this approach is not suitable in a drug discovery setting to screen a large number of compounds, it may nonetheless be useful in a development scenario with limited number of compounds.

Our study is unique in a sense that we have also used up to 5% DMSO (Fig. 5) as opposed to up to only 1% DMSO used in other studies (Taylor et al., 1997; Okumu et al., 1997). This was done with an aim to maximize the possibility of solubilizing poorly soluble compounds. The permeability of a hydrophilic marker compound, mannitol, was elevated with 5% DMSO in the AP side (Fig. 5). However, the permeability of transcellulary transported lipophilic compounds, SCH-A, SCH-B, progesterone, and propranolol (Fig. 5) as well as an actively transported compound, D-glucose, did not increase. In fact, there was a drop in the permeability of SCH-A, SCH-B, and progesterone with an increasing concentration of DMSO. This supports the hypothesis that perhaps the slight reduction in the TEER and other not so obvious morphological changes as a result of treatment with 5% DMSO in the presence of 4% BSA in the BA side do not increase the transport of lipophilic compounds transported through transcellular pathway. As far as slight reduction in permeability with 5% DMSO is concerned, it is likely that the increasing concentration of DMSO, a highly permeable solvent, decreases binding of the compound to BSA (Irollo et al., 1987), thereby, reducing the driving force necessary for ideal in vivo sink conditions. Further studies are needed to elucidate the underlying mechanism with regard to the effect of DMSO on the binding of compounds to BSA.

The TEER values at the end of the experiment (2 h) dropped dramatically ($> 30\%$ of initial value) with 5% DMSO in the AP side, and in the absence of BSA in the BA side; therefore 5% DMSO alone could not be used. Whereas, the TEER values in the presence of 4% BSA in the BA side and 5% DMSO in both AP and BA sides were lowered at the end of experiment (2 h) but remained within an acceptable range (within 80% of the initial TEER).

To the best of our knowledge, this is the first study showing that the DMSO concentration as high as 5% in the AP side in the presence of 4% BSA in the BA side can be used with Caco-2 monolayer to solubilize relatively insoluble compounds without any appreciable damage to CaCo-2 monolayer and increase in the permeability of transcellulary transported compounds. In as far as small hydrophilic compounds that are water soluble and primarily transported through the paracellular route are concerned, DMSO is not required for solubilization. Therefore, it is prudent not to use 5% DMSO in the AP side while evaluating the permeability of hydrophilic compounds.

5. Conclusion

Reliable estimates of permeability were attained for compounds that exhibited extensive retention by the Caco-2 monolayers and non-specific binding to in vitro apparatus by including bovine serum albumin in the basolateral side. The inclusion of the BSA in the basolateral side resulted in a considerable improvement in recovery and consequently permeability values for highly protein bound lipophilic compounds, SCH-A, SCH-B, and progesterone, which exhibited poor recovery. The permeability for relatively less lipophilic and structurally diverse compounds exhibiting good recovery, SCH-E, propranolol, and mannitol, remained relatively unaffected by the inclusion of bovine serum albumin in the basolateral side. The finding that up to 5% DMSO in the apical side with 4% BSA in the basolateral side can be used with the Caco-2 monolayers could help in obtaining a reliable estimate of intrinsic permeability of otherwise relatively insoluble and lipophilic compounds. In our view, the low permeability values associated with the poor recovery should be interpreted with caution because in some of these cases the permeability obtained is likely a conservative estimate of actual permeability.

Acknowledgements

The authors would like to thank Mitchell N. Cayen, Ph.D. and Ronald E White, Ph.D. for a critical review of the manuscript and valuable suggestions. The authors would also like to thank Laura Norton for excellent technical assistance.

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