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Research paper

Effect of bovine serum albumin on drug permeability estimation across Caco-2 monolayers

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Abstract

The purpose of this study was to explore approaches to more accurately assess Caco-2 permeability of poorly water-soluble new chemical entities (NCEs) in an effort to determine their biopharmaceutics classification system (BCS) permeability class with a higher level of confidence. The transport of reference compounds and NCEs (Sch-Y, Sch 56592) was studied across Caco-2 monolayers in the absence or presence of varying percentage of bovine serum albumin (BSA) in the receiver chamber. The inclusion of 0.5–4% BSA in the receiver chamber caused a 4–5-fold increase in Sch-Y P_{app}, while Sch 56592 P_{app} was not significantly influenced. Amongst reference solutes, the P_{app} ratio (+BSA/ctrl) was significant (1.3-fold) only for diltiazem (log PC = 2.7, plasma protein binding = 78%), but the prediction of human oral absorption for such drugs was not affected by the presence of BSA in receiver. In summary, the use of 4% BSA in the receiver chamber during transport studies can dramatically affect the estimated Caco-2 P_{app} and BCS permeability ranking of highly lipophilic NCEs, as in the case of Sch-Y with a log PC of 4.0. For Sch-Y, this is presumably due to improved sink conditions and/or a reduction in non-specific drug adsorption to plastic wells. In contrast, the permeability classification of Sch 56592 (log PC = 2.4) based on estimated Caco-2 P_{app} values is not affected by the presence of receiver BSA.

Keywords: Caco-2; Permeability estimation; Bovine serum albumin; Oral absorption

1. Introduction

With the advent in recent years of combinatorial chemistry and high throughput screening assays, an early prediction of bioavailability is critical in the drug selection process to promote lead candidates from discovery to development. The assessment of drug absorption is one essential part in estimating bioavailability. For orally administered compounds, the use of Caco-2 monolayers (human colon adenocarcinoma cell line) [1,2] as an in vitro human absorption surrogate is well established. There are indeed numerous examples in the literature of the successful application of Caco-2 monolayers for the prediction of or correlation with human absorption [3,4].

The purpose of the present study was to explore approaches to more accurately assess the Caco-2 permeability and biopharmaceutics classification system (BCS) permeability class of poorly water-soluble new chemical entities (NCEs), rather than to extend our current understanding of using Caco-2 monolayers to predict permeability. In many cases, new chemical entities are very lipophilic and water-insoluble [5]. These poorly water-soluble compounds are often problematic in Caco-2 transport studies due to their inherent solubility limitation, compromise in sink conditions, and non-specific adsorption. This often results in inaccurate estimation of Caco-2 permeability, and the extent of human absorption being severely underestimated. The present study demonstrates that in Caco-2 transport studies with highly lipophilic compounds, the presence of bovine serum albumin (BSA) in the receiver compartment can greatly improve the accuracy of the permeability values obtained.

Based on early phase preformulation data, 2 poorly water-soluble compounds of unrelated pharmacological class were studied. Sch-Y, an imidazole derivative, is a H_3 receptor antagonist with pK_a of 6.45, log PC of 4.0, and solubility of 5 μg/ml in pH 7.4 phosphate buffer. Sch 56592 is a broad-spectrum triazole antifungal agent and has pK_as of 4.64 and 3.58, log PC of 2.4, and 12 μg/ml solubility in pH 7.4 modified Ringer's buffer [6,7]. The transport of reference compounds (polyethylene glycol 4000, mannitol, cimetidine, phenytoin, diltiazem) as outlined in the BCS [8–10] was also studied in the presence

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of BSA. From the correlation of our Caco-2 permeability values with human oral absorption data (from the literature) [9,10] for these reference compounds, it was possible to accurately classify Sch-Y and Sch 56592 on a permeability diagram as reported by Amidon et al. [8].

2. Materials and methods

2.1. Materials

^{3}H-Sch-Y (specific activity, 1.73 mCi/mg) and ^{14}C-Sch 56592 (specific activity, 78 μCi/mg) were provided by Schering–Plough Radiochemistry (Kenilworth, NJ). The ^{3}H-label on Sch-Y was in a non-exchangeable position of the molecule so that washout of the tritium will not be a concern. Radiolabeled reference molecules (D-^{3}H-mannitol, D-^{14}C-mannitol, ^{3}H-diltiazem, ^{14}C-5,5-diphenylhydantoin, ^{3}H-cimetidine, and ^{14}C-polythylene glycol 4000) were obtained from Dupont NEN (Boston, MA) and Amersham Co. (Downers Grove, IL). Sch-Y and Sch 56592 drug substance were supplied by Schering–Plough Chemical Research (Kenilworth, NJ). Unlabeled compounds and BSA were purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture media and supplies were obtained from Gibco (Grand Island, NY). Transwell inserts (0.4 μm, 12 mm diameter), and cluster plates were obtained from Corning-Costar Corp. (Cambridge, MA).

2.2. Caco-2 cell culture

The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD) at passage 17. Cells were cultured at 37°C in 95% air/5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM with 4.5 g/l D-glucose) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 2 mM L-glutamine, and 1% penicillin–streptomycin [1,2]. Cells were subcultured every 7 days by treatment with 0.05% trypsin-0.53 mM EDTA, and plated on collagen-coated (rat tail collagen, type I) polycarbonate Transwell filters at a density of 60,000–100,000 cells/cm^2. The transepithelial electrical resistance (TEER, Ω cm^2) of cultures was monitored with an EVOM epithelial voltohmeter (World Precision Instruments, Sarasota, FL). Physiologically and morphologically well developed confluent Caco-2 monolayers (at least 21 days old) as observed under light microscopy and with TEER values of ~300 Ωcm^2 were used for drug transport studies.

2.3. Drug transport across Caco-2 monolayers

Prior to each experiment, Caco-2 monolayers on Transwell inserts were washed with pH 7.4 modified Ringer's solution (MRS) containing 1 mM $CaCl_2$, 5.3 mM KCl, 0.4 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 3.3 mM NaH_2PO_4, 137 mM NaCl, 10 mM HEPES, and 25 mM D-glucose. The TEER was then measured to gauge the integrity of the monolayers.

2.3.1. Apical–basal transport of Sch-Y and Sch 56592

Transport was initiated by adding 0.5 μCi of ^{3}H-Sch-Y or ^{14}C-Sch 56592, and 0.5 ml of unlabeled drug saturated MRS to the apical (donor) chamber of Transwell inserts. The basolateral (receiver) chamber was bathed with 1.5 ml MRS in the absence or presence of 0.5%, 1%, 2%, and 4% bovine serum albumin (BSA). The transport of D-mannitol (1 mM unlabeled mannitol with 1 μCi/ml ^{3}H-or ^{14}C-mannitol) as a paracellular marker [11] was monitored simultaneously. The Transwell units were agitated on a Rotomix orbital shaker (Thermolyne, Dubuque, IA) that was housed in a 37°C incubator. For apical–basal flux study, 200 μl of receiver fluid was sampled at 0.5, 1, 1.5 and 2 h post-dosing, and counted in a liquid scintillation spectrometer (Wallac, Gaithersburg, MD). The volume withdrawn was replaced with an equal volume (200 μl) of pre-equilibrated MRS containing varying percentages (0, 0.5%, 1%, 2%, 4%) of BSA. The integrity of cell monolayers was assessed by mannitol permeability, and TEER was also measured at the end of each transport experiment for comparison with initial values. Following the transport study, each Transwell filter with cells was washed with ice-cold MRS buffer to wash out extracellular label; the filter was then cut, placed in 0.5 ml of 0.5% Triton X-100 for 30 min and vortexed in order to lyse the cells. The radioactivity incorporated into cells or adsorbed on the filter was then estimated.

2.3.2. Apical–basal transport of diltiazem, cimetidine, phenytoin, PEG 4000, and mannitol

The same transport protocol as described for Sch-Y and Sch 56592 was used for the reference compounds. Diltiazem, cimetidine, phenytoin, and PEG 4000 were studied at a donor concentration of 0.1 mM unlabeled drug with 1 μCi/ml ^{3}H-diltiazem, -cimetidine, or ^{14}C-phenytoin, -PEG 4000, respectively. For D-mannitol, the donor concentration was 1 mM unlabeled mannitol with 1 μCi/ml ^{3}H-mannitol. The basolateral compartment was bathed with 1.5 ml MRS in the absence or presence of 0.5%, 1%, 2%, and 4% BSA.

2.4. Data analysis

The steady-state flux was estimated from the slope of the linear portion of a plot of cumulative amount of drug appearing in receiver fluid vs. time. The apparent permeability coefficient (P_{app}, cm/s) was calculated from the observed flux (dQ/dt) normalized against the surface area of filter membrane (A, 1.13 cm^2) and the initial soluble radiolabeled drug concentration (C_o) in donor fluid. Statistical significance was tested by two-tailed Student's t-test or one-way analysis of variance (ANOVA) (Fisher PLSD), and set at $P < 0.05$.

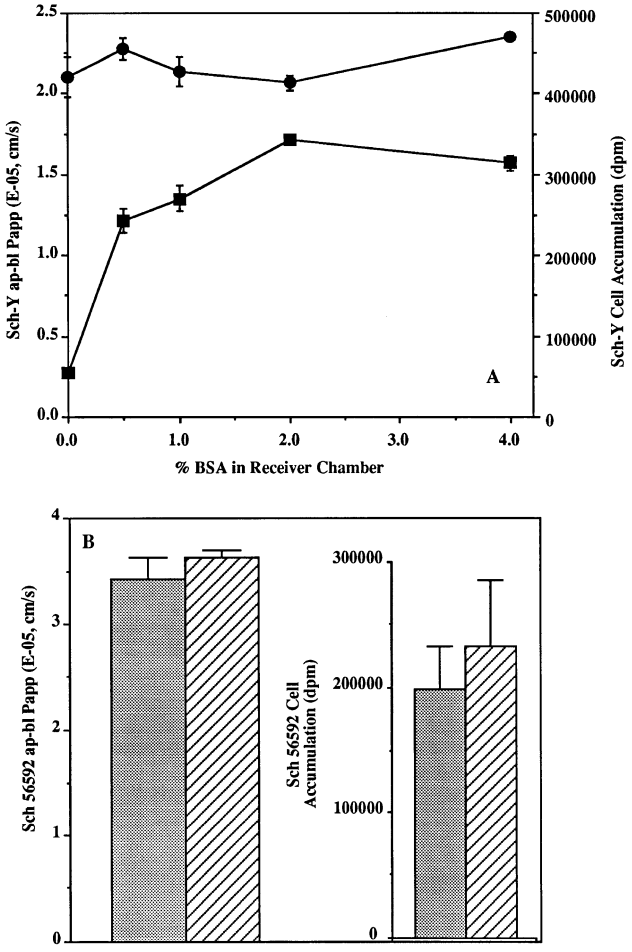

Fig. 1. Panel A: Sch-Y apical–basal P_{app} (■) and cell accumulation (●) as a function of bovine serum albumin percent in receiver chamber. Panel B: Sch 56592 apical–basal P_{app} and cell accumulation in the absence (▤) or presence (▨) of 4% bovine serum albumin in receiver chamber. Error bars represent SEM for $n = 3$–4. Wherever it is not seen, the error bar is smaller than the size of the symbol.

3. Results

3.1. Sch-Y and Sch 56592 permeability: effect of BSA in receiver

The effect of increasing percentage of BSA in the receiver (basolateral) chamber on the apical–basal permeability of Sch-Y across Caco-2 monolayers is shown in Fig. 1A. In the absence of BSA in the receiver chamber (control), the apical–basal apparent permeability coefficient (P_{app}) for Sch-Y was 0.3×10^{-5} cm/s. The addition of 0.5% BSA caused a 4-fold increase ($P < 0.05$) in Sch-Y P_{app} to 1.2×10^{-5} cm/s. Thereafter, Sch-Y permeability increased with increasing percentage of BSA in receiver, and attained a plateau P_{app} value of 1.6×10^{-5} cm/s with 4% BSA. Therefore, the effect of further increasing the percentage of BSA in the receiver chamber was not explored in subsequent experiments. The amount of ^{3}H-Sch-Y accumulated within Caco-2 monolayers at the end of flux studies remained unchanged ($P > 0.05$) with increasing percentage (0–4%) of BSA in receiver (Fig. 1A).

In the case of Sch 56592, the apical–basal permeability in the absence of BSA was 3.4×10^{-5} cm/s. Interestingly in this case, the presence of 4% BSA in the receiver chamber had no significant effect ($P > 0.05$) on Sch 56592 P_{app} (3.6×10^{-5} cm/s) (Fig. 1B). The accumulation of ^{14}C-Sch 56592 within Caco-2 monolayers also remained unchanged ($P > 0.05$) in the absence or presence of BSA in the receiver chamber (Fig. 1B inset).

3.2. Permeability of reference compounds: effect of BSA in receiver

Table 1 illustrates the apical–basal P_{app} values of reference compounds in the absence or presence of 4% BSA in the receiver chamber. In either case, higher P_{app} values ($\geq 3 \times 10^{-5}$ cm/s) were obtained for diltiazem and phenytoin (Table 1). Cimetidine exhibited a permeability value comparable to that of mannitol. Except in case of diltiazem, the P_{app} value of solutes was not significantly different when estimated with or without receiver BSA. Furthermore, there was only an insignificant change in mannitol (paracellular marker) permeability when estimated in the presence of varying percentages (0.5, 1, 2, 4%) of BSA in the receiver chamber.

The correlation of Caco-2 P_{app} values (estimated in the absence or presence of 4% BSA in the receiver chamber) with human oral absorption data (from the literature) for these reference molecules was strikingly similar in both cases, as shown in Fig. 2.

3.3. Influence of lipophilicity on Caco-2 P_{app} values estimated in the presence or absence of receiver 4% BSA

The correlation of log PC values of reference molecules, Sch Y and Sch 56592 with the ratio of Caco-2 P_{app} estimated in the presence or absence of receiver BSA (in the current study) is shown in Fig. 3. The P_{app} ratio (+BSA/ctrl) was significantly higher (5.3-fold) in case of Sch-Y when the

Table 1
Apparent permeability coefficient (P_{app}) values for apical–basal transport of reference compounds across Caco-2 monolayers in the absence (−) or presence (+) of 4% BSA in receiver chamber[a]

Solute	log PC[b]	P_{app} ($\times 10^{-5}$ cm/s)[c]	
		(−)	(+) 4% BSA
PEG 4000	−5.1	0.02 ± 0.00	0.02 ± 0.00
Mannitol	−3.1	0.15 ± 0.01	0.20 ± 0.01
Cimetidine	0.4	0.31 ± 0.05	0.23 ± 0.03
Phenytoin	2.5	2.94 ± 0.42	3.99 ± 0.27
Diltiazem	2.7	4.97 ± 0.26	6.71 ± 0.04*

[a] *$P < 0.05$, significantly different from control.
[b] Logarithm of the n-octanol/pH 7.4 buffer partition coefficient of reference compounds from the literature [4,8].
[c] Mean ± SEM for $n = 3$–4.

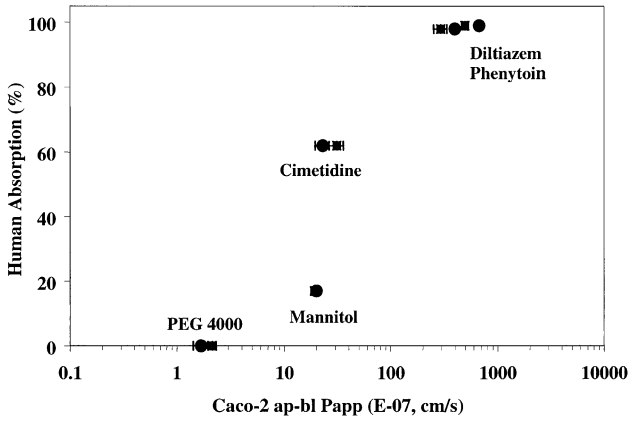

Fig. 2. Correlation of Caco-2 P_{app} values of reference compounds estimated in absence (■) or presence (●) of 4% BSA in receiver chamber with human oral absorption data (from the literature). Error bars represent SEM for $n = 3$–4. Wherever it is not seen, the error bar is smaller than the size of the symbol.

intrinsic log PC approached a value of 4.0 (Fig. 3). The P_{app} ratio (+BSA/ctrl) was also significant (1.3-fold), albeit to a lesser extent, for lipophilic diltiazem (log PC = 2.7). A similar trend was reported by Aungst et al. [14] for compounds with log PC > 3.0.

3.4. Caco-2 monolayer integrity

The integrity of cell monolayers during Sch-Y and Sch 56592 flux studies was monitored by mannitol permeability, and TEER measurements. The P_{app} values of ^{14}C-mannitol (during Sch-Y flux) and ^{3}H-mannitol (during Sch 56592 flux) in the absence or presence of varying percentages of BSA in receiver are shown in Table 2. The corresponding TEER (2 h) values as a percentage of initial are summarized as well (Table 2). The TEER of Caco-2 monolayers during flux studies with reference compounds (with or without BSA) was also not compromised and remained stable ($P > 0.05$) over the experimental period.

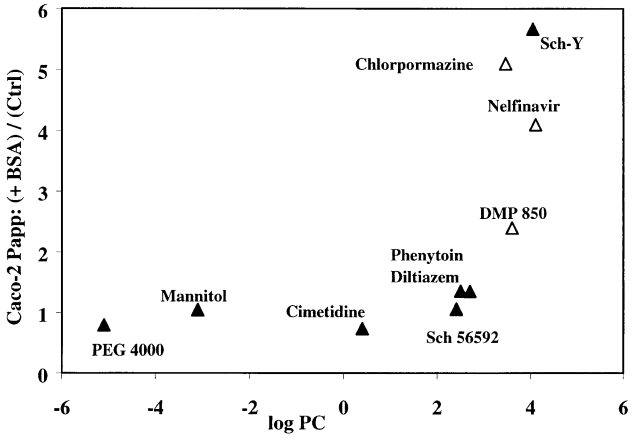

Fig. 3. Correlation of log partition coefficient of reference molecules, Sch-Y and Sch 56592 with ratio of Caco-2 P_{app} values estimated in the presence or absence of receiver 4% BSA (▲). The correlation was compared with data (△) from Aungst et al. [14].

4. Discussion

The present study highlights that in Caco-2 transport studies with very lipophilic and poorly water-soluble NCEs, the presence of BSA in the receiver chamber (during flux studies) is useful in overcoming the associated problems of compromise in sink conditions, and non-specific adsorption. This allows a more accurate assessment of permeability values of NCEs, and in turn, predicts their extent of human oral absorption from a typical correlation curve (of Caco-2 P_{app} values with human oral absorption), such as one shown in Fig. 2.

An important finding in this study is that Sch-Y P_{app} was 5.3-fold higher when estimated in the presence of 4% BSA in the receiver chamber compared to control (without BSA) (Fig. 1A). This led to an extent of human absorption that is greater than 90%, based on a correlation curve with human oral absorption data. However, BSA did not significantly affect Sch 56592 P_{app} (Fig. 1B) or the correlation of human oral absorption data (from the literature) with Caco-2 permeability values for reference compounds (Fig. 2).

Amongst reference solutes, the P_{app} ratio (+BSA/ctrl) was significant (1.3-fold) only for the lipophilic (log PC = 2.7) and highly protein bound (plasma protein binding = 78%) diltiazem [12], albeit to a lesser extent compared to Sch-Y (Fig. 3). It is to be emphasized that despite the difference in diltiazem P_{app} values (estimated with or without receiver BSA), the correlation with human oral absorption data (from the literature) was strikingly similar in either case (Fig. 2). A similar conclusion was also obtained with the other reference solutes (Fig. 2).

The effect of BSA on Caco-2 monolayers during flux studies with Sch-Y, Sch 56592, or reference solutes was assessed by concomitant mannitol (a paracellular marker) [11] permeability and/or TEER measurements. A significant drop in monolayer TEER (% initial value) associated with an increase in mannitol permeability may suggest that integrity of the epithelial barrier is compromised. As shown in Table 2, there was no significant ($P > 0.05$) drop in Caco-2 TEER (% initial value) associated with an increase in mannitol permeability during flux studies with Sch-Y or Sch 56592. The mannitol permeability did increase during Sch 56592 permeation in the presence of 4% BSA; however, the TEER was not significantly different (with BSA), whereby the increased permeability may be attributed to inherent cell monolayer intercellular tight junctional spaces. Moreover, for a moderately hydrophobic molecule such as Sch 56592 (log PC = 2.4) that is likely to opt for the transcellular diffusion pathway, the paracellular permeation will be relatively less. During flux studies for the reference solutes, mannitol permeability was not measured; however, there was no significant ($P > 0.05$) drop in Caco-2 TEER

Table 2
Mannitol permeability coefficient (P_{app}) and TEER during apical–basal transport of Sch-Y, Sch 56592, and reference compounds across Caco-2 monolayers in the absence or presence of varying percentages of BSA in receiver chamber

Drug	% BSA in receiver	Mannitol P_{app} ($\times 10^{-6}$ cm/s)[a]	TEER (% initial value)[a]
Sch-Y	0	1.52 ± 0.10	104 ± 5
	0.5	0.94 ± 0.05	105 ± 7
	1	0.96 ± 0.11	101 ± 5
	2	1.10 ± 0.22	115 ± 8
	4	1.47 ± 0.22	123 ± 2
Sch 56592	0	1.55 ± 0.38	102 ± 5
	4	3.02 ± 0.10	109 ± 14
PEG 4000	0	ND[b]	91 ± 5
	4	ND	117 ± 14
Mannitol	0	1.50 ± 0.10	104 ± 5
	4	2.00 ± 0.10	123 ± 2
Diltiazem	0	ND	111 ± 4
	4	ND	115 ± 5
Phenytoin	0	ND	111 ± 7
	4	ND	108 ± 2
Cimetidine	0	ND	101 ± 1
	4	ND	103 ± 8

[a] Mean ± SEM for $n = 3$–4.
[b] ND, not determined.

(% initial value) suggesting that cell monolayer integrity was not compromised during these flux studies in the presence of BSA.

The results are in general agreement with the work of Krishna et al. [13] with compounds that exhibit extensive non-specific binding, and of Aungst et al. [14] with HIV protease inhibitors and other lipophilic compounds. As reported by Aungst et al. [14], reservoir albumin significantly increased absorptive Caco-2 P_{app} values of only the most lipophilic (log PC > 3.0) and highly protein bound (>95%) compounds – chlorpromazine, DMP 850 and nelfinavir (compared in Fig. 3). In case of DMP 851 (log PC = 3.81), the presence of receiver albumin caused a drastic 38-fold increase in P_{app} by reducing cellular drug accumulation, and may also reduce or prevent compound adsorption to plastic wells [14]. The present study does not include molecules within the log PC range of 3–4; nevertheless, our findings conclusively demonstrate that for highly lipophilic compounds such as Sch-Y (log PC = 4.0), the presence of receiver BSA will give a more accurate estimate of Caco-2 permeability for predicting absorption. In contrast, permeability classification of the relatively less lipophilic (compared to Sch-Y) Sch 56592 (log PC = 2.4) based on estimated Caco-2 P_{app} values is not affected by the presence of receiver BSA. The amount of ^{3}H-Sch-Y or ^{14}C-Sch 56592 accumulated within Caco-2 monolayers at the end of flux studies remained unchanged ($P > 0.05$) in the absence or presence of BSA in the receiver (Fig. 1A, Fig. 1B inset). It appears that for the highly lipophilic Sch-Y at least, the 5-fold higher P_{app} estimation in the presence of 4% receiver BSA may be due to improved sink conditions and/or a reduction in non-specific drug adsorption to plastic wells.

A correlation curve as in Fig. 2 may be useful in assigning a permeability ranking to new drug entities based on Caco-2 P_{app} values. The confidence level of such a prediction will depend to a great extent on the accurate in vitro estimation of Caco-2 permeability under experimental conditions that mimic the physiological state. This is best exemplified by Sch-Y, a highly lipophilic drug (log PC = 4.0) with very poor aqueous solubility. In the absence of BSA in the receiver chamber, Sch-Y P_{app} was 0.3×10^{-5} cm/s, corresponding to approximately 60% human oral absorption if extrapolated from Fig. 2. However, with 4% BSA in receiver, Sch-Y P_{app} was 1.6×10^{-5} cm/s which would then predict an extent of human absorption that is greater than 90%. The BCS guidance defines a high permeability drug as one with >90% extent of human absorption (based on administered dose). Therefore, without albumin in the transport system, Sch-Y may be erroneously classified as low solubility–low permeability class IV compound under the current BCS. However, the inclusion of BSA during the permeability estimations will allow a more correct assessment of Sch-Y as a low solubility–high permeability class II molecule. In sharp contrast, the classification of Sch 56592 (log PC = 2.4) as a high permeability class II molecule (based on Caco-2 P_{app} value) is not affected by the inclusion of BSA in the receiver chamber during transport studies.

In summary, a methodology is proposed for conducting Caco-2 transport studies of poorly water-soluble NCEs in an effort to more accurately assess their permeability values. This involves the use of 4% bovine serum albumin in the receiver chamber. The proposed methodology can dramatically affect the estimated Caco-2 P_{app} and permeability ranking (under BCS guidance) of highly lipophilic NCEs, as in the case of Sch-Y with a log PC of 4.0. In some cases, BSA can also be beneficial in accurately estimating Caco-2 P_{app} values of solutes with log PC ≥ 2.7 and high plasma protein binding (e.g. diltiazem), despite not affecting the prediction of human oral absorption for such drugs. A limitation of the proposed methodology is that if such transport studies are conducted in classical diffusion chambers (stirring provided by gas-bubbling), the surfactant property of BSA gives rise to an undesirable frothing and foaming effect. However, this does not occur when the studies are performed in a stirred Transwell system, as in the current research.

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