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Development of a more rapid, reduced serum culture system for Caco-2 monolayers and application to the biopharmaceutics classification system

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Abstract

The objectives were: (1) to develop a more rapid, reduced serum culture system for Caco-2 monolayers, relative to the traditional 21-day, 10% fetal bovine serum (FBS) system; and (2) to determine the biopharmaceutical drug classification of an oral therapeutic agent using this new system. Caco-2 cells were grown in the six well format on polycarbonate filters, in medium containing 2% iron supplemented calf serum (sCS) and a combination of growth factors and hormones. After 4 days in culture, permeabilities of three marker compounds (metoprolol, mannitol, and taurocholate) across monolayers were determined, and compared to permeabilities from the traditional 21-day, 10% FBS system, using cells at similar passage number. Cell morphology, degree of cell differentiation, and the presence of two efflux pumps were assessed. The 2% sCS model was also used to classify the permeability of an oral therapeutic agent as high or low. No difference in permeability was observed for metoprolol transport ($P = 0.38$) between the two culture methods, and the values obtained were independent of passage number of the cells. Mannitol permeability was about 2-fold higher from the 2% sCS system, as compared to the 10% FBS system. Taurocholate permeability was low indicating the 2% sCS culture at 4 days lacked this particular active transporter capability. Electron micrographs of cells grown in the 2% sCS system at 4 days revealed the presence of microvilli and tight junctions. P-glycoprotein and an efflux pump for furosemide were functionally present. The 2% sCS system indicated the oral therapeutic agent as highly permeable, which agreed with the 10% FBS system. This new system provides a rapid, accurate, and economical option for passive permeability determination, and appears to be applicable to the proposed Biopharmaceutics Classification System (BCS).

Keywords: Permeability; Caco-2 cells; Reduced-serum cell culture; Biopharmaceutics Classification System; Oral absorption

1. Introduction

Traditional Caco-2 cell culture for permeability studies requires a 3-week period to attain differentiated monolayers. Medium changes during this

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culture period are cumbersome and expensive, since the medium is supplemented with 10% fetal bovine serum (FBS). It would be desirable to grow differentiated Caco-2 cells that maintain structural and functional characteristics in less time and at less expense.

The role of serum in cell culture medium is to provide hormones, growth factors, attachment factors, and other components required for cell growth and differentiation. The exact composition of serum is undefined and can vary from lot to lot. Serum added to the culture medium can be reduced or replaced with an appropriate mixture of hormones, growth factors, transport proteins, and vitamins that is optimized for a particular cell line (Mather, 1984). Not only does reduced-serum or serum-free defined medium eliminate lot to lot variability, it significantly reduces expense.

A number of reduced-serum or serum-free culture environments for epithelial derived cell lines have been described (Halleux and Schneider, 1991; Baten et al., 1992; Belford et al., 1995; Oguchi et al., 1995). In one report, a serum free primary culture of fetal rat intestinal epithelial cells was successfully developed using Ham's F-12 medium, supplemented with bovine serum albumin, epidermal growth factor (EGF), insulin, cholera toxin, transferrin, and hydrocortisone (Fukamachi, 1992). These factors induced selective epithelial cell proliferation, void of fibroblast contamination. Structurally, these cells developed tight junctions, desmosomes, and microvilli, but enzymatic activity of alkaline phosphatase was undetectable, indicating incomplete cellular differentiation. Chopra et al. (1987) were able to serially passage human fetal normal epithelial cells by supplementing the medium with insulin, transferrin, EGF, and cholera toxin. Results from this investigation demonstrated the effectiveness of insulin in cell multiplication. The remainder of the supplements were found to be associated with morphologic differentiation.

In this study, two objectives were pursued. The first was to compare permeabilities through Caco-2 monolayers grown for 4 days in 2% supplemented calf serum (sCS) to permeabilities through monolayers grown for 21 days in the traditional 10% FBS medium. Metoprolol, mannitol, and taurocholate permeabilities were determined to compare the transcellular, paracellular, and active transport characteristics of monolayers from 2% sCS and 10% FBS growth conditions. Ultrastructural analysis, brush border alkaline phosphatase enzyme expression, and efflux pump activity were also carried out to examine the cellular differentiation in the 2% sCS culture system. The second objective was to apply the developed 2% sCS model to classify the permeability of an oral therapeutic agent as high or low.

2. Experimental

2.1. Materials

Radiolabeled [^{14}C]mannitol (specific activity of 51.5 mCi/mmol) and [^{3}H]taurocholate (specific activity of 3.47 Ci/mmol) were obtained from DuPont NEN (Boston, MA). [^{3}H]vinblastine sulphate (specific activity of 12.5 Ci/mmol) was obtained from Amersham Life Science (Piscataway, NJ). Metoprolol tartrate and other drug substances were USP grade. All other reagents were analytical grade. Caco-2 cells were obtained from ATCC (Rockville, MD). The following were purchased from Sigma (St. Louis, MO): Hank's balanced salt solution (HBSS), bovine insulin, human transferrin, dexamethasone, iron sCS, cholera toxin, cold taurocholate, and phosphate buffered saline (PBS). FBS, HEPES buffer, nonessential amino acids (NEAA), and penicillin/streptomycin were purchased from Biofluids (Rockville, MD). Dulbecco's modified Eagle's medium (high glucose) and Ham's F-12 were obtained from GIBCO-BRL (Grand Island, NY). Epidermal growth factor and bovine pituitary extract were purchased from UBI (Lake Placid, NY). Ascorbic acid phosphate magnesium salt η-hydrate was obtained from Wako (Japan). Tissue culture treated 10 cm plates and six well cell cluster dishes with polycarbonate Transwell® (3 μm pore size) filters were purchased from Corning-Costar (Cambridge, MA). The therapeutic agent used as a model compound was used as received from GlaxoWellcome.

2.2. Cell culture

Caco-2 cells at passage numbers 20–50 were seeded onto Transwell® filters. For the traditional culture conditions, cells were grown in DMEM supplemented with 10% FBS, 1% NEAA, 50 U/ml penicillin and 50 μg/ml streptomycin, at a seeding density of 3.77×10^5 cells per 4.71 cm^2. Medium was changed every 48 h. Cells were cultured for 21 days at 37°C, 90% relative humidity, and 5% CO_2, then used for permeability studies. For the 2% sCS culture condition, cells were plated at a seeding density of 1.00×10^6 cells per 4.71 cm^2, and grown in a 1:1 mixture of DMEM/F-12, with 2% iron supplemented calf serum and seven additional factors: bovine insulin (10 μg/ml), human transferrin (10 μg/ml), dexamethasone (10^{-8} M), EGF (50 ng/ml), ascorbic acid (50 μg/ml), cholera toxin (25 ng/ml), and bovine pituitary extract (50 μg/ml). Cells were cultured using 2.5 ml of supplement containing medium on the basolateral side, and 0.2 ml of plain DMEM/F-12 medium on the apical side, to promote polarization. Medium was changed after 48 h, then daily for the remainder of the culture period. These cells were cultured for 4 days, at 37°C, 90% relative humidity, and 5% CO_2, then used for permeability studies.

2.3. Permeability studies: marker compounds and efflux pumps

Permeability studies were conducted in HBSS containing 10 mM HEPES buffer (solution pH 6.8) at 37°C and 50 oscillations per min. Monolayer integrity was monitored using transepithelial electrical resistance (TEER) in HBSS and [^{14}C]mannitol permeability. Donor concentrations were 0.438 mM metoprolol tartrate, 1.5 μM mannitol, and 6.5 μM taurocholate (0.06 μM of which was [^{3}H]taurocholate). Transport studies were conducted using Transwell® inserts in the apical to basolateral direction, at intervals of 5, 10, and 15 min. Apical volume was 1.5 ml, and basolateral volume was 2.6 ml. Metoprolol was quantified using HPLC. [^{14}C]mannitol and [^{3}H]taurocholate were quantified using a scintillation counter. In each study, mass balance was determined and ranged from 90 to 110%. Permeability in each experiment was calculated using Eq. (1):

$$P = \frac{dM/dt}{A \cdot C_d} \quad (1)$$

where P is permeability, dM/dt is rate of drug mass accumulation in the receiver compartment, A is area, and C_d is donor drug concentration.

Apical to basolateral and basolateral to apical permeabilities of 4 nM [^{3}H]vinblastine and 10 μM furosemide were determined to assess the presence of efflux transporters in the 2% sCS system at day 4. For basolateral to apical studies, media was sampled in the receiver compartment at each time interval, and replaced with fresh HBSS.

2.4. Ultrastructure comparisons

Caco-2 cells at passage number 27 and 36 from the 10% FBS and the 2% sCS culture systems, respectively, were used for ultrastructure studies. After the appropriate time in culture, filters were rinsed three times with sterile PBS, and fixed using 2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2). After 1 h, cells were rinsed in 0.2 M sodium cacodylate buffer, and stored at 4°C until embedded for transmission electron microscopic evaluation using a JEOL JEM 1200 EX II Microscope. Cells were post-fixed using Osmium.

2.5. Growth study

Using six well cluster dishes, Caco-2 cells were seeded at an initial density of 1.20×10^5 cells per 9.40cm^2 well. Cells were cultured using either 10% FBS or 2% sCS media. Medium was replaced every 48 h. At various days after seeding, cells ($n = 3$) were rinsed with PBS, then harvested using trypsin/EDTA for counting. Cells were stained with Trypan blue dye exclusion, and counted on a hemacytometer. Data was collected through 14 days.

2.6. Alkaline phosphatase activity

Caco-2 cells (passage 35) were plated onto polycarbonate Transwell® filters and brush border

fragments were isolated at 4 days (2% sCS system) or 21 days (10% FBS system). Medium was removed and cells were rinsed three times with PBS. The cells from three filters were scraped and combined for each analysis. Brush border fragments were isolated according to the procedure of Howell et al. (1992). Alkaline phosphatase was assayed using the method described by Bessey et al. (1946), with *p*-nitrophenyl phosphate as substrate. Enzyme units are expressed as nmol of substrate hydrolyzed per min. Enzyme activity is expressed as units per mg of cellular protein. Cellular protein was assayed spectrophotometrically using the Bio-Rad protein assay kit (#500.0002 Bio-Rad Laboratories, Richmond, CA).

2.7. Application to the BCS

To implement the BCS, the permeabilities of 15 diverse compounds were determined to construct a relationship between percent fraction dose absorbed in humans and Caco-2 permeability in the 2% sCS system. Permeability studies were conducted in HBSS containing 10 mM HEPES buffer (solution pH 6.8) at 37°C and 50 oscillations/min. Transport studies were conducted using Transwell® inserts in the apical to basolateral direction. Apical volume was 1.5 ml, and basolateral volume was 2.6 ml. Compounds (donor concentrations) studied were: PEG 4000 (4 μM), atenolol (500 μM), mannitol (1.5 μM), furosemide (10 μM), disopyramide phosphate (343 μM), chlorpheniramine maleate (1020 μM), nicardipine HCl (58 μM), aspirin (1800 μM), indomethacin (140 μM), metoprolol tartrate (438 μM), dexamethasone (15 μM), diltiazem HCl (266 μM), theophylline (1000 μM), lisuride hydrogen maleate (11 μM), and coumarin (445 μM). Except for radiolabelled PEG 4000 and mannitol, compounds were quantified by HPLC. Generally, the donor concentrations were derived from the highest dose divided by 1000 ml. For chlorpheniramine maleate, a higher donor concentration was needed due to UV detection limitations. Mannitol permeability was monitored with each compound to ensure monolayer integrity. All mannitol permeabilities were less than 3.25×10^{-6} cm/s.

To classify the permeability of an oral therapeutic agent, the 2% sCS and 10% FBS cell culture and permeability studies were conducted as described above. Transport studies were conducted in both the apical-to-basolateral (AP–BL) and the basolateral-to-apical (BL–AP) directions, using a donor concentration of 0.54 mM of the oral therapeutic agent. Drug was quantified using HPLC. [^{14}C]mannitol permeability was determined in the AP–BL direction to monitor monolayer integrity during the time course of the experiment. Metoprolol tartrate was used as a high permeability reference. A drug permeability greater than metoprolol was classified as high permeability. In each study, mass balance was greater than 95%.

In addition, a phase-solubility approach was used to determine the solubility of the oral therapeutic agent between pHs 1.5 and 8. A solubility threshold value of 0.60 mg/ml was needed to classify the therapeutic agent as highly soluble, based on its largest dosage strength according to the guidelines in the SUPAC IR Guidance (Amidon et al., 1995).

It should be noted that detailed methodology for BCS implementation is currently not well developed. The methods described above are an attempt to implement the draft BCS in this laboratory; however, a comprehensive validation of any one permeability method would perhaps require a multi-laboratory evaluation.

3. Results and discussion

3.1. Permeability studies: marker compounds and efflux pumps

Table 1 summarizes the permeability values obtained from the two culture conditions. Relative to monolayers grown for 21 days in 10% FBS, monolayers grown for 4 days in 2% sCS yielded practically equivalent permeability values for passive compounds (metoprolol and mannitol), over the studied 25–50 passage range (Fig. 1).

No difference in permeability was observed between the 10% FBS system at 21 days and the 2% sCS system at 4 days for metoprolol transport

(analysis of variance, ANOVA, $P = 0.38$). These results demonstrate the applicability of the 2% sCS culture to a highly permeable transcellularly transported compound.

Statistically, permeability of mannitol across the two systems was different with the 2% sCS at 4 days > 10% FBS at 21 days. From Table 1, mannitol permeability values were 2.81 (± 0.16) and 1.46 (± 0.13) $\times 10^{-6}$ cm/s for the 2% sCS and 10% FBS systems, respectively. However, since a major utility of Caco-2 monolayers is their ability to differentiate between high permeability and low permeability compounds, mannitol permeability from the 2% sCS system at day 4 can be considered to be similar to mannitol permeability from the 10% FBS system at day 21. Like the 10% FBS system, the 2% sCS system discriminated between a highly permeable (metoprolol) and lowly permeable (mannitol) compound. Additionally, mannitol permeability from the 2% sCS system (2.81×10^{-6} cm/s) was within the range of mannitol permeability values from other laboratories using the traditional 10% FBS approach (Cogburn et al., 1991; Rubas et al., 1993; Adson et al., 1994; Surrendran et al., 1995; Walter and Kissel, 1995; Kagedahl et al., 1997; Yu et al., 1997; Anderle et al., 1998; Chao et al., 1998; Gres et al., 1998). For example, a typical mannitol value in the 10% FBS system is 3.23×10^{-6} cm/s (Rubas et al., 1993), although can be as high as 5.50×10^{-6} cm/s (Surrendran et al., 1995). It should be noted that permeability values of lowly permeable compounds, such as mannitol, differ between laboratories (Artursson et al., 1996). Comparisons of mannitol permeability from the 2% sCS system to mannitol permeability in this laboratory (Table 1) and other laboratories (Cogburn et al., 1991; Rubas et al., 1993; Adson et al., 1994; Surrendran et al., 1995; Walter and Kissel, 1995; Kagedahl et al., 1997; Yu et al., 1997; Anderle et al., 1998; Chao et al., 1998; Gres et al., 1998) indicate that the new system is comparable to the traditional culture approach.

TEER values in HBSS at room temperature ranged from 502 to 1119 (Ω cm^2) for the 2% sCS system, and 633–1114 (Ω cm^2) for the 10% FBS system over the studied passage numbers. These TEER values are obtained after subtracting a filter resistance of 610 Ω cm^2.

Permeability of taurocholate from the 10% FBS system at day 21 was nearly 10-fold higher than from the 2% sCS system at day 4. This difference indicates less cellular differentiation in the 2% sCS system at 4 days. Permeability values for taurocholic acid across Caco-2 cells grown in 10% FBS vary between laboratories (Hidalgo and Borchardt, 1990; Chandler et al., 1993; Kagedahl et al., 1997; Yee, 1997). Expression levels of the bile acid transporter in Caco-2 cells diminish with passage age and culture conditions (Kagedahl et al., 1997; Yu et al., 1997). Because of this underlying variability, including diminished bile acid transporter expression observed here in the 10% FBS system, comparison of taurocholate permeabilities from the 10% FBS and 2% sCS systems is difficult. However, these taurocholate permeabilities indicate reduced cellular differentiation at 4 days in the 2% sCS system.

In addition to permeabilities from the 10% FBS system at day 21, permeabilities from the 10% FBS system at day 4 were also determined. Caco-2 monolayers at passage 25, grown in 10% FBS for 4 days, showed unacceptable variability. The permeability of mannitol was 4.78 (± 2.18) $\times$

Table 1
Mean permeability of marker compounds through Caco-2 monolayers

Method	Metoprolol permeability $\times 10^6$ (cm/s)	Mannitol permeability $\times 10^6$ (cm/s)	Taurocholate permeability $\times 10^6$ (cm/s)
10% FBS (Day 21)[a]	41.9 (± 1.6)	1.46 (± 0.13)	10.3 (± 1.3)
2% sCS (Day 4)[a]	40.0 (± 1.4)	2.81 (± 0.16)	1.61 (± 0.10)

[a] Results are mean ($\pm$ S.E.M.) for $n = 33$ for each metoprolol, mannitol, and taurocholate.

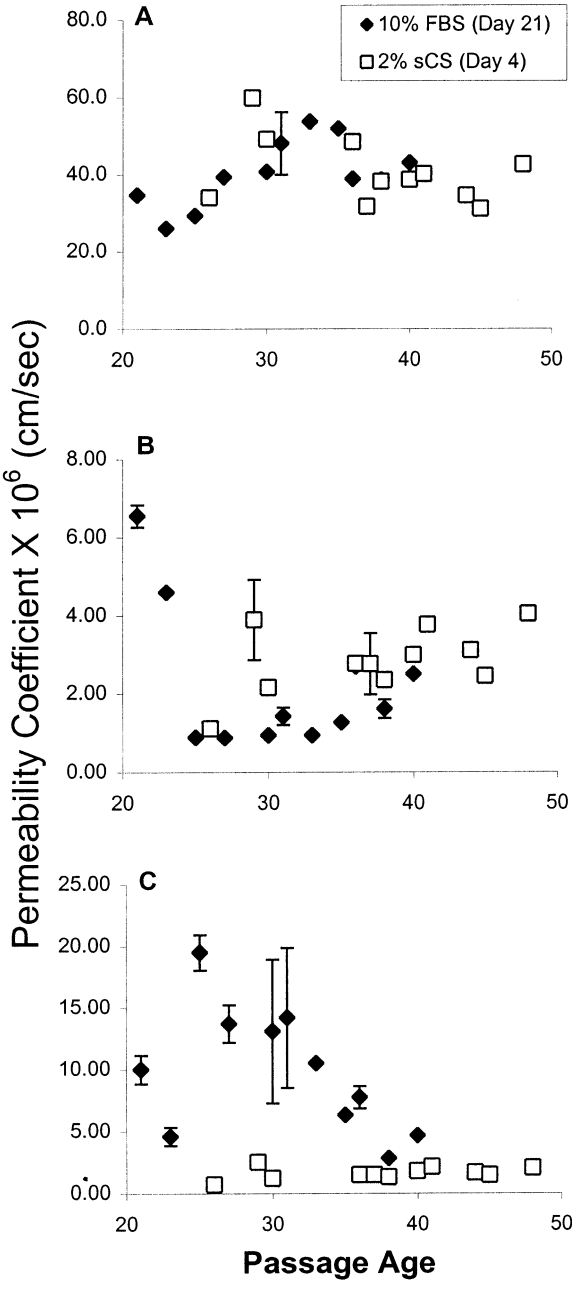

Fig. 1. Mean permeability ($\pm$S.E.M.) of metoprolol, mannitol, and taurocholate across Caco-2 cells grown in 2% supplemented calf serum (sCS) for 4 days, and in 10% fetal bovine serum (FBS) for 21 days. For metoprolol (A), similar permeabilities resulted. (ANOVA, $P = 0.38$) For mannitol (B), permeability from the 2% sCS system was about 2-fold higher than from the 10% FBS system. For taurocholate (C), active transport was absent from the 2% cSC system, while transport of taurocholate was variable from the 10% FBS system.

10^{-6} cm/s. This high variability is not surprising considering the common protocol for producing functional monolayers in 10% FBS requires about 21 days in culture. Variable mannitol permeability values can be attributed to incomplete monolayer formation. This result is consistent with Bailey et al. (1996), who showed unacceptably high permeability values for mannitol prior to day 20 for monolayers grown in 10% FBS. These results indicate that the traditional 10% FBS culture system is not optimized for rapid Caco-2 cell culture. Given the use of Caco-2 monolayers in drug delivery evaluation in the last 10 years, a more optimal media, such as this report's 2% sCS approach, would be advantageous in terms of time saving and cost. In addition to mannitol, metoprolol permeability at day 4 from 10% FBS was slightly high. Metoprolol permeability at day 4 was 53.9 (± 0.53) $\times 10^{-6}$ cm/s. Taurocholate transport was not expressed from the 10% FBS system at 4 days. Hence, its permeability was a low and variable 2.66 (± 1.54) $\times 10^{-6}$ cm/s. These results indicate that monolayers grown in 2% sCS form suitable monolayers faster than those grown in 10% FBS.

Regarding efflux pumps, the 2% sCS system was used to assess the permeability of the known P-glycoprotein substrate vinblastine (Hunter et al., 1993). Transport of 4 nM vinblastine was 3.33 (± 0.07) $\times 10^{-6}$ cm/s in the apical to basolateral direction, and 13.3 (± 0.7) $\times 10^{-6}$ cm/s in the basolateral to apical direction, thus demonstrating polarized efflux in the 2% sCS system. Polarized permeability of 10 μM furosemide, a substrate for a second secretion system (Flanagan and Benet, 1999), was also observed using the 2% sCS system. Permeability of furosemide was 3.40 ($\pm$ 0.27) $\times 10^{-6}$ cm/s in the apical to basolateral direction, and 10.0 (± 0.1) $\times 10^{-6}$ cm/s in the basolateral to apical direction.

3.2. Growth study

Fig. 2 plots the cell growth curve over 14 days, from an initial density of 1.20×10^5 cells per 9.40 cm^2 well. Growth rates are nearly identical, with the 2% sCS system slightly more rapid than the 10% FBS system. Caco-2 cells grown in 2% sCS

reached confluence at day 9. Cells grown in 10% FBS reached confluence at day 12. In agreement with Hidalgo et al. (1989), cell density decreased initially for 10% FBS, due to reduced plating efficiency in these culture conditions.

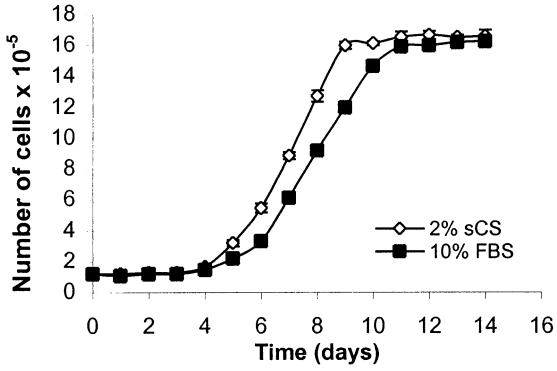

Fig. 2. Growth curve for cells grown in 2% supplemented calf serum (sCS) and in 10% fetal bovine serum (FBS). Initial seeding density was 1.20×10^5 cells per 9.40 cm^2 well. Caco-2 cells reached confluence at day 9 in 2% sCS and at day 12 in 10% FBS.

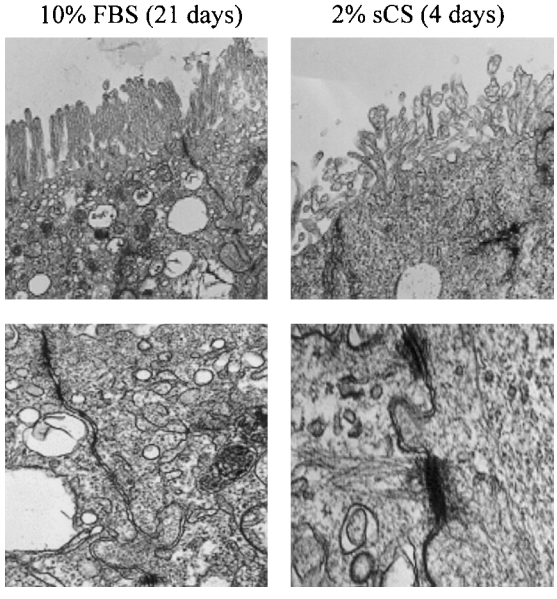

Fig. 3. Transmission electron micrographs of Caco-2 cells grown in different culture conditions. Upper panels: apical membrane of Caco-2 cells. Microvilli are present from each culture condition. Magnification: 9000 × . Lower panels: tight junctions and desmosomes are evident from each culture condition. Magnification left to right: 30 000 × ; 37 500 × .

Table 2
Comparison of alkaline phosphatase activity under different culture conditions[a]

Method	Alkaline phosphatase activity (nmol/min per mg protein)
10% FBS (Day 21)	87.72 (± 7.08)
2% sCS (Day 4)	2.46 (± 0.79)

[a] Values are mean (± S.E.M.) for $n = 3$.

3.3. Ultrastructure comparison

Ultrastructure comparisons were determined through transmission electron microscopy (TEM). Fig. 3 illustrates the microvilli, tight junctions, and desmosomes from Caco-2 cells grown in 10% FBS at day 21, as well as cells grown in the 2% sCS at 4 days. The 10% FBS system at 21 days provided columnar cells with a more organized apical microvillular surface. Microvilli were partially developed in the 2% sCS system at 4 days. Tight junctions and desmosomes were present and equally apparent in both the 10% FBS and 2% sCS systems. These results demonstrate the 2% sCS system reproduced many of the morphological and differentiation characteristics (Louvard et al., 1992) from the 10% FBS system.

3.4. Alkaline phosphatase determination

Activity of alkaline phosphatase at the Caco-2 brush border is a biochemical marker of differentiation (Pinto et al., 1983; Howell et al., 1992; Yu et al., 1997). Table 2 summarizes the alkaline phosphatase activity present in the brush border under each culture condition. Alkaline phosphatase activity from the 10% FBS system at 21 days $\gg$ 2% sCS at 4 days. These results are in agreement with those of Howell et al. (1992) and Pinto et al. (1983), which show increased alkaline phosphatase activity with time in culture.

3.5. Application to the BCS

The 2% sCS at day 4 was used to determine the permeabilities of 15 compounds to construct a

Table 3
Percent fraction dose absorbed in humans vs. Caco-2 permeability (2% sCS model)[a]

Compound	Permeability coef. (cm/s) $\times 10^6$ AP–BL	% fraction dose absorbed in humans	Ref.
PEG 4000	1.69 ($\pm$0.06)	0	Artursson and Karlsson, 1991
Atenolol	2.62 ($\pm$0.17)	50	Artursson and Karlsson, 1991
Mannitol	2.81 ($\pm$0.16)	16	Artursson and Karlsson, 1991
Furosemide	3.33 ($\pm$0.07)	61	Ponto and Schoenwald, 1990
Disopyramide	4.24 ($\pm$0.05)	85.3	Lima et al., 1984
Chlorpheniramine	16.0 ($\pm$1.9)	80	Dollery, 1991
Nicardipine	19.8 ($\pm$0.7)	100	Delchier et al., 1988
Aspirin	22.2 ($\pm$0.9)	100	Artursson and Karlsson, 1991
Indomethacin	38.4 ($\pm$2.2)	100	Dollery, 1991
Metoprolol	40.0 ($\pm$1.4)	94.5	Dollery, 1991
Dexamethasone	40.3 ($\pm$2.1)	100	Artursson and Karlsson, 1991
Diltiazem HCl	42.4 ($\pm$1.1)	99	Echizen and Eichelbaum, 1986
Theophylline	61.0 ($\pm$0.9)	96	Hendeles et al., 1977
Lisuride	61.1 ($\pm$1.3)	100	Humpel et al., 1981
Coumarin	141 ($\pm$6)	100	Ritschel et al., 1979

[a] Results are mean ($\pm$S.E.M.) for $n = 3$, except for metoprolol ($n = 33$) and mannitol ($n = 33$).

relationship between percent fraction dose absorbed in humans versus Caco-2 pemeability. The percent fraction dose absorbed in humans versus 2% sCS Caco-2 permeability values are summarized in Table 3 and Fig. 4. These results suggest the applicability of the 2% sCS system for classifying drug permeability as high or low.

In Table 4, the permeability of the oral therapeutic agent was the same from the 10% FBS and the 2% sCS model. Its permeability was classified as high, since all permeability values obtained for the compound were greater than all metoprolol permeabilities (Table 1). Mannitol permeabilities from the 2% sCS and 10% FBS system were 3.07 ($\pm$0.10) and 1.46 ($\pm$0.09) $\times 10^{-6}$ cm/s, respectively. These results also conclude that: (1) the 2% sCS model is able to predict apparent passive permeability; and (2) mannitol permeability in the 2% sCS model is about 2-fold higher than in the 10% FBS system.

Solubility at each pH was classified as high, since solubility exceeded 0.60 mg/ml for each of the pHs studied (data not shown). Based on the results of solubility and permeability determinations, the oral therapeutic agent was classified as highly soluble and highly permeable.

4. Discussion

This 2% sCS approach provides considerable time and resource savings relative to the conventional 21-day system, which uses 10% FBS. The new culture method has a materials cost which is

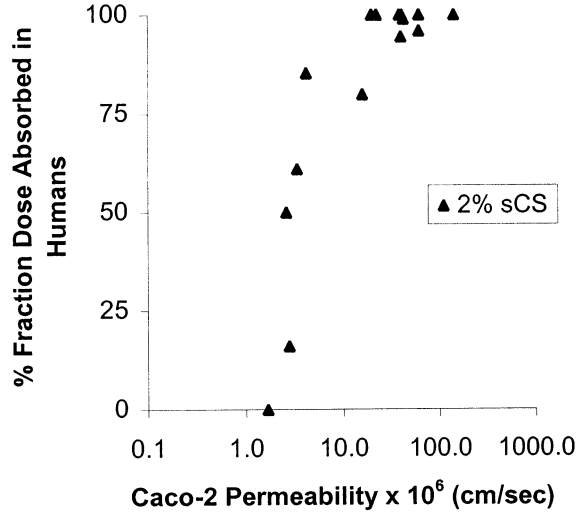

Fig. 4. Percent fraction dose absorbed in humans vs. Caco-2 permeability in the 2% supplemented calf serum (sCS) system.

Table 4
Oral therapeutic agent permeability from 10% fetal bovine serum (FBS) and 2% supplemented calf serum (sCS) Caco-2 models[a]

Culture method	Therapeutic agent permeability $(cm/s) \times 10^6$	
	AP–BL	BL–AP
10% FBS	65.6 ($\pm$0.6)	54.0 ($\pm$0.3)
2% sCS (Day 4)	66.4 ($\pm$2.4)	59.8 ($\pm$0.7)

[a] Values are mean ($\pm$S.E.M.) for $n = 3$.

one-sixth that of the traditional 10% FBS protocol. Time and labor costs are also significantly saved. It should be noted that a commercially available 3 day system for growing Caco-2 monolayers (BIOCOAT®, Becton Dickinson Labware, Franklin Lakes, NJ) can be purchased; however, this system is proprietary, and significantly more expensive than the approach described here.

Additionally, there are vast variabilities in permeability values reported using Caco-2 monolayers from different laboratories, particularly in the low permeable range (Cogburn et al., 1991; Rubas et al., 1993; Adson et al., 1994; Surrendran et al., 1995; Walter and Kissel, 1995; Artursson et al., 1996; Briske-Anderson et al., 1997; Yu et al., 1997; Anderle et al., 1998; Chao et al., 1998; Gres et al., 1998). FBS is obtained from biological sources and used in many cell culture systems. The role of this serum in cell culture medium is to provide hormones, growth factors, attachment factors, and other components required for cell growth and differentiation. It is well established that the lot to lot variation in FBS results in variable growth and differentiation of cells in culture. Briske-Anderson et al. (1997) attributed variability in the Caco-2 system to several possibilities, including serum variation that may favor the selection of a predominant clone over the course of passages. Engle et al. (1998) showed the Caco-2 line expresses a combination of colonocyte and enterocyte phenotypes. Hence, the intra- and interlaboratory variability associated with the 10% FBS growth protocol may be attenuated with the use of a reduced-serum system such as 2% sCS.

In conclusion, a more rapid, reduced-serum culture system for Caco-2 monolayers was developed. Passive permeabilities from this 2% sCS system at 4 days were comparable to permeabilities from the 10% FBS system at 21 days. The 2% sCS was applied to an oral therapeutic agent, and yielded the same permeability and permeability classification as the more cumbersome 10% FBS system.

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