Development of a 7-Day, 96-Well Caco-2 Permeability Assay with High-Throughput Direct UV Compound Analysis

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Purpose. The aim was to replace the traditional 21-day Caco-2 permeability protocol by a more high-throughput assay.
Methods. Caco-2 cells were seeded at high density in 96-well plates in novel cell culture boxes. After 7 days, drug permeability studies were performed. Samples were analyzed by a new UV detection method.
Results. With increased cell seeding density, functional Caco-2 monolayers with polarized efflux transporters were established after 7 days in 96-well polycarbonate filter plates in standard medium. For faster feeding and to eliminate medium replacement in each individual well, plates were completely submerged in medium in novel cell culture boxes, and only medium outside the plate was exchanged. For high-throughput sample analysis, a novel UV-transparent transport buffer was established that allowed direct quantification of permeated drug from its UV absorption. *In vitro* permeability studies analyzing 22 passively absorbed drugs in the new model correlated well with reported human permeability values ($r^2 = 0.8725$).
Conclusions. The new 7-day, 96-well Caco-2 permeability model tight to UV analysis offers considerable time, cost, and resource savings compared to the traditional model. It has a potential for automation and makes it possible to determine the permeability of passively diffusing compounds and to classify them according to the BCS in a truly medium- to high-throughput mode.

KEY WORDS: Caco-2 cells; permeability; Biopharmaceutics Classification System; high-throughput; oral absorption.

INTRODUCTION

Over the last decade, the Caco-2 model has been widely accepted by pharmaceutical companies and by regulatory authorities as a standard permeability-screening assay for drug intestinal permeability (1,2). The model uses the polyclonal human colon carcinoma cell line Caco-2, which forms tight cell monolayers on filter supports with clearly defined apical (mucosal) and basolateral (serosal) sites with enterocyte-like properties (3). This allows studying the major absorptive processes in the small intestine for drugs, such as passive paracellular and transcellular diffusion or the study of active uptake and of efflux mechanisms. Based on permeability studies in the Caco-2 model, drugs are also classified according to the Biopharmaceutics Classification System (BCS) (4).

In its traditional form, each of the three consecutive steps in the Caco-2 model, (a) cell growth and feeding, (b) transport study, and (c) subsequent sample analysis, is laborious, time consuming, expensive, and low throughput. To adapt the model to the needs of modern high-throughput screening, a number of efforts have been undertaken by various authors and companies to simplify the model and/or increase its throughput. These approaches include (a) reduction of the growth time of the cells from 21–28 days to 3–5 days either by use of special media and collagen coatings (Biocoat) or by replacement of the Caco-2 cells with faster growing cells such as MDCK cells (5–7), (b) automation of the permeation study (8), (c) simultaneous analysis of drug permeability by cassette dosing or sample pooling (9,10), (d) reduction of costs by lowering the FBS concentration in the medium or by reducing the number of replicates run in the experiment (11,12), and (e) the recent introduction of 96-well plates to replace the 12- and 24-well culture plates (13). However, despite these modifications, most of the traditional Caco-2 cell culture and permeability assay conditions remained unchanged, and the overall throughput capacity is still limited. To enable higher throughput, it will be necessary to optimize each step of the model and to seamlessly link them together to avoid the generation of new bottlenecks. For example, if the number of permeability assays that can be performed in parallel is the bottleneck, replacement of 12-well plates by 96-well plates may shift the bottleneck to analytic if it cannot cope with the enormous increase in the number of samples. Other major bottlenecks are the feeding conditions, in particular the separate replacement of apical and basolateral medium in individual wells, the generally accepted 3-week growth time for the cell monolayers, and the lack of automation compatibility in most setups. To increase throughput, we addressed in this study the growth time for functional cell monolayers, the seeding and feeding conditions, and the sample analysis in the 21-day Caco-2 model and found that they can be replaced by less laborious, faster, and less costly approaches.

MATERIALS AND METHODS

Chemicals

All cell culture media were supplied by Gibco BRL. [1-^{14}C]Glycyl-L-Proline (2.04 GBq/mmol), D-[1-^{14}C]mannitol (2.07 GBq/mmol), L-[4-^{3}H]phenylalanine (962 GBq/mmol), [4-^{14}C]testosterone (2.07 GBq/mmol), and tauro[carbonyl-^{14}C]cholic acid (2 GBq/mmol) were purchased from Amersham. [^{3}H]Polyethylene glycol (MW 400, 15 Ci/mmol), [^{14}C]creatinine (MW 113.12, 55 mCi/mMol), L-[1-^{14}C]leucine (MW 131.2, 55 mCi /mmol), and [^{14}C]urea (MW 60.1, 55 mCi/mmol) were from American Radiolabeled Chemicals. [^{14}C]Saquinavir was from Roche Welwyn (Ro-31-8959/006, MW 766.95, 115 μCi/ml, 85.3 μCi/mg). All radioactive chemicals had purity >96%. Ritonavir was kindly provided by Abbott. Saquinavir mesylate and glycofurol 75 were from Roche. All other reagents were from Sigma and Fluka and of high purity or analytic grade.

Cell Culture

Cell seeding and cell growth in the traditional 12-well Transwell® system was performed as described previously (14). For the 96-well system, Caco-2 cells (passage 103–112)

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ABBREVIATIONS: HTS, high-throughput screening; P_C, permeability coefficient; GMW, mixture of Glycofurol 75, methanol, and water (80/10/10) (v/v).

were maintained at 37°C in 75 cm^2 flasks (Sarstedt) (50,000 cells/flask) in DMEM/MEM/Pen-Strep supplemented with 10% fetal bovine serum (FBS) and Glutamax in an atmosphere of 5% CO_2 and 95% relative humidity. Medium was exchanged every 2–3 days. After reaching 70–80% confluence, cells were detached by Trypsin-EDTA treatment (5 ml/flask for 10 min at 37°C), centrifuged for 5 min at 800 rpm and adjusted to a density of 5×10^5 cells/ml. For cell seeding, sterile 96-well polycarbonate filter plates (Millipore, Multi-Screen Plates, 0.4 μm polycarbonate filter, TC) (0.32 cm^2/filter) were mounted in ethylene oxide–sterilized, disposable, stackable Hosta PET cell culture boxes (Herba Plastic, Switzerland) (Fig. 1), and 45 ml of cell culture medium was added below the plate until its surface was approximately 0.5 cm above the level of the filters. One hundred microliters of the cell suspension was added per well (5×10^4 cells/filter). After incubation for 2 days, 75 ml of cell culture medium was added until the plate was completely submerged in medium. After incubation for 2 days at 37°C, 90 ml of the medium outside the plate was replaced by fresh, prewarmed medium. The medium inside the individual wells of the plate (~30 ml total) was not removed. After 3 days, medium was replaced again, and cells were used for permeation experiments either the same day (after an incubation for at least 3 h with fresh medium) or within the next 2 days.

Permeability Studies

Permeability studies in Transwells® were performed as described previously (14). Studies in 96-well plates were conducted in 40 mM Bis-Tris/120 mM Tris-base buffer (pH adjusted to 7.4 or 6.5 with 5 N HCl) containing 0.1 mg/ml of $CaCl_2$, NaCl, KCl, and $MgCl_2$ (each), 1.1 mg/ml glucose, 0.15% (w/v) Pluronics F68, and 0.03% (w/v) Cremophor RH40 (UV buffer). Because the filter plates did not fit into the commercially available 96-well plates, new 96-well rectangular Teflon plates were created that served as receiver and donor compartments in AP-to-BL and BL-to-AP permeability studies, respectively (Roche workshop, Basel) (Fig. 2). These plates had spacers at each corner to keep the filters 1 mm above the bottom of the receiver plate. Stock solutions (10 mM) of drugs were prepared by dissolving drug in Glycofurol 75/methanol/water (80/10/10) (v/v) (GMW). Alternatively, drugs not soluble in GMW were dissolved in water (i.e., propranolol). Stocks were stored at –20°C until use. Sample preparation and all liquid-handling steps were performed either manually or using a Packard Multiprobe II Robotic Liquid Handling System.

Before the permeability studies, cell culture medium was completely removed from the 96-well cell culture plate. The cell monolayers were washed twice by submerging the plate into 2 × 200 ml of prewarmed UV buffer, pH 7.4, in cell culture boxes. Transport studies were conducted in triplicates or quadruplicates in the rectangular Teflon plates containing 0.14 ml of prewarmed UV buffer (Fig. 2). Donor volume and drug concentrations were 0.14 ml and 0.01–0.3 mM in UV buffer (pH 7.4 or pH 6.5), respectively. Lucifer yellow (2 μg/ml) was added to the donor compartment to monitor the integrity of the monolayer. Drug samples were prepared by diluting the stock solutions in UV buffer in 2-ml deep-well microtiter plates [maximum GMW content 3% (= 0.3% methanol)]. Samples were centrifuged at 3200 × *g* in a Heraeus Omnifuge centrifuge before use to remove precipitated material. Because precipitated material was removed, the absolute drug amount in the supernatant was not always known. A buffer control was included on each plate. In apical-to-basolateral studies, the 96-well plate was moved after 30 min at 37°C to another 96-well rectangular Teflon plate with 140 μl of prewarmed UV buffer per well. In basolateral-to-apical studies, 100 μl of the apical transport buffer was replaced by 100 μl of prewarmed fresh UV buffer. After another 90 min (sample point at 120 min), 100-μl samples of the donor and receiver samples were collected and analyzed as described below. During incubation, plates were covered with a lid and sealed with a tape to avoid liquid evaporation. To avoid contact between tips and cell monolayer during manual collection of apical medium, a newly designed 96-hole plastic frame was placed on top of the plate, which stopped descending tips approximately 1 mm above the cell monolayer (not shown).

Sample Analysis

Labeled compounds and lucifer yellow were quantified using a Packard TopCount.scintillation counter and a Spec-

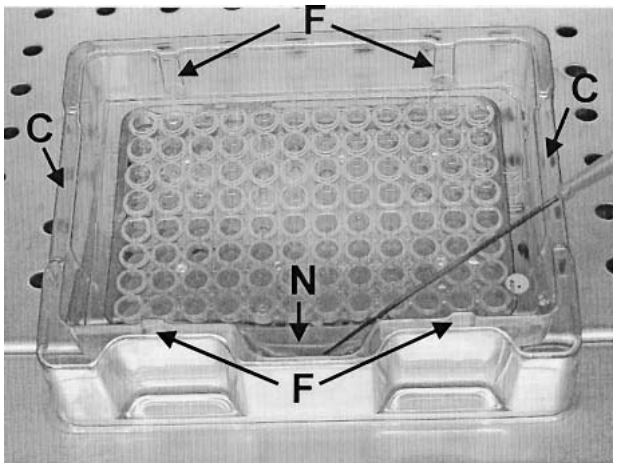

Fig. 1. In-house-designed cell culture boxes for seeding and faster feeding of Caco-2 cells in 96-well filter plates. The box had four fixation points (F) that were used to mount the filter plate in the box and prevented the plate from floating in medium. A notch (N) in front of the box allowed access to the space below the plate, and the two openings at the sides (C) free ventilation during incubation. For cell feeding, only medium outside the plate (~¾) was replaced.

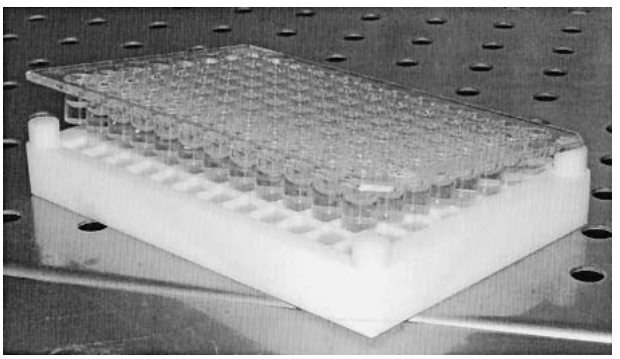

Fig. 2. Carrier plate for Caco-2 permeability studies. An in-house-developed 96-well Teflon plate with rectangular wells was used for transport experiments (for better presentation the filter plate in the picture has been lifted slightly).

traMax Gemini fluorescence reader at 435 nm/515 nm (Ex/Em), respectively (14). For UV measurement, 100 μl of each sample was transferred to a 96-well quartz plate (Hellma, Switzerland) and mixed with 20 μl of 5 N HCl for 5 min on a microplate shaker. The absorption spectrum of each sample was determined using a SpectraMax Plus UV reader (190–600 nm, 5-nm steps). After subtraction of the spectrum of the buffer control, the absorption maximum for each drug was identified and used for subsequent calculations.

Calculation of Permeability Coefficients (P_c)

Drug permeability was calculated using Eq. (1):

$$P_c = \frac{1}{A \times C_D} \times \frac{dQ_R}{dt} = \frac{1}{A \times C_D} \times \frac{d(C_R \times V_R)}{dt} \quad (1)$$

where P_c is permeability coefficient (cm/s), A is the surface area of the insert membrane (cm^2), C_D and C_R are the concentrations in the donor and receiver compartments (mg/ml), V_R is the volume of the receiver compartment (ml), dQ_R/dt is mass transported in time interval dt (mg/s). Because drug UV absorption is directly proportional to drug concentration in solution

$$E = C \times \varepsilon \times d \quad \text{(Lambert–Beer)} \quad (2)$$

where C = drug concentration (mol/L); ε = extinction coefficient [l/(mol·cm)]; and d = length of light path (cm).

Equation (1) can be transformed into

$$P_c = \frac{\varepsilon \times d}{A \times E_D} \times \frac{d(E_R \times V_R)}{dt \times \varepsilon \times d} = \frac{1}{A \times E_D} \times \frac{d(E_R \times V_R)}{dt} \quad (3)$$

where E_D and E_R are the measured UV absorption of the drug in the donor and receiver compartments, respectively.

For calculations, donor concentration C_D was adjusted for mass loss in each observed time interval. In each study, mass balance (sum of amount recovered from the apical and basolateral compartment) was determined and typically ranged from 90% to 110%. P_C values were calculated automatically from UV absorption spectra using a software package developed in house. Results are expressed as mean values ± SD. The value of n is defined as the number of wells per compound.

RESULTS

Cell Seeding and Feeding on 96-Well Plates

In conventional Caco-2 assay systems, cells seeded on filter supports have to be fed every 2–3 days for 3 weeks. When 96-well plates are introduced and automation is not available, then feeding becomes a very tedious procedure. Therefore, a different setup for feeding of the cells was investigated. For cell seeding, the 96-well plates were first mounted in novel disposable, transparent cell culture boxes, medium was added below the plate, and cells were seeded in 100-μl volumes/well. After 2 days at 37°C, the plate was completely covered with medium, and for feeding, only medium outside the plate was replaced with fresh medium every 2–3 days (Fig. 1, Position N).

Millipore provided the 0.4-μm polycarbonate filter plates used in our setup. Their dimensions are similar to standard MultiScreen plates from Millipore but differ from marketed 96-well filter plates (MultiScreen™ Caco-2) in that they have a larger surface area (0.32 cm^2) and no basolateral access ports. Permeability studies performed with 10-μM radiolabeled compounds 3 weeks after seeding in either 12-well Transwell™ plates or in 96-well plates yielded very similar results (Table I). Mannitol and testosterone (both passive transport) could be classified in both systems as low- and high-permeability compounds, respectively. For saquinavir, a directed efflux was observed that could be inhibited by a 10-fold excess of the P-gp inhibitor ritonavir. In both permeability systems, a clear directed transport of the actively transported compounds Gly-Pro, phenylalanine and taurocholic acid could not be detected. Coating of the filters in the 96-well plate with rat tail collagen type II was not necessary for cell attachment and growth and even resulted in higher SD for permeability data (data not shown). Furthermore, with the same equipment, no differences were observed in the quality of cell monolayers between plates submerged in medium and

Table I. Effect of Time in Culture and of Plate Format on Drug Permeability Across Caco-2 Monolayers[a]

	Permeability ($\times 10^6$ cm/s)								
	12-Well plate (3 weeks)			96-Well plate (3 weeks)			96-Well plate (1 week)		
	AP > BL	BL > AP	Ratio BL > AP/ AP > BL	AP > BL	BL > AP	Ratio BL > AP/ AP > BL	AP > BL	BL > AP	Ratio BL > AP/ AP > BL
Saquinavir	1.4 (±0.3)	9.9 (±0.4)	7.1	2.4 (±0.7)	13.9 (±2.4)	5.8	2.4 (±0.2)	13.3 (±1.2)	5.5
Saquinavir + Ritonavir	3.0 (±0.5)	3.8 (±0.8)	1.3	4.8 (±1.0)	5.0 (±0.9)	1.0	6.6 (±0.3)	4.9 (±1.4)	0.7
Gly-Pro	4.7 (±0.5)	7.3 (±0.8)	1.6	4.2 (±1.4)	4.8 (±1.1)	1.1	2.3 (±0.6)	2.1 (±0.9)	0.9
Mannitol	3.0 (±1.1)	2.3 (±0.5)	0.8	3.6 (±0.9)	2.4 (±0.8)	0.7	1.0 (±0.4)	1.4 (±0.40)	1.4
Phenylalanine	17.7 (±1.7)	10.5 (±1.3)	0.6	22.3 (±3.2)	10.0 (±1.8)	0.4	13.5 (±1.5)	15.5 (±1.8)	1.1
Taurocholic acid	1.7 (±0.5)	1.9 (±0.5)	1.1	1.8 (±0.9)	0.6 (±0.3)	0.3	1.1 (±0.6)	0.6 (±0.4)	0.5
Testosterone	61.8 (±3.2)	56.7 (±2.8)	0.9	28.0 (±2.1)	18.9 (±1.8)	0.7	31.6 (±4.1)	38.5 (±3.2)	1.2

[a] Caco-2 cells were seeded in 12-well Transwell™ plates at 0.6×10^5 cells/cm^2 or in 96-well polycarbonate filter plates at 0.6×10^5 cells/cm^2 (3 week) or 1.6×10^5 cells/cm^2 (1 week) and grown for the time indicated. Permeability assays were performed in quadruplicate with 10 μM solutions of labeled drug compound at pH 7.4. Samples were added either to the apical or basolateral chamber, and appearance in the receiver compartment was measured. Values represent average P_c values ± SD.

plates where apical and basolateral medium was not in contact and was replaced separately (data not shown).

Reduction of Growth Time

At a seeding density of 0.5×10^5 cells/cm^2, it takes at least 7–10 days in the traditional 3-week Caco-2 model until the cells are confluent (2,15). We investigated if higher cell seeding densities in combination with the standard medium could be used to attain differentiated monolayers in less time. Cells were seeded at 1.6×10^5 cells per cm^2 on untreated polycarbonate filters in 96-well plates and grown for up to 10 days using the new incubation boxes and feeding conditions described above. Seven days after seeding, cell density was 0.4×10^6 cells/cm^2. Lucifer yellow permeability reached 0.5 ($\pm$ 0.35) $\times 10^{-6}$ cm/s after 5 days and stayed at that level for the next 5 days, indicating integrity of the monolayer only 5 days after seeding (data not shown). Permeability values, SD, and BCS classification of several other test compounds after 1 week were comparable to those obtained after 3 weeks in the traditional and in the 96-well Caco-2 systems. Polarized permeability of saquinavir and its inhibition by ritonavir was also similar to the 3-week systems (Table I). Trials with higher seeding densities (3×10^5 cells/cm^2 and 6×10^5 cells/cm^2) did not change the results or speed up monolayer formation (data not shown). Therefore, in subsequent studies, cells were seeded at 1.6×10^5 cells/cm^2 and used for permeability experiments after 7–10 days.

Sample Analysis by Direct UV Measurement

To handle the several hundreds of samples resulting from a single 96-well plate, we investigated if standard analytic methods such as high-pressure liquid chromatography (HPLC) or liquid chromatography mass spectrometry (LC-MS/MS) could be replaced by a simple direct UV measurement. Because standard transport media used in permeability studies have a significant intrinsic UV absorption, a Bis-Tris/Tris buffer (UV buffer) was established. The unacceptable high UV absorption of this buffer at pH 7.4 in quartz plates decreased on acidification with HCl and allowed the detection of compounds with an absorption maximum above 210 nm (Figs. 3 and 4). At the Bis-Tris/Tris concentrations used the buffer was isotonic, and its high buffer capacity avoided significant changes in pH in the pH range 6–8.5 even when stronger acidic or basic drugs were tested. Addition of the two non–UV-absorbing surfactants, Pluronics F68 and Cremophor RH40, improved the solubility of lipophilic compounds and reduced drug loss due to adsorption to surfaces (data not shown). Cell monolayer integrity and transport of efflux pump substrates were not affected by the buffer for up to 4 h at 37°C in permeability studies. Finally, stock solutions of compounds were prepared in GMW, which had solubilization properties similar to DMSO but increased the UV absorption of samples much less (Fig. 3). An example for the linear relationship between UV absorption and concentration of a lipophilic drug in the UV buffer is shown in the insert in Fig. 3.

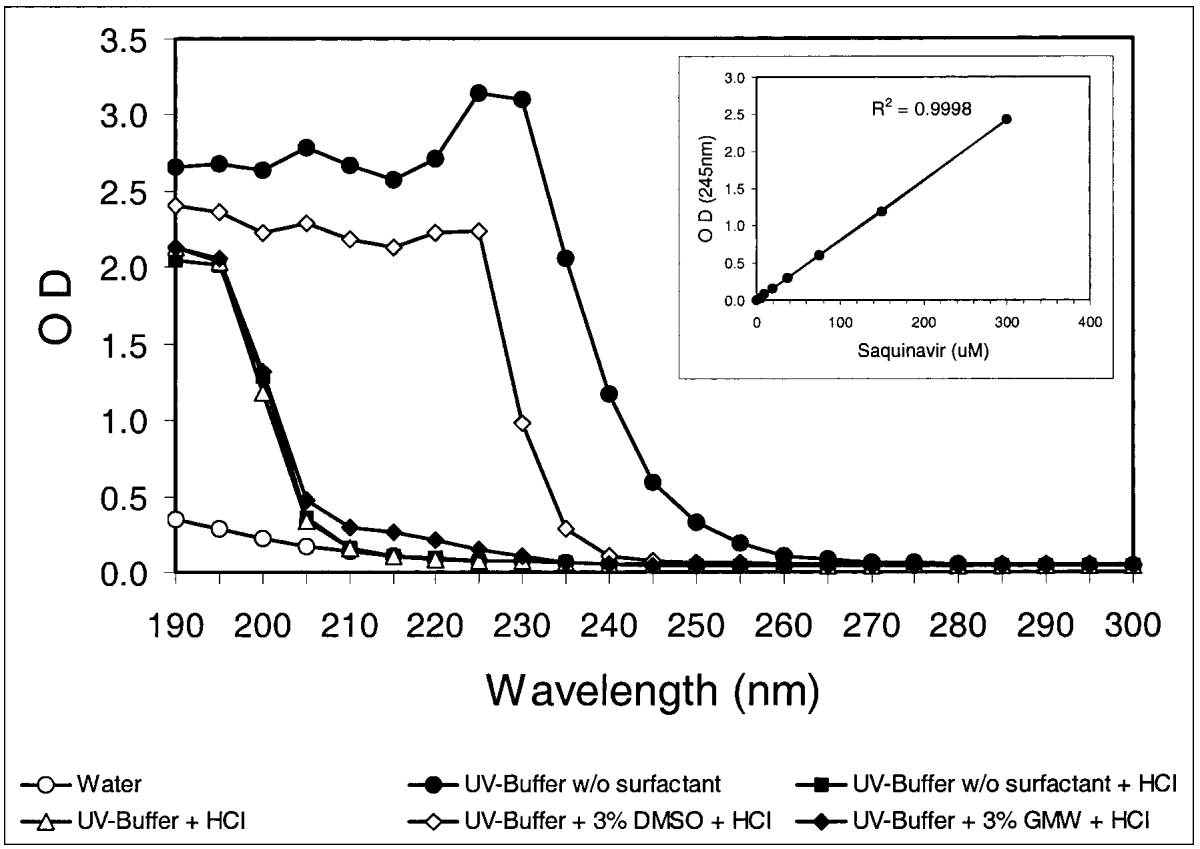

Fig. 3. Influence of excipients on the UV absorption of the Bis/Tris buffer (UV buffer) used for Caco-2 permeability studies. The UV spectrum of water or of UV buffer alone or in the presence of various excipients was determined in the range of 190–300 nm. Insert: Serial dilutions of saquinavir in UV buffer were measured at 245 nm, and the OD was plotted against the saquinavir concentration.

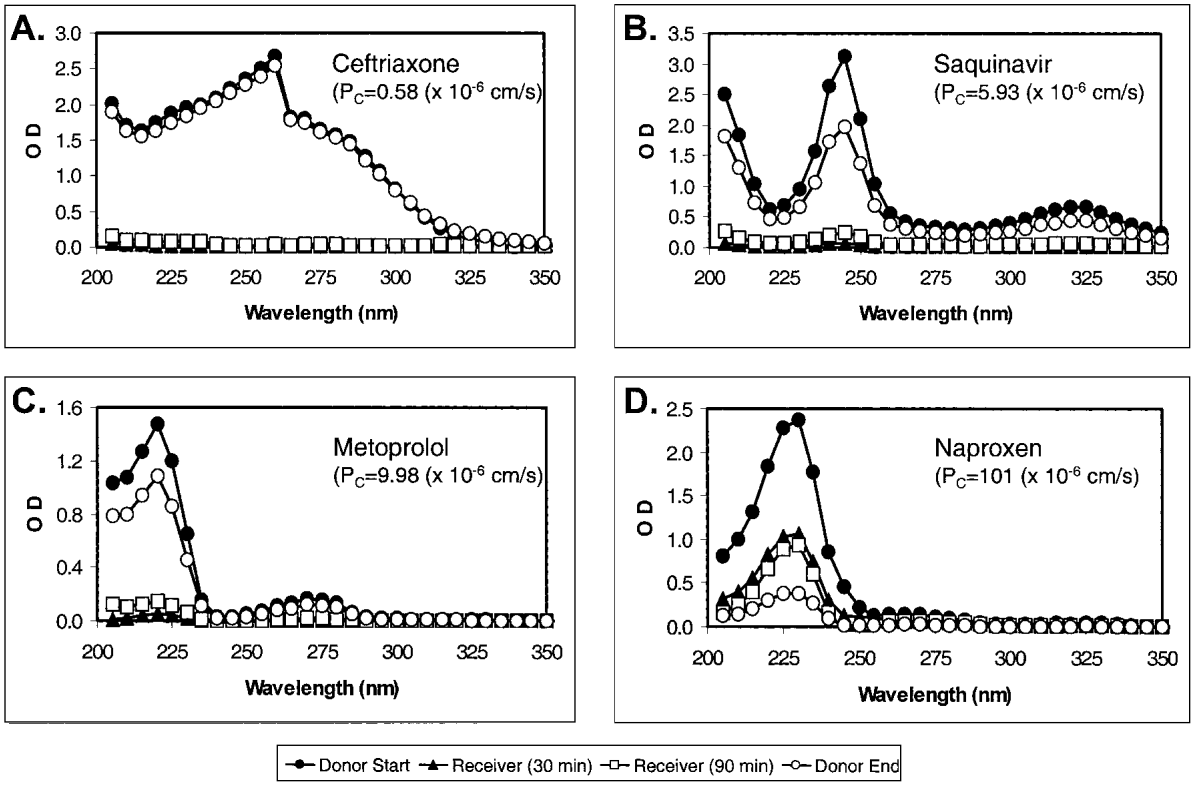

Fig. 4. UV spectra of compounds in samples from permeability studies. Compounds were dissolved in UV buffer, and a Caco-2 permeability study was performed (AP (pH 6.5)-to-BL(pH 7.4)). The UV spectra of the starting solution (donor start), of the receiver compartment at two different time points (30 min and 90 min), and of the donor compartment at the end of the experiment (donor end) were determined and plotted after subtraction of the spectrum of the buffer control.

Permeability Studies

Drug permeability studies across Caco-2 cell monolayers were performed 1 week after seeding using the new 96-well rectangular Teflon plates (Fig. 2) and the sample analytics described before. A typical result of a permeability study with UV detection of compounds is depicted in Fig. 4. For low-permeability compounds, such as ceftriaxone, almost no difference was observed between the drug's start and end concentration in the donor compartment, and only very low amounts of compound appeared after 90 min in the receiver compartment (Fig. 4A). For compounds with higher permeability, drug concentration decreased in the donor compartment, and its appearance in the receiver compartment was clearly detectable (Fig. 4B–D). The interassay variability (day-to-day) of the assay was low as shown in Fig. 5A. The intraassay standard deviation (SD) of P_C values of drugs increased with decreasing P_C values (Fig. 5B). At permeability coefficients $>5 \times 10^{-6}$ cm/s, SD was typically below 20% and even below 10% at P_C values exceeding 10×10^{-6} cm/s. In the P_C range 1–5 $\times 10^{-6}$ cm/s, SD increased but was still below 40%. Only for low-permeability compounds ($P_C < 1 \times 10^{-6}$ cm/s) did SD exceed 40%.

The 96-well Caco-2 model was validated using 28 compounds with reported human intestinal permeability (Table II). Drugs were applied to the donor (apical) side of Caco-2 monolayers in UV buffer at pH 7.4 or at pH 6.5, and drug transport to the receiver (basolateral) compartment (pH 7.4) was determined. The *in vivo*/*in vitro* correlation coefficient (r^2) for the permeability of compounds at pH 7.4 and pH 6.5 was 0.6711 (n = 26) and 0.7685 (n = 20), respectively. Two compounds, L-dopa and benserazide, were not included in the calculation because their UV spectra changed during the permeability study and calculated P_C are most likely not correct. Four other compounds, lisinopril, α-methyldopa, ranitidine, and terbutaline, also could not be included in the calculation at pH 6.5 because their permeability was too low for detection in the receiver compartment ($P_C = 0$).

Several of the compounds tested are known substrates for active transporters such as the amino acid transporter or the di/tripeptide transporter (amiloride, amoxicillin, L-dopa, L-leucine, lisinopril, α-methyldopa, phenylalanine) (3,16,17). Removal of these carrier-mediated absorbed drugs from the test set improved the *in vivo*/*in vitro* permeability correlation. The correlation coefficient (r^2) calculated from the log values was 0.8834 (n = 20) at pH 7.4 and 0.8391 (n = 16) at pH 6.5. Inclusion of the two amino acids L-leucine and phenylalanine into the data set did not change the correlation significantly (Fig. 6).

DISCUSSION

Recently, 96-well filter plates have been introduced to increase the throughput of Caco-2 permeability assessment in drug discovery. These plates definitively increase the capacity to screen compounds in parallel; however, their use may also generate new bottlenecks such as insufficient analytic capacity for resulting samples or a need for robotic systems for efficient liquid handling. Moreover, a switch to 96-well plates does not address other existing shortcomings of the assay such

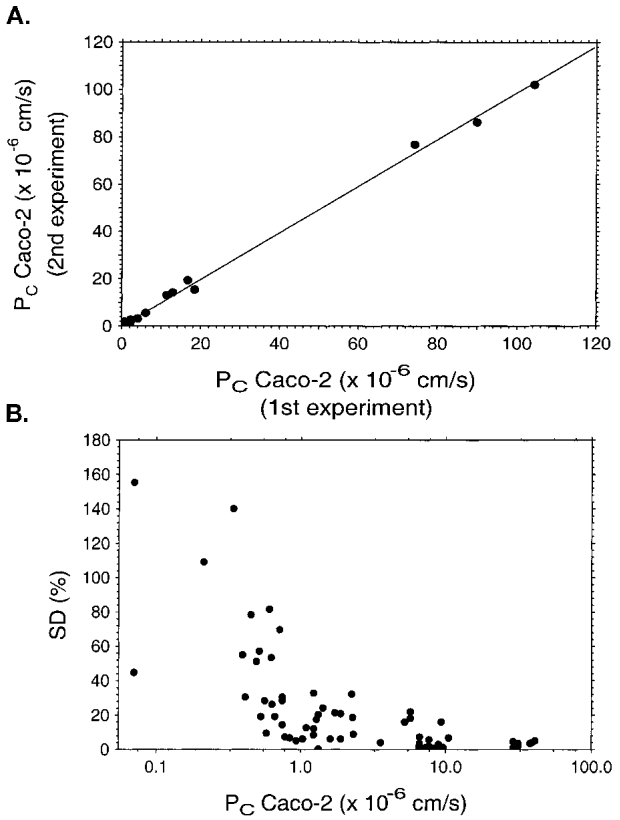

Fig. 5. Inter- and intraassay variability of the 7-day 96-well Caco-2 permeability assay. The permeability of 23 compounds across Caco-2 cell monolayers in the apical (pH 6.5)-to-basolateral (pH 7.4) direction was tested in quadruplicate on different occasions and the day-to-day (r^2 = 0.9973) (A), and well-to-well variability was determined (B).

as the long culturing period of at least 3 weeks before transport experiments can be performed or the tedious feeding procedure.

As an alternative to the laborious and time-consuming standard feeding procedure and to avoid an expensive robotic solution, we established a new cell culture box for cell seeding and feeding (Fig. 1). After a 2-day cell-seeding phase, the plate in this box is completely submerged in medium, and no separation exists between individual wells or apical and basolateral medium. For feeding, only medium outside the plate is replaced every 2–3 days. This procedure is simple, fast (about 2 min per 96-well plate), eliminates the need to separately replace apical and basolateral medium, and reduces the risk of damaging the filters (dropout rate <2%). On the basis of total filter area, the amount of fresh medium available for cell growth and differentiation in our system (~3 ml of medium/cm^2) is comparable to that in most other setups. Drug transport studies performed with Caco-2 cells grown in either the traditional Transwell™ or the new 96-well system yielded similar results for passively and actively transported compounds (Table I). Therefore, a separation of basolateral and apical compartments during cell growth with potential accumulation of certain growth factors in one compartment does not seem to be a prerequisite for the development of tight and differentiated monolayers. Moreover, as described recently for another accelerated Caco-2 permeability model, an uneven hydrostatic pressure does not seem to be important for cell polarization in our model (6). In the current setup, cell feeding is still performed manually. However, the system is, with minor modifications, automation compatible and will require only low-tech one-channel pipettes for medium exchange. For further increase in throughput, one might use the same seeding and feeding technique; however, consider boxes that can hold more than one plate.

In this study, we could show that a simple increase in cell seeding density from the traditional 0.5×10^5 cells/cm^2 to 1.5×10^5 cells/cm^2 can reduce the time required to obtain confluent and polarized monolayers from 3 weeks to only 1 week. In contrast to previously reported modified protocols for short-term culture that require specialized media (5,6,12,15), our model works with the traditional 10% FBS culture medium. Permeability coefficients of test compounds after 1 week were comparable to those obtained with the standard protocol after 3 weeks (Table I). Cellular efflux pumps for saquinavir were also present, indicating a polarized expression of at least some transporters in the monolayer after 7 days. The cell density on filters after 7 days was 4×10^5 cells/cm^2. Therefore, only about 3 cell divisions are required to achieve confluent monolayers. Because the division time of our Caco-2 cells is about 20–24 h, it can be expected that filters are already covered with cells after 3–4 days. This is consistent with an observed low LY permeability 5 days after seeding (data not shown).

A permeability study in a single 96-well plate generates 384 samples that have to be analyzed, assuming that samples are collected from donor start, donor end, and from the receiver compartment at two different times. To reduce the analytic workload, fewer sampling points, cassette dosing, or sample pooling have been considered by others; however, in the long run standard analytic sequential methods such as HPLC or LC-MS/MS may not be able to handle the vast number of samples in a reasonable time or at reasonable expenses (9,10,18,19). As an alternative, we established a simple UV method that can be used for most compounds from drug discovery. The method is inexpensive, allows the fast and parallel analysis of samples from permeability studies, and requires only a 96-well UV reader for drug quantification. Because standard transport buffers (HEPES, HBSS, MES, FCS, etc.) on their own have a too high intrinsic UV absorption, a Bis-Tris/Tris buffer was established for UV detection of drug in transport studies. This buffer had, after acidification, a very low intrinsic UV absorption and allowed the detection of dissolved drugs (Figs. 3 and 4). Because some compounds (i.e., prodrugs) may be degraded in the acidic environment and change their UV absorption spectrum with time, all samples were measured after a defined period of time. The buffer composition and the high buffer capacity in the pH range 6–8.5 prevents significant pH shifts when acids, bases, or salts are dissolved at the concentrations required for UV detection (up to 300 μM) and avoids the formation of potentially less soluble salts. The buffer is isotonic, nontoxic to the cells, and contains sufficient amounts of supplements to perform transport studies for up to 4 h without loss of the barrier function of the monolayer. The two surfactants added, Pluronics F68 and Cremophor RH40, improved the recovery of lipophilic drugs without affecting the UV absorption of the buffer (Fig. 3), passive drug transport across monolayers, or active transport of saquinavir by efflux pumps (data not

Table II. Comparison of Reported Human Effective Permeability with Caco-2 Cell Permeability Coefficients of Various Compounds[a] in the 7-Day 96-Well Model

No.	Compound	P_{eff} human perfusion ($\times 10^{-4}$ cm/s)	P_c (Caco-2) ($\times 10^{-6}$ cm/s)	
			pH 7.4 (donor)	pH 6.5 (donor)
1	Amiloride	1.6[a]	1.13 (±0.72)	0.57 (±0.48)
2	Amoxicillin	0.3[b]	0.01 (±0.01)	1.63 (±0.69)
3	Antipyrine	4.5[b]	54.30 (±2.98)	53.31 (±3.54)
4	Atenolol	0.2[b]	1.73 (±0.66)	0.25 (±0.22)
5	Benserazide	3.8[d]	1.85 (±0.52)[g]	0.37 (±0.28)[g]
6	Carbamazepine	4.3[b]	62.23 (±3.98)	56.03 (±4.46)
7	Cimetidine	0.3[c]	0.59 (±0.35)	0.53 (±0.37)
8	Creatinine[i]	0.3[b]	1.55 (±0.72)	2.15 (±0.81)
9	Desipramine	4.4[b]	43.00 (±2.01)	7.59 (±1.05)
10	Enalaprilat	0.2[b]	1.85 (±0.79)	nm[f]
11	Furosemide	0.05[b]	0.31 (±0.09)	0.43 (±0.05)
12	Hydrochlorothiazide	0.04[b]	0.42 (±0.33)	0.23 (±0.22)
13	Ketoprofen	8.4[b]	24.36 (±1.82)	101.64 (±1.27)
14	L-Dopa	3.4[b]	0.01 (±0.01)[g]	—[g]
15	L-Leucine[i]	6.2[b]	15.50 (±1.68)	69.51 (±3.68)
16	Lisinopril	0.33[e]	1.27 (±0.83)	0
17	α-Methyldopa	0.2[b]	0.15 (±0.09)	0
18	Metoprolol	1.3[b]	31.77 (±2.73)	9.98 (±0.99)
19	Naproxen	8.3[b]	53.07 (±2.91)	125.79 (±3.71)
20	PEG400[i]	0.56[c]	3.12 (±1.53)	3.49 (±1.24)
21	Phenoxymethylpenicillin	0.26[d]	1.90 (±1.19)	nm[f]
22	Phenylalanine	3.4[c]	18.34 (±3.16)	76.52 (±5.31)
23	Piroxicam	7.8[c]	28.85 (±2.81)	116.34 (±1.21)
24	Propranolol	2.9[b]	47.20 (±2.56)	12.08 (±1.18)
25	Ranitidine	0.27[c]	0.67 (±0.39)	0
26	Terbutaline	0.3[b]	1.71 (±0.79)	0
27	Urea[i]	1.4[b]	4.82 (±1.12)	4.51 (±0.90)
28	Verapamil	6.7[b]	44.67 (±3.61)	7.58 (±0.68)

[a] From Ref. (17).
[b] From Ref. (25).
[c] From Ref. (28).
[d] From Ref. (29).
[e] From Ref. (23).
[f] Not measured.
[g] Change in UV spectrum during incubation with Caco-2 cells.
[h] The Caco-2 permeability was conducted at 100–300 μM donor drug concentration in the apical-to-basolateral direction. The pH in the donor compartment was adjusted to pH 7.4 or 6.5, and that in the receiver compartment to pH 7.4. Results are the mean ± SD (n = 3).
[i] Labeled compound used.

shown) (20). DMSO stock solutions of compounds, which are often used to facilitate compound handling in early phases of drug discovery, were replaced by GMW, which showed less UV absorption but similar solubilization properties (Fig. 3). At the final concentrations used, Glycofurol 75 (≤ 3%) and methanol (≤ 0.3%) are nontoxic to the cells and do not enhance drug permeability (21,22).

For drug quantification, the UV spectra in donor and receiver compartments are determined, and the spectrum of the corresponding buffer control is subtracted (Fig. 4). The absorption at the maximum can then be used to calculate permeability coefficients for the drugs because drug UV absorption is directly proportional to drug concentration in solution (Lambert-Beer's law) (Fig. 3). The applicability and accuracy of this detection method depend on both the drug's permeability coefficient and its UV absorption at the maximum. Compounds with insufficient UV absorption (i.e., mannitol or urea) or too low a solubility in the UV buffer (i.e., danazol) are not suitable. Also compounds that exhibit changes in the absorption spectrum during permeability experiments as a result of enzymatic or chemical degradation can not be used. Examples are benserazide and L-dopa, which showed changes in UV spectrum in our studies. In our setup, drugs with $P_C < 1 \times 10^{-6}$ cm/s required UV absorption values >1, and compounds with $P_C > 10 \times 10^{-6}$ cm/s UV absorption values > 0.2 to become detectable in the receiver compartment after a 90-min incubation. In general, compounds with flat UV absorption spectra were more difficult to quantify than compounds with sharp peaks, in particular in the low-UV range (<230 nm). Therefore, depending on the drug's extinction coefficient and permeability, the accuracy of the method varies. For high-permeability compounds the SD of the P_C is usually very low (Fig. 5B). For low-permeability compounds the SD is higher and depends on the UV absorption of the start solution: if it is high, the SD can still be low (i.e., ceftriaxone, SD = 29%) (Fig. 4), if it is low, the SD is,

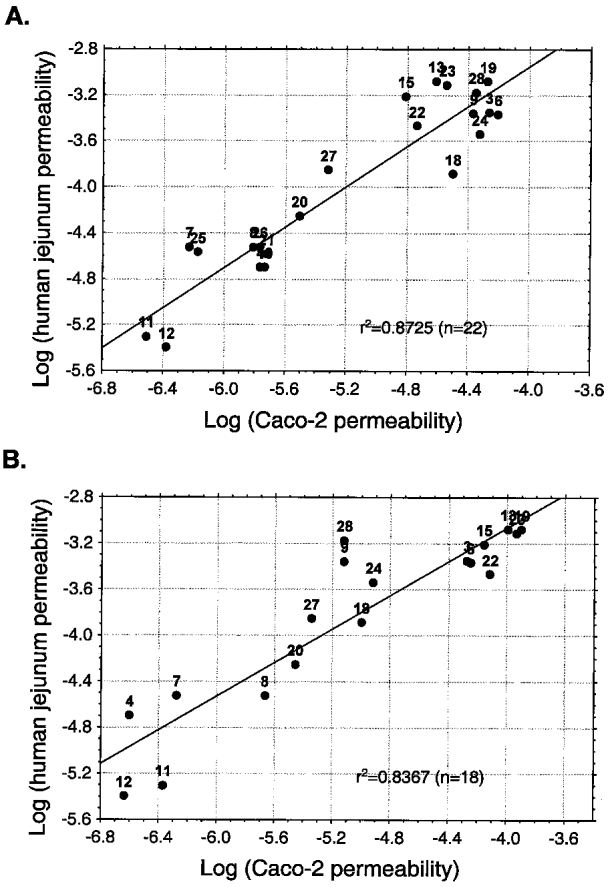

Fig. 6. *In vitro*/*in vivo* permeability correlation for passively transported drugs. Reported human permeability coefficients were correlated with *in vitro* permeability coefficients obtained from the 7-day 96-well Caco-2 permeability assay at pH 7.4 (A) or pH 6.5 (B) in the donor compartment and pH 7.4 in the receiver compartment (apical-to-basolateral direction). (For compound number, see Table II.)

in most cases, high (i.e., atenolol, SD = 109%). At present, it is not clear if the two currently commercially available 96-well insert systems (Millipore, Becton Dickinson) can be used with our analytic method because the filter area available for permeation is three times lower, and permeated compound will be more diluted because the basolateral volume is two to four times higher.

The new 96-well Caco-2 permeability model and the analytic method were validated using drugs with known human permeability (Table II and Fig. 6). Our *in vitro* results correlated well with *in vivo* human intestinal permeability studies, and correlation coefficients (r^2) for passively absorbed drugs were 0.8725 (n = 22) and 0.8367 (n = 18) at pH 7.4 and pH 6.5 in the donor compartment, respectively. As expected, correlation coefficients were lower when carrier-mediated absorbed drugs were included in the data set because these carriers may quantitatively not be expressed adequately in Caco-2 cells to reflect the *in vivo* situation (3). These data are in excellent agreement with a previous report by Sun *et al.* (23). Permeability values for individual compounds also correlate well with other reported data. On average, values for low-permeability compounds are lower than in other accelerated models (5,6,12). The observed effect of medium pH on the permeability of weak acids and bases is also in agreement with previous studies and may affect their BCS classification as discussed previously (Table II) (23,24). In the current setup (AP, pH 6.5; BL, pH 7.4), a compound is regarded as a BCS high-permeability drug if its P_C is $>10 \times 10^{-6}$ cm/s and as a low-permeability drug if it is below that limit.

The assay is running now in our laboratory for about 3 years, and a throughput of 100–200 compounds per week with manual handling is feasible without significant efforts. For standardization and for control of intra- and interassay variability, six standards (paracellular, transcellular, active and/or pH-dependent transport) (ceftriaxone, furosemide, saquinavir, metoprolol, propranolol, naproxen) and a buffer control are run in triplicates in a typical permeability assay on each plate together with 25 test samples. A mass balance is performed for each well. The latter is of particular importance for highly lipohilic compounds, where a physical loss due to nonspecific adsorption may lead to an underestimation of the transport. The introduction of the two surfactants into the UV buffer partially addresses this issue; however, the handling of compounds with logD > 3 is a general problem in all current Caco-2 models and will require further studies.

During routine analysis of compounds, we noticed three additional advantages of the assay. First, in bidirectional permeability studies, P_c values can be directly compared at a point in time because the buffer volume on both sides is identical. In standard permeability assays, this is often not possible because sink conditions are exceeded faster in one of the compartments. Second, for the small number of compounds that can not be directly determined by the UV method, samples are still available for conventional analytics. Third, at the high drug concentrations required for UV detection, it is likely that active transporters are saturated and that passive diffusion is the predominant process. However, this also indicates that the model may not be suitable for studying cellular transport mechanisms, and this issue has to be addressed in additional experiments.

In summary, the 7-day, 96-well Caco-2 permeability model tight to UV analytics was demonstrated to be a valuable alternative to the traditional model. It offers significant reductions in costs, time, and resources and has the potential for automation. The improved methodology for cell feeding and growth, the 96-well format, and the simple UV analytics make it a valuable tool for truly medium- to high-throughput screens for passive permeability determination. Furthermore, the excellent *in vivo*/*in vitro* permeability correlation with human intestinal permeability suggests that the model can be used as a reliable tool for drug permeability screening, for rank ordering of compounds, and for the classification of drugs according to the BCS. This can help to identify permeability-limited absorption problems, to select the right pharmaceutical dosage form, and to better estimate the overall development costs early on in development (25–27).

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