



## Biopharmaceutics Classification System (BCS)



### Validation of the Caco-2 cell monolayer system for determining the permeability of drug substances according to the Biopharmaceutics Classification System (BCS)

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#### Summary

The German Federal Institute for Drugs and Medical Devices (BfArM) recently announced a revision to the drug approval process (bioavailability and bioequivalence) as laid down in article 21 of the German Drug Law (AMG). The update was based work carried out by an expert group at the European Agency for the Evaluation of Medicinal Products (EMA) and published as the "Note for Guidance on the Investigation of Bioavailability and Bioequivalence - CPMP/EWP/QWP/1401/98" (referred to in the following as "Note for Guidance"). After a transitional period of three months that ended on June 26, 2003, the new announcement came into force, replacing

the previous 9th announcement on bioavailability and bioequivalence.

The first German conference on the subject of the Biopharmaceutical Classification System (BCS) was held in March 2003. The conference, which was attended by delegates from BfArM, from the German Pharmaceutical Industry Association (BPI), from pharmaceutical companies and from contract research organizations, provided an opportunity to summarize German experience of the BCS and to formulate common expectations of the system in Germany. The first biowaiver was issued by the BfArM in 2002 for the drug compound Sotalol hydrochloride. Work on this initial pilot scheme

involved close collaboration between the Committee on Generic Compounds at the BPI and members of BfArm. BfArm representatives told delegates that ten requests for biowaivers have been received over the last two years and that in 50 % of the cases the request has been granted.

The BfArm decisions were based on the EMEA Note for Guidance, which is itself modeled on the guidance published in 2000 by the Food and Drug Administration (FDA) of the U.S. Department of Health and Human Services under the name "Waiver of In Vitro Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System". In contrast to the FDA guidance, the EMEA guidance does not specifically exempt highly permeable, highly soluble compounds (so called FDA class I compounds). In principle, the Note for Guidance allows biowaivers to be issued for all classes of compounds provided that appropriate supporting data is provided. According to the EMEA Note for Guidance, a request for a biowaiver that is based on the use of alternative in vitro experiments should incorporate a complete characterization of the drug substance (e.g. in vitro permeability, solubility and physicochemical characterization) and the drug product (content, release etc.), and should take particular account of the effects of excipients used in the formulation. If the metabolic stability of the drug substance is an issue, the report submitted with the request must contain experimental data collected by the applicant or drawn from the published literature.

The conference agreed that given the time and costs associated with in vivo bioequivalence studies, the number of requests for biowaivers is expected to rise. It was estimated that the costs of the in vitro methods and solubility measurements are about one tenth or one fifteenth of the costs associated with a full in vivo bioequivalence study (approx. 150,000 Euro). Furthermore, the time required to acquire the data and to compile the requisite documentation would decrease from months to weeks. Further scientific work leading to a more differentiated view of drug classification in terms of solubility and permeability is desirable.

## 1. INTRODUCTION

The US FDA and the European agency EMEA have issued guidelines under which pharmaceutical companies can request a waiver from in vivo bioequivalence studies based on the Biopharmaceutics Classification Systems (BCS) [1,

2]. The theoretical basis of the BCS system was first described in 1995 [3].

BCS [4, 5] is a scientific framework for classifying substances according to their aqueous solubility and their intestinal permeability. The BCS also takes account of the dissolution of the drug product and hence covers the three main factors which govern the rate and extent of drug absorption from immediate-release (IR) solid oral dosage forms (e.g. tablets, capsules):

- dissolution rate
- solubility
- permeability

According to the BCS, drug substances can be classified as belonging to one of four classes:

- Class 1: high solubility and high permeability
- Class 2: low solubility and high permeability
- Class 3: high solubility and low permeability
- Class 4: low solubility and low permeability

As the pH solubility profile of the test compound during passage through the gastrointestinal tract may influence drug dissolution rate and permeability, the solubility profile should be determined in the pH range of 1-7.5 at  $37 \pm 1$  °C. In addition, the stability of the drug compound at different concentrations of physiological fluids that mimic gastrointestinal fluid and gastric juice must be taken in account. The drug substance is classified as highly soluble, when the highest dose strength is soluble in 250 mL of aqueous media over the pH range 1-7.5.

Both the FDA and EMEA [1,2] recommend the use of monolayers of suitable epithelial cells for classifying the permeability of drug compounds. One epithelial cell line that has been widely used as a model system for measuring intestinal permeability is the Caco-2 cell line [6-8]. By determining the permeability in the Caco-2 system a correlation was established between drug permeability and the fraction absorbed in humans.

The dependence of the permeability values on the following parameters was also studied: inter-day precision, the effects of drug application on apical-to-basolateral absorption and on basolateral-to-apical absorption, number days in culture, and passage number.

A main element of the study was to measure the activity of the efflux transporter p-glycoprotein (pgp), which was detected in the Caco-2 cells

using known p<sub>gp</sub> substrates such as rhodamine 123 and digoxin in the presence and absence of the known p<sub>gp</sub> inhibitor verapamil.

The results confirm that the Caco-2 cell model can readily differentiate highly permeable compounds, which are known to be well absorbed after oral dosing in humans, from less permeable compounds that are poorly absorbed. The in vitro permeability assays were carried out on a batch of Caco-2 cell monolayers that had met our own stringent quality control criteria (see table 1).

## 2. MATERIALS AND METHODS

### Cell culture

Caco-2 cells were obtained by Across Barriers from the German Cell Culture Collection DSMZ, DSMZ-No.: ACC 169. Aliquots of passage 3 were stored in liquid nitrogen. All cell cultures tested negative for mycoplasma (PCR testing) prior to cryoconservation.

Dulbecco's Modified Eagle Medium (DMEM), non-essential amino acids (NEA), and gentamicin sulfate were purchased from Biochrom KG (Berlin, Germany), while the Trypsin-EDTA solution was from Sigma Chemicals (Deisenhofen, Germany). Fetal calf serum (FCS) was supplied by Greiner Labortechnik (Frickenhausen, Germany). The reference compounds were from local suppliers and were of the highest chemical-grade purity.

The Caco-2 cells were cultured at 37 °C, 90 % humidity, 10 % CO<sub>2</sub> in cell culture flasks in DMEM medium containing 10 % FCS and antibiotics. The medium was changed three times a week, and cells were passaged once per week until confluence was about 80 %. Caco-2 cells were detached by means of trypsin-EDTA solution and sown with 10<sup>4</sup> cells/cm<sup>2</sup> on Transwell™ clear filters (Costar™, Wiesbaden,

Germany) with an area of 1.13 cm<sup>2</sup> and a 0.4 μm pore size. Monolayer growth was monitored weekly by measuring the transepithelial electrical resistance (TEER).

### Permeability studies

Immediately before the experiment, the cells were washed twice with KRB buffer, and the KRB buffer was then replaced by KRB buffer containing the test compound. After 30-35 minutes pre-incubation, samples were withdrawn from the donor and acceptor compartments. Sample withdrawal was taken as defining the start of the experiment (t = 0 min). During the transport study further samples were withdrawn from the acceptor compartments at defined times. The volumes withdrawn were refilled by fresh KRB or KRB with the corresponding inhibitor. When not being sampled, the monolayers were incubated in a CO<sub>2</sub> incubator. At the end of the study a second sample was taken from each donor compartment. Finally, the TEER was remeasured; a TEER value above 200 Ω·cm<sup>2</sup> indicating monolayer tightness.

The volumes of the apical and basolateral compartments used in the transport experiments were 500 μL and 1500 μL respectively. The recovery of the test compounds was calculated by comparing pre-incubation samples with samples withdrawn at the end of the transport study.

The quality of a batch of cell monolayers was monitored by measuring the transepithelial electrical resistance (TEER) of a subgroup of randomly selected monolayers and by measuring their permeability with respect to three test compounds. Quality control measurements were performed at least three times for each cell transport condition (e.g. transport direction, presence or absence of inhibitors).

The following quality criteria have to be met before the Caco-2 monolayer batches are released for permeability studies with test compounds:

Test item	Quality criterion (to be met by each individual monolayer)
Number of cell passage	< 50
Age of monolayer	14-30 days
Low permeability	P <sub>app</sub> of fluorescein (ab) < 1·10 <sup>-6</sup> cm/s
High permeability	P <sub>app</sub> of propranolol (ab) > 5·10 <sup>-6</sup> cm/s
Expression of p-glycoprotein	P <sub>app</sub> of rhodamine 123 (ba) > 4·10 <sup>-6</sup> cm/s
Tightness of barrier before transport	TEER > 200 Ω·cm <sup>2</sup>
Tightness of barrier after transport	TEER > 200 Ω·cm <sup>2</sup>

Tab. 1: Quality Control of Caco-2 cell monolayer batch

## Analysis of samples

The fluorescent compounds were analyzed by fluorescence measurement (Victor<sup>2</sup>, Wallac) and radio-labeled compounds were measured by liquid scintillation counting (Wallac 1450 Microbeta, Wallac). All other compounds were analyzed by LC-PDA (Waters Alliance 2790). All test methods had been validated for each compound.

## Permeability calculation

The apparent coefficient of permeation ( $P_{app}$ ) was calculated using the following equation:

$$\text{Eq. 1} \quad P_{app} = \frac{dQ}{dt} \cdot \frac{1}{m_0} \cdot \frac{1}{A} \cdot V_{Donor}; [\text{cm/s}]$$

$dQ/dt$	permeability rate (steady state transport rate) obtained from the transport-time profile of the substrate [e.g. counts/s]
$A$	area of the exposed cell monolayer [cm <sup>2</sup> ]
$m_0$	the original mass of the marker substance in the donor compartment [for e.g. counts]
$V_{Donor}$	volume of donor compartment [cm <sup>3</sup> ].

## 3. RESULTS

### Correlation of Caco-2 permeability coefficients and "fraction absorbed" data for model compounds

To demonstrate that Caco-2 cell monolayer permeability correlates well with in vivo absorption, the  $P_{app}$  values for 19 model compounds were plotted against their published fractional absorption values (Fa) in humans (figure 1). The sources of human absorption data are listed in references [9-15].

The reference compounds fluorescein and rhodamine 123 which were used to demonstrate monolayer tightness and p-gp activity respectively were not included in fig. 1 because the relevant Fa data was not available in the literature.

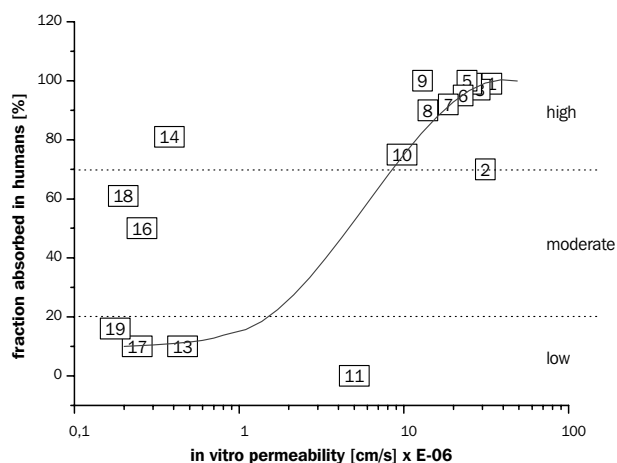


Fig. 1: Comparison of the apparent permeation coefficients of model drugs measured in the Caco-2 cell monolayer system and the fraction absorbed in humans. Data were determined at cell passage 14.

The permeability coefficient of [<sup>3</sup>H]PEG4000 (compound 11), which has a known low Fa value (zero marker), was measured to be 5.00E-06 cm/s in the Caco-2 system. This high value may have been due to impurities with lower molecular weight, or to the instability of the radioactive labeling (PEG Supplier, personal communication). Furosemide (compound 18), which showed a low permeability in the Caco-2 cell monolayer system, is absorbed in vivo in a narrow absorption window (duodenum). In the case of radio-labeled digoxin (compound 14), the literature values for the fraction absorbed in humans varied because of its interaction with p-gp.

### Caco-2 monolayers exhibit apical efflux due to p-glycoprotein

To further validate the suitability of the Caco-2 in vitro model, the monolayers were tested for p-gp activity. Rhodamine 123 and digoxin are known substrates of p-gp, and verapamil is a known p-gp inhibitor [14].

The ratio of the  $P_{app}$  values from the basolateral side (which corresponds to the blood circulation side) to the apical cell side (representing the intestinal lumen) to the transport rate in the reverse direction (i.e.  $P_{app}$  (ab)) depends upon the transport mechanism operating.

Transport mechanism	$P_{app}$ (ba) / $P_{app}$ (ab)
absorption (active)	<< 1
diffusion (passive)	≈ 1
secretion (active)	>> 1

Tab. 3: ratios of permeability coefficients

No.	Compound	$P_{app}$ ab [cm/s]	BCS permeability class	Fa [%]	Reference
1	propranolol	3.42E-05	high	99/90	7, 12
2	carbamazepin	3.18E-05	high	70	8
3	antipyrine	2.91E-05	high	97	7
4	naproxen	2.58E-05	high	99	7
5	verapamil	2.42E-05	high	100	7
6	metoprolol	2.29E-05	high	95	7
7	ketoprofen	1.86E-05	high	92	7
8	citalopram	1.40E-05	high	90	9
9	[ <sup>14</sup> C]caffeine	1.35E-05	high	100	7
10	[ <sup>3</sup> H]clonidin	9.75E-06	high	75/95	7, 8
11	[ <sup>3</sup> H]PEG4000	5.00E-06	low	0	10, 11, 13
12	fluorescein	5.17E-07	low	-	-
13	[ <sup>3</sup> H]PEG400	4.54E-07	low	10	13
14	[ <sup>3</sup> H]digoxin	3.78E-07	low / efflux substrate	81	7
15	rhodamine 123	3.61E-07	low / efflux substrate	-	-
16	[ <sup>3</sup> H]atenolol	2.57E-07	low	55/50	11, 12
17	[ <sup>3</sup> H]PEG900	2.41E-07	low	10	10, 13
18	furosemide	1.98E-07	low	61	7, 12
19	[ <sup>14</sup> C]mannitol	1.77E-07	low	16	7, 11

Tab. 2: Comparison of the apparent permeation coefficients of model drugs and the BCS permeability class. The  $P_{app}$  values are mean values from experiments performed in triplicate. Data were determined at cell passage 14.

The ratios for selected compounds are summarized in fig. 2.

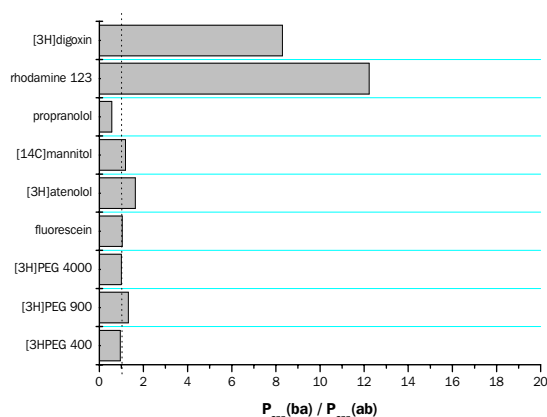


Fig. 2: Ratios of  $P_{app}$  (ba) to  $P_{app}$  (ab) of the apparent permeation coefficients of model drugs in the Caco-2 cell monolayer system. The data are mean data of experiments performed in triplicate. Data were determined at cell passage 14.

At a specific Caco-2 cell monolayer passage, the apparent permeation coefficients of digoxin were measured with and without 40  $\mu$ M verapamil in the

assay buffer. The data are summarized in figure 3. Without inhibitor, digoxin exhibits a substantial  $P_{app}$  (ba) to  $P_{app}$  (ab) transport polarity ratio of 12.5. In the presence of the pgp inhibitor, verapamil, the transport polarity of digoxin is reduced to a value of 1.3. These results confirm that pgp is acting to enhance apical efflux activity.

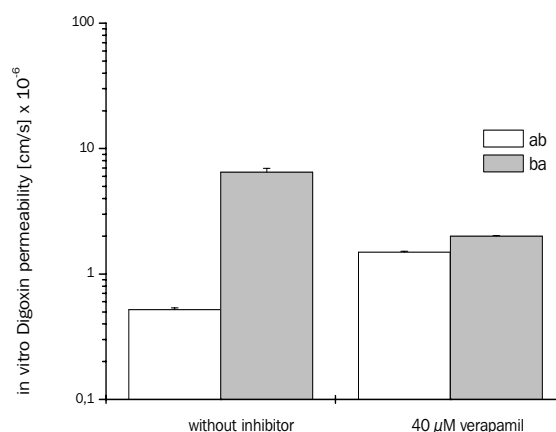


Fig 3: Comparison of the apparent permeation coefficients of digoxin in the absence and presence of 40  $\mu$ M verapamil. The data are mean data from experiments performed in triplicate. Data were determined at cell passage 11.

## Caco-2 cell passage number and its impact on the permeability of selected compounds

Figures 4 and 5 show the permeability of fluorescein, propranolol and rhodamine 123 as a function of various cell passage numbers in relation to the quality criteria listed in table 1. The permeability results do not appear to be dependent on passage number.

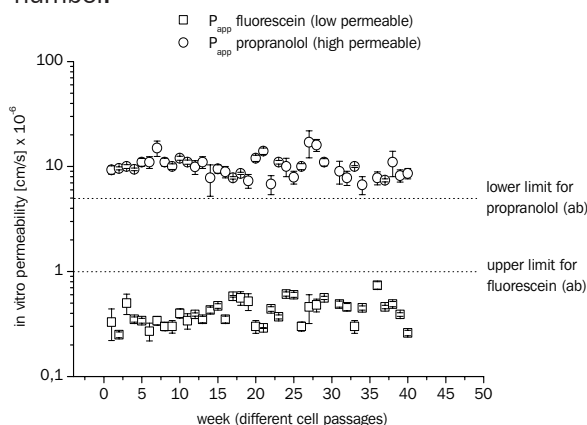


Fig. 4: Comparison of the apparent permeation coefficients of the model drugs propranolol and fluorescein for different passages. The values are mean values from experiments performed in triplicate.

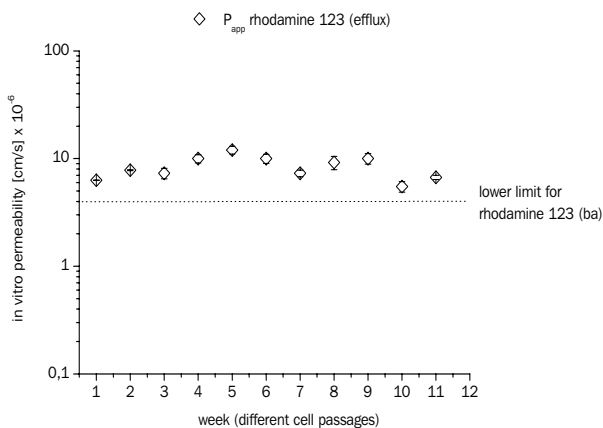


Fig. 5: Comparison of the apparent permeation coefficients of rhodamine 123 measured at different cell passage numbers. The values are mean values from experiments performed in triplicate.

## Inter-day variations of Caco-2 permeability data

The Caco-2 monolayer permeability of the two low permeability compounds atenolol and mannitol and the high permeability compound propranolol were determined on two different days at cell passage 14.

Figure 6 shows the permeability for monolayers cultured for 16 or 18 days and for 20 days. The permeability coefficients of the three model compounds tested and the TEER values of the cell monolayer did not show any dependence of the number of days in culture.

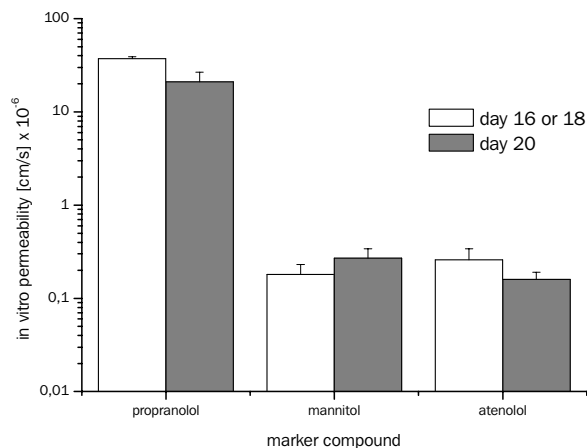


Fig. 6: Comparison of the apparent permeation coefficients of selected compounds measured at cell passage 14 on different days. Permeability coefficients were determined for mannitol and atenolol on day 16, for propranolol on day 18, and were compared to the data obtained on day 20. The values are mean values from experiments in triplicate.

## Batch-to-batch variability

The Caco-2 monolayer permeability of four low permeability compounds was determined after two different passages.

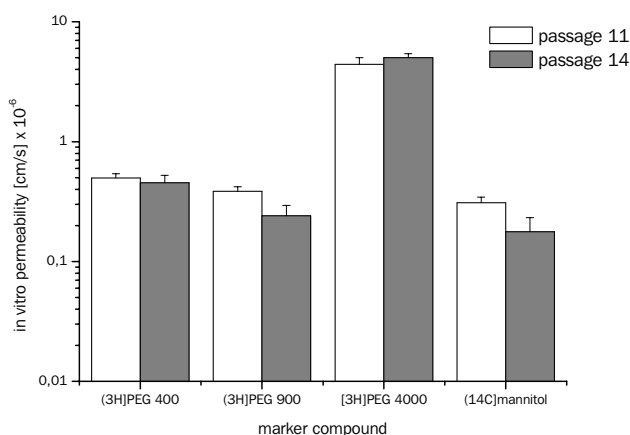


Fig. 7: Comparison of the apparent permeation coefficients of selected compounds measured at cell passage 11 and cell passage 14. The values are mean values from experiments in triplicate.

The permeability coefficients of the four model compounds tested and the TEER values of the cell monolayers did not show any dependence on cell passage number. This result accords with the long-term characterization depicted in fig. 4.

## 4. CONCLUSIONS

Several parameters of the Caco-2 cell permeability assay were evaluated to assess the robustness of the system.

(i)

Caco-2 cell monolayers can be used to study compounds with a wide range of permeabilities ranging from  $P_{app}$   $1.77E-07$  cm/s (e.g. mannitol) to  $3.42E-05$  cm/s (e.g. propranolol), enabling the Caco-2 system to be used to discriminate between high and low permeability compounds as classified in the BCS.

(ii)

Caco-2 cell monolayers show directional transport for the pgp substrates digoxin and rhodamine, with a significantly higher basal-to-apical transport than in the reverse direction. Directional transport can be inhibited by the pgp inhibitor verapamil at a concentration of  $40 \mu\text{M}$ .

(iii)

The  $P_{app}$  values of many of the model compounds correlate well with their absorption in humans.

(iv)

Caco-2 cell monolayers demonstrate only a very weak inter-day variability for monolayers in culture. The inter-day variability is always lower than the measured inter-compound differences.

(v)

Caco-2 cell monolayers can be used for more than 30 passages without there being a noticeable change in the permeability of the model compounds.

The present Caco-2 system is therefore a valid tool with which to determine the gastrointestinal permeability of a test compound in accordance with the FDA's Biopharmaceutics Classification System.

## 5. REFERENCES

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