Pharmaceutical Research, Vol. 25, No. 7, July 2008 (© 2008)
DOI: 10.1007/s11095-008-9556-9

Research Paper

Optimisation of the Caco-2 Permeability Assay Using Experimental Design Methodology

Mark Lakeram,[1] David J. Lockley,[2] Ruth Pendlington,[2] and Ben Forbes[1,3]

Received July 17, 2007; accepted February 5, 2008; published online February 22, 2008

Purpose. This study used a Box-Behnken experimental design to optimise the experimental conditions in the Caco-2 assay for a series of *p*-hydroxybenzoate ester compounds (log *P* 1.96–5.69), as highly lipophilic compounds are not handled well in this system.
Methods. Caco-2 cells, passage 55–70, were cultured on Transwell™ cell culture supports and permeability assays were performed on day 21. A three level three factorial experimental design was used to optimise the experimental conditions.
Results. Addition of BSA (4% *w*/*v*) in the medium increased the apparent permeability coefficients (Papp) of each of the parabens except the octyl ester. Increasing the stirring rate by 100 rpm increased Papp for all the parabens. Use of simulated intestinal fluid either increased (fasted state) or decreased (fed state) the Papp of methyl–butyl parabens.
Conclusions. The optimised conditions were; 1.55% *w*/*v* BSA, 215 rpm stirring rate and 3.02 mM sodium taurocholate in the simulated intestinal fluid; where octyl paraben (log *P* 5.69) had an Papp of $33.93 \pm 1.84 \times 10^{-6}$ cm/s, reflecting its rapid absorption in man. This study provides a systematic optimisation of the Caco-2 permeability assay to avoid the underestimation of the intestinal permeability of compounds with log $P > 3$.

KEY WORDS: apparent permeability; caco-2; factorial design; lipophilic; parabens.

INTRODUCTION

Apparent permeability in Caco-2 cell monolayers is predictive of the absorption of orally ingested compounds in man (1–3). However, the trend towards the discovery of more lipophilic drug candidates presents difficulties in the estimation of their permeability *in vitro*. Lipophilic compounds are transcellularly absorbed and tend to be highly permeable (4) but poorly soluble compounds (5). In addition to the drug delivery challenge, the estimation of permeability of highly lipophilic compounds is problematic in cell culture permeability screens. The first difficulty is that such compounds often have limited solubility in test solutions, but this can be overcome by the use of co-solvents or solubility enhancers in the test solutions. Further problems are that permeability is underestimated due to factors such as non-specific binding to the experimental apparatus and the effect of the unstirred water layer.

A number of attempts have been made to improve cell culture systems to propitiate the measurement of the permeability of lipophilic compounds (6). Adaptations include the addition of albumin to the receiver compartment (7), stirring to reduce the effect of the unstirred water layer (8) and the use of solubilisers in the donor compartment (9). In this study, each of these strategies was evaluated independently for the same lipophilic series of compounds, before experimental design software was employed to define an optimised set of conditions.

In the preliminary experiments, problems associated with the use of simple salt solutions as the donor solution were addressed by the use of both fasted and fed state simulated intestinal fluid (SIF) solutions. This approach has been reported previously (10–12). The unstirred water layer/aqueous boundary layer adjacent to the surface of the cell membrane, which is a significant barrier to the absorption of lipophilic solutes (13), was minimised by agitation. To enhance solubility in receiver fluid, maintain sink conditions and reduce non-specific binding of highly lipophilic compounds, bovine serum albumin (BSA) was added to the receiver compartment (14–16). Each of these strategies for improving the *in vitro* assay can be related to conditions that exist *in vivo*. The use of fasted and fed state simulated intestinal fluids in the donor chamber are designed to mimic to the intestinal lumenal environment. The stirring rate is representative of gut motility and the BSA in the receiver chamber reproduces the presence of albumin in blood.

The three experimental variables identified as contributing to the underestimation of permeability were studied systematically for their effect on the permeability of a homologous series

[1] Pharmaceutical Science Division, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, UK.
[2] Safety and Environmental Assurance Centre, Unilever Colworth, Sharnbrook, Bedfordshire MK44 1LQ, UK.
[3] To whom correspondence should be addressed. (e-mail: ben.forbes@kcl.ac.uk)

of small molecular weight lipophilic compounds. These were methyl, ethyl, propyl, butyl, heptyl and octyl parabens which are benzoic acid esters which vary in the length of their alkyl chain (Fig. 1). The safety of these widely-used preservative compounds has been questioned (17–19), but a critical review has dismissed the suggestion that the weak estrogenic activity of parabens might modulate or disrupt the endocrine system, and the compounds have been deemed safe (20).

In this study, a three level experimental design using response surface methodology was employed to optimise the Caco-2 assay system for the measurement of lipophilic compound permeability. The relationship between one or more response variable and a set of quantitative parameters can be examined using response surface methods such as the Box-Behnken design (21). Application of experimental design has not been reported in conjunction with the Caco-2 cell permeability assay, but affords an efficient means of optimising experimental conditions systematically as only three levels are required for each factor. The system ensures that all factors are never simultaneously set at their high levels, therefore, all the design points fall within the safe operating zone.

The aim of this study was to: (a) evaluate physiologically-relevant experimental approaches to overcome the challenges of screening the intestinal permeability of lipophilic compounds *in vitro*, (b) use experimental design methodology to optimise the selected experimental factors in the Caco-2 model when lipophilic compounds are tested, and (c) measure the permeability of lipophilic compounds under the optimised conditions compared to more standard conditions for Caco-2 cells.

MATERIALS AND METHODS

MATERIALS

Caco-2 cells were obtained from ATCC and were used from passage 55. Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin, non-essential amino acids, L-glutamine, 0.25% trypsin–0.2% EDTA, gentamicin (50 mg/ml), Hanks' Balanced Salt Solution (HBSS), Bis-(*p*-nitrophenyl) phosphate (BNPP), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), morpholinoethansulphonic acid (MES), sodium taurocholate were purchased from Sigma-Aldrich (Dorset, UK). Lecithin (Epikuron 200, 97% phosphatidyl choline) was obtained from Lucas Meyer, (Germany). Transwell® 12-well plates were obtained from Corning Costar Corporation (Buckinghamshire, UK) and cell culture apparatus and 162 cm^2 flasks were from Fisher (Leicestershire, UK).

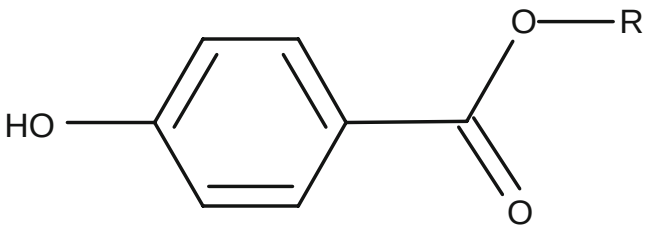


Fig. 1. Chemical structure of *p*-hydroxybenzoate esters, where R = methylparaben (CH_3), ethylparaben (C_2H_5), propylparaben (C_3H_7), butylparaben (C_4H_9), heptylparaben (C_7H_{15}) and octylparaben (C_8H_{17}).

Methyl (MP), ethyl (EP), propyl (PP), butyl (BP), heptyl (HP) and octyl (OP) paraben were purchased from Avocado Research Chemicals (Lancashire, UK). [14C]-propyl and [14C]-octyl parabens were purchased from BioDynamics (Northamptonshire, UK). [14C]-mannitol was purchased from Amersham Biosciences (Buckinghamshire, UK). StarScint was from Perkin Elmer (Buckinghamshire, UK). Dimethyl sulfoxide was purchased from Sigma-Aldrich (Dorset, UK) and acetonitrile analytical grade was from Rathburn Chemicals (Scotland, UK). The parabens were dissolved in DMSO and diluted with 300 μM BNPP in HBSS or simulated intestinal fluids. The final paraben concentration was 50 μM in 1% *v/v* DMSO. These test solutions were used for the paraben permeability studies.

Simulated Intestinal Fluids (SIF)

Caco-2 cell-compatible fasted and fed state SIF transport solutions were manufactured as reported previously (12). The molar ratio between lecithin and sodium taurocholate was fixed at 1:2 to mitigate bile salt toxicity. The fasted state SIF contained lecithin 1.5 mM and sodium taurocholate 3.0 mM in HBSS at a pH of 6.5. The fed state SIF contained lecithin 7.5 mM and sodium taurocholate15 mM in HBSS at a pH of 6.0. The SIF solutions were made by adding the lecithin and sodium taurocholate to HBSS under continuous stirring until all the components were dissolved. The solution was made up to volume and the pH was adjusted using a 1 M solution of MES.

Caco-2 Cell Culture

Caco-2 cells (passage 55–70) were grown in 162 cm^2 cell culture flasks and subcultured weekly on achieving near-confluence by a 1:5 split. Cell culture growth medium was DMEM supplemented with 10% *v/v* fetal bovine serum, 1% *v/v* L-glutamine, 1% *v/v* non-essential amino acids and 0.1% *v/v* gentamicin. Cells were grown in a humidified atmosphere of 5% CO_2: air at 37°C. Medium was changed every 2 to 3 days. For permeability studies the cells were seeded at a density 1×10^5 cells/well on Transwell polycarbonate cell culture supports. These cells were provided with fresh growth medium three times a week until the time of use. The cells were used at 21 days in culture. In preparation for experiments, growth medium was removed and replaced twice with HBSS at 37°C to wash the cell layers; 0.5 ml to the apical chamber and 1.5 ml to the basolateral chamber. The cells were then equilibrated with 300 μM of the organophosphate carboxylesterase inhibitor bis-(*p*-nitrophenyl) phosphate (BNPP) in HBSS (transport medium) for 15 min at 37°C; 0.5 ml in the apical chamber and 1.5 ml in the basolateral chamber. BNPP was used to inhibit enzymatic hydrolysis of the ester compounds that would otherwise confound the permeability measurements (22).

Caco-2 Permeability Experiments

All experiments were conducted at 37°C. For absorptive (apical to basolateral) permeability, transport was initiated by adding 0.5 ml of the test solution to the apical (donor) chamber of Transwell inserts with 1.5 ml transport medium in the absence or presence of BSA in the basolateral (receiver)

Table I. Presentation of 17 Experiments (BB1–BB17) with Coded Values for Factor Levels for the Box-Behnken Experimental Design

	Factor and Factor Level		
Experiment	*a*	*b*	*c*
1	−1	−1	0
2	−1	+1	0
3	−1	0	−1
4	−1	0	+1
5	0	−1	0
6	0	−1	+1
7	0	0	0
8	0	0	0
9	0	0	0
10	0	0	0
11	0	0	0
12	0	+1	0
13	0	+1	+1
14	+1	−1	0
15	+1	+1	0
16	+1	0	−1
17	+1	0	+1

chamber. For secretory (basolateral to apical) permeability, transport was initiated by adding 1.5 ml of the test solution to the basolateral (donor) chamber with 0.5 ml transport medium in the apical (receiver) chamber. Samples (100 µl) were withdrawn from the receiver chamber at 20, 40, 60, 90 and 120 min and from the donor chamber at 0 and 120 min. The volume withdrawn was replaced with fresh transport medium. Paraben concentration was measured by HPLC.

The cell layers were stirred using an orbital shaker. The integrity of the monolayer was confirmed by taking trans-epithelial electrical readings (TER) before and after the experiment and by measuring the permeability of the paracellular marker compound [14C]-mannitol. Radiolabelled samples (100 µl) were added to 10 ml of StarScint scintillation cocktail. The amount of [14C] isotope was measured using a Beckman Coulter LS6000TA (Buckinghamshire, UK), counted for 5 min per sample.

The apparent permeability coefficient (Papp, cm/s) at the end of the experiment for each paraben was calculated according to the following equation:

$$\text{Papp} = \mathrm{d}Q/\mathrm{d}t \times (1/C_{\mathrm{o}} \cdot A)$$

Where $\mathrm{d}Q/\mathrm{d}t$ is the gradient of the slope of flux vs time, C_{o} is the initial drug concentration applied in the donor chamber (µmoles) and A is the surface area of the Transwell® filter (cm^2).

HPLC Analysis of Parabens

Quantification of MP, EP, PP, BP, HP and OP was achieved using a HPLC system equipped with a HP 1050 modular liquid chromatography system with a multi wavelength detector. A reversed phase Hypersil ODS C_{18} column (150 × 4.6 mm I.D., 5 µm) protected by an ODS 20 mm pre-column was used. The mobile phase was a 0.02 M potassium phosphate solution, pH 3.0, and acetonitrile in the following ratios, (retention time in minutes in brackets): 50:50 *v*/*v*—MP (2.9), EP (3.5), PP (4.4) and BP (5.9), 15:85 *v*/*v*—HP (3.5) and OP (4.1). Injection volume was 20 µl. Flow rate was 1.0 ml/min, with detection at 254 nm. LOD was less than 1 µM and LOQ ranged from approximately 2–3 µM.

Experimental Design

A three-level three-factorial Box-Behnken experimental design (constructed using Design-Expert 5) was used to evaluate the effects of the selected independent variables on the response (permeability), to optimise the Caco-2 drug permeability assay for lipophilic compounds. This design is suitable for exploration of quadratic response surfaces and for construction of second order polynomial models, thus helping to optimize a process while using a small number of experimental operations. For the three-level three-factorial Box-Behnken experimental design, a total of 17 experimental runs (Table I) were used. The model generated contains quadratic terms explaining the non-linear nature of response. The design consists of replicated centre points and a set of points lying at the mid-point of each edge of the multidimensional cube that defines the region of interest. The model is of the following form:

$$y = m_0 + m_1 a + m_2 b + m_3 c + m_4 ab + m_5 ac + m_6 bc + m_7 a^2 + m_8 b^2 + m_9 c^2 \quad (1)$$

where y is the selected response, m_0–m_9 are the regression coefficients, a, b and c are the factors studied. Box-Behnken experimental design is an orthogonal design; therefore, the factor levels are evenly spaced and coded for low, medium and high settings, as −1, 0 and +1 (23,24).

Factors studied in the Box-Behnken experimental design were concentration of BSA in the receiver chamber, stirring rate and the concentration of sodium taurocholate in the simulated intestinal fluid used in the donor chamber (Table II). Each factor was set at limits determined to be the lowest and highest values (either experimentally viable or based on physiological relevance). The selected response was the apparent permeability (cm/s) of propylparaben, the compound in the mid-range of the lipophilic series. For the optimisation experiments radiolabelled parabens ([14C]-propyl and [14C]-octyl parabens) were used and analysed as described for [14C]-mannitol.

Table II. Factors and Factor Levels Investigated in Box-Behnken Experimental Design

	Level		
Factor	−1	0	+1
a: BSA concentration (%)	0	2	4
b: Stirring rate (rpm)	25	125	225
c: SIF[a], NaTC concentration (mM)	3	9	15

[a] Lecithin concentration (mM) was kept at the 1:2 ratio, and pH was set at 6.25 (the value between the fasted and fed state pH) for all levels.

Statistical data analyses were performed using analysis of variance (ANOVA) with SPSS 11.0.

RESULTS

The apparent permeability for mannitol under all of the experimental conditions studied was within the range 0.99–2.01 × 10^{-6} cm/s. Transepithelial electrical remained >500 Ω cm^2 in all experiments and did not change over the course of experiments by >15%. Under the 'non-optimised' conditions often used for screening permeability in Caco-2 cell monolayers, the apparent permeability of compounds in the paraben series was inversely proportional to log *P*, with no transport measured for HP and OP (Fig. 2). The absorptive and secretory permeability of the parabens across the Caco-2 cells were equivalent for each of the parabens studied (data not shown).

Preliminary Investigations

Three factors that have the potential to increase the apparent permeability of lipophilic compounds were selected for study because they could be altered simultaneously and they can be related to a physiological condition in man. These factors were the presence of albumin present in blood, gut motility and the gastrointestinal lumenal environment. To establish the influence of each of these conditions on parabens permeability they were first investigated independently for effects across the entire paraben series with analysis of variance (ANOVA) applied at a 5% significance level (Table III).

The addition of 4% BSA to the receiver chamber increased the apparent permeability for PP and BP by 4.0 and 5.8 × 10^{-6} cm/s, respectively (Fig. 2a). There was measurable transport for HP with a Papp value of approximately 6 × 10^{-6} cm/s, but no transport of OP. The stirring rate was increased from 50 to 150 rpm; this increased the apparent permeability for all the parabens resulting in similar Papp for MP–BP of approximately 40 × 10^{-6} cm/s and Papp for HP and OP of 10.1 and 5.8 × 10^{-6} cm/s, respectively (Fig. 2a). The use of the two types of SIF produced variable results (Fig. 2b). For MP there was an increase and for EP there was a decrease in apparent permeability. For all the parabens, permeability was higher when fasted state SIF was used as the donor solution compared to fed state SIF. With PP and BP the fed state SIF caused a reduction in the Papp and the fasted state SIF caused an increase compared to the use of HBSS as the donor solution; this effect was more noticeable for BP. The SIFs increased the transport of both HP and OP compared to HBSS, i.e. increased the transport to become measurable.

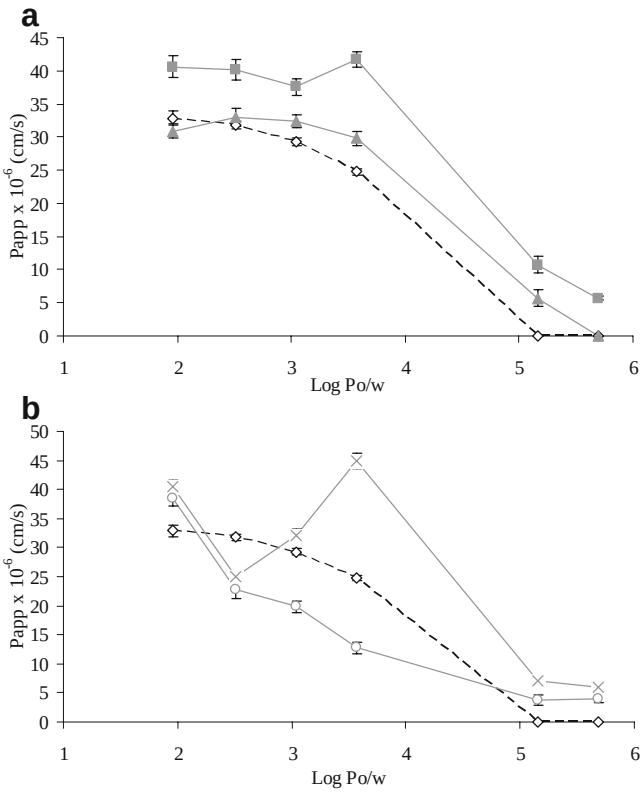


Fig. 2. Relationship between the log *P*o/w and the apparent permeability (Papp×10^{-6} cm/s) for *p*-hydroxybenzoate esters across Caco-2 in the apical to basolateral direction under standard conditions, stirring at 50 rpm using HBSS as transport medium (*diamonds*) with the adaptations **a** 4% BSA in the receiver chamber (*triangles*), stirring at 150 rpm (*squares*); **b** fasted simulated intestinal fluid in the donor chamber (cross), fed simulated intestinal fluid in the donor chamber (*circles*). Data represent mean±SD, *n*=3.

Optimisation of Conditions

The optimisation exercise was based on PP, the paraben in the mid-range of the lipophilicities studied and the lowest log *P* compound for which all the factors had a significant effect. The permeability of PP was measured in the 17 experimental runs of the Box-Behnken experimental design and ranged from 13.9–37.0 × 10^{-6} cm/s. As the response data was a rate (cm/s), the model carried out an inverse transformation.

The use of a Box-Behnken design accommodates non-linear relations between the factors and response, Eq. 2 contains both linear terms (*a*, *b*, *c* to the power of 1) and quadratic terms (*a*, *b*, *c* to the power 2). The negative value for *c* indicated that as this factor increased the response decreased. This corresponded with the effects shown in Fig. 2 and reported previously (12), whereby the change in SIF from fasted to fed state exerted a negative effect on apparent permeability. The *bc* term (stirring rate and simulated intestinal fluid) in the equation indicated this combined factor significantly effected the response ($p = 0.0001$); whereas the combined factors with BSA ($ac + ab$) were not significant compared to the individual factors. The quadratic terms a^2 and b^2 had an antagonistic effect whilst c^2 had a synergistic effect on the response.

$$y = 23.39 + 0.028a + 6.65b - 3.64c - 4.29ab - 0.72ac + 1.58bc - 1.69a^2 - 1.07b^2 + 3.39c^2 \quad (2)$$

An insignificant lack of fit for the model was obtained (ANOVA; $p = 0.0591$). The correlation coefficient (R^2) revealed that 99.31% of the variability of the experimental data could be explained using the model. To estimate the quantitative effects of the factors, a Student's *t*-test was performed. A factor is considered to influence the response if the response differs significantly from zero and the *p*-value is less than 0.05. The only factor found not to influence the

Table III. Analysis of Variance for the Basolateral to Apical Direction, 4% BSA in the Receiver Chamber, Fasted and Fed State Simulated Intestinal Fluids and 150 rpm Stirring Rate Experiments Compared with the Apical to Basolateral Direction Permeability Experiment (Non-significant Effects of Experimental Conditions on Apical to Basolateral Permeability Values are Italicised)

Compound	B–A	4% FBS	Stirring (150 rpm)	Simulated Intestinal Fasted Fluid	Simulated Intestinal Fed Fluid
Methylparaben	*0.167*	*0.153*	0.006	0.003	0.008
Ethylparaben	*0.926*	*0.340*	0.002	0.001	0.002
Propylparaben	*0.076*	0.020	0.001	0.037	0.000
Butylparaben	*0.460*	0.003	0.000	0.000	0.000
Heptylparaben	–	0.003	0.000	0.000	0.004
Octylparaben	–	–	0.000	0.000	0.002

response, with a factor effect of 0.56 and a *p*-value of 0.5941, was when the BSA concentration and simulated intestinal fluid concentration were combined (*ac* from Eq. 1).

To assess the change of the response surface, three-dimensional (3D) plots for the measured responses were constructed based on the model polynomial functions (Fig. 3). Since the model has more than two factors, one factor was held constant for each diagram, each of which illustrates the relationship between two factors.

When the simulated intestinal fluid was held constant a dome shaped plot was observed (parabola opening downwards). This Type I plot (Fig. 3a) featured a linear increase in apparent permeability along one axis (stirring rate) while along the other axis (BSA concentration) there was an increase only up to a maxima and a reduction in apparent permeability thereafter. This indicated that stirring rate and BSA concentration were a positive influence up to a critical point where high apparent permeability was favoured after which the response may decline. When the stirring rate was held constant a saddle shape was observed (Fig. 3b), as a result of the interaction between a negative factor (simulated intestinal fluid) and the positive influence of BSA. In this Type IV plot the surfaces are smooth showing increase/decrease in one axis and decrease/increase in the other axis. When the BSA concentration was held constant an inverted dome-shaped plot (Fig. 3c) was observed (parabola opening upwards). In this Type II plot a decrease in apparent permeability is observed up to a certain critical value of simulated intestinal fluid, while the stronger influence of stirring rate showed an increase from the lowest to the highest value.

The optimal experimental parameters were calculated using canonical analysis, which allows a compromise among various responses and searches for a combination of factor levels that jointly provide an optimised set of responses by satisfying the requirements for each response set. The optimal calculated experimental parameters were:

- BSA in the receiver chamber (*a*) 1.55%
- Stirring rate (*b*) 215 rpm
- Simulated intestinal fluid used in the donor chamber (*c*) 3.02 mM sodium taurocholate

To confirm the validity of the calculated optimal parameters and the predictive power of the program, the apparent permeability for PP and OP under the optimal conditions of factors were measured. For PP a predicted Papp of 39.6×10^{-6} cm/s was obtained whilst the actual observed response was $40.4\pm2.05\times10^{-6}$ cm/s, this residual of +0.8 indicates the potential error in the optimisation. For OP a Papp of $33.93\pm1.84\times10^{-6}$ cm/s was measured.

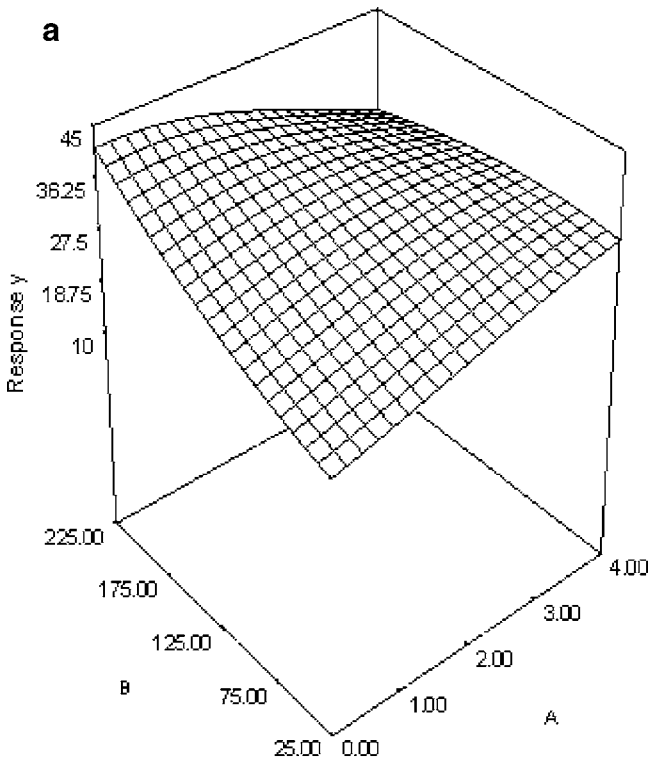
DISCUSSION

Despite the success of the Caco-2 drug absorption model since it was introduced as an *in vitro* tool for studying intestinal transport (25), measurement of the permeability of very lipophilic compounds is problematic in Caco-2 systems. This is due to inherent solubility limitations, non-specific adsorption and difficulties in maintaining sink conditions. This often renders experiments impracticable or low *in vitro* measurements leading to underestimation of human intestinal absorption. In this study, the systematic optimisation of Caco-2 assay conditions is undertaken to define a system in which the transport of highly lipophilic compounds can be studied.

Passive intestinal permeability measured *in vitro* classically increases, peaks and declines in a bell-shaped curve with increasing compound lipophilicity (26). Under 'non-optimised' conditions, the profile of the permeability of parabens vs log *P* (Fig. 2a) correspond to the declining portion (peak permeability to minimal permeability) of this parabolic curve. The similarity between absorptive and secretory transport indicated that the transport of the parabens is passive with an absence of ester hydrolysis or active transport in the presence of BNPP as demonstrated previously (21).

The addition of 4% BSA to the receiver chamber mimics conditions *in vivo* where blood contains approximately 4% albumin. The protein binds lipophilic compounds increasing solubility, helps to maintain the donor-to-receiver concentration gradient and prevents non-specific adsorption. The impact of BSA on permeability is dependent on compound lipophilicity; no effect on permeability was observed for the least lipophilic parabens (MP and EP), there was an increase in the permeability of PP, BP and HP, but OP permeability remained unmeasurable. Similar results have been reported for two new chemical entities; the permeability of compound Sch-Y (log *P* 4.0) was increased whereas the permeability of compound Sch 56592 (log *P* 2.4) was not affected by the use of 4% BSA (16).

The stirring rate of the experiment *in vitro* is representative of gut motility *in vivo*. The unstirred water layer is approximately 30–100 μm thick in man (27). An increased stirring rate reduces the influence (thickness) of the unstirred water layer at the epithelial interface and enhances mixing in the donor and receiver chambers. Increasing the stirring rate by 100 rpm increased the apparent permeability for each of the parabens. For MP, EP, PP and BP the resultant apparent permeability was similar at $\sim40\times10^{-6}$ cm/s. The permeability



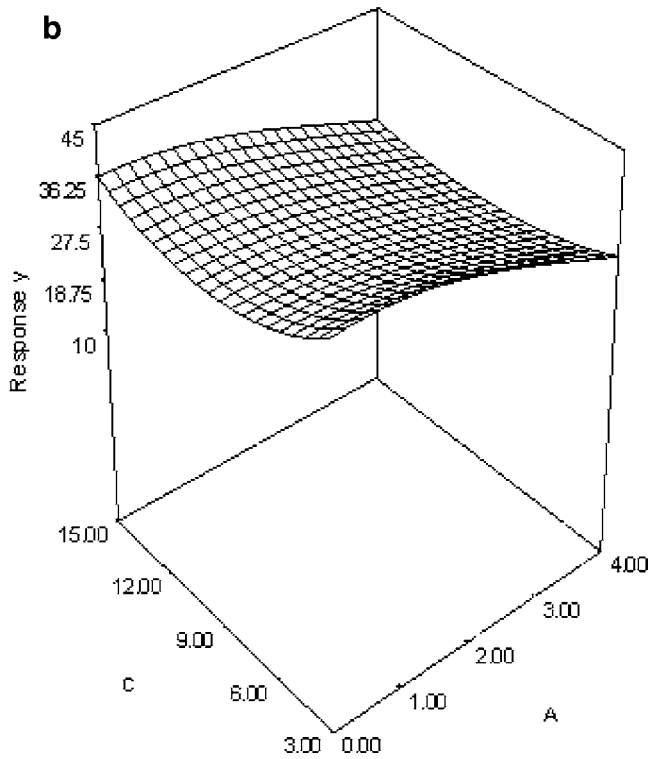


Fig. 3. Response surface plots (3D) showing the effect of the factors on response y=Papp$\times 10^{-6}$ cm/s, *A* BSA concentration (%), *B* Stirring rate (rpm) and *C* NaTC concentration (mM). **a** Fixed level: NaTC concentration=3.02 mM, **b** Fixed level: Stirring rate=215 rpm, **c** Fixed level: BSA concentration=1.55%.

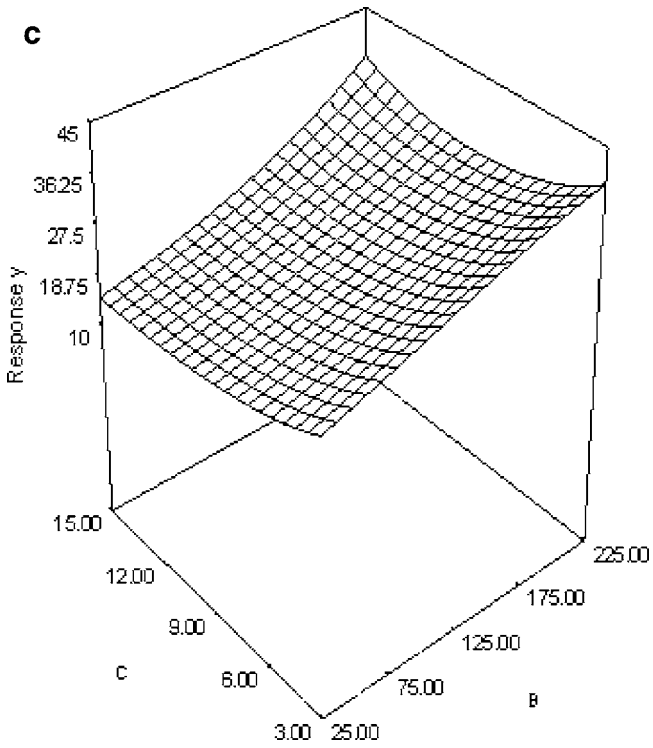


Fig. 3. (continued)

of HP and OP, although not as high as for the other parabens, became measurable providing a distinct improvement from the unmodified system. These findings concord with previous reports that the permeability of highly permeable drugs such as testosterone (28) and grepafloxacin (29) is dependent on stirring conditions in Caco-2 experiments.

The environment in the donor chamber was altered using SIFs, which mimic the intestinal luminal environment *in vivo* in the fed and fasted states. Parabens partition between the bile salt-lecithin mixed micelles of the SIF solutions and the continuous phase will affect both the solubilising capacity of the solution and the paraben concentration in each phase. The fasted and fed state SIFs affected the permeability of each of the parabens studied without affecting the barrier properties of the cell layer, i.e. mannitol permeability and TER were not altered. The fasted state SIF increased the permeability of each of the parabens, except for EP, whereas the general trend for the fed state simulated intestinal fluid was a reduction in the apparent permeability. The effect of SIFs on permeability (which is often reduced) have been reported for the fasted (11,12) and the fed state (12) for a variety of compounds. For the highly lipophilic parabens HP and OP, not only did their permeability become measurable, the recovery of test compound from the apical and baso-lateral chambers was greatly improved to ~70% and 80%, respectively.

Having established the impact of conditions in the donor (SIF) and receiver (BSA) chambers and on the system as a whole (stirring), the inter-relationship of these factors was explored to optimise the conditions. Experimental design methodology provides an efficient and economic strategy for determining the optimum conditions which will generate permeability values predictive of those found in man, i.e.

correct the underestimation of the permeability of lipophilic compounds. Box-Behnken factorial experimental design was used to generate a set of test conditions for cell permeability assays. To our knowledge, this is the first time that such an approach has been reported for the optimisation of cell culture permeability screening methods. Design-Expert 5 software was used to determine model reliability and provide an estimation of the quantitative effects of the different levels of the investigated factors. The levels of these factors were predicted to obtain an optimal response with reference to set constraints.

Under optimised conditions, the mean observed permeability (response) for PP was Papp 40.4×10^{-6} cm/s, which was close to the predicted value of 39.6×10^{-6} cm/s. The optimisation of the conditions (factors) affecting the permeability of PP was achieved rapidly with a minimal number of experimental runs. The optimal set of conditions were then used to determine the apparent permeability for the most lipophilic compound studied, OP, which despite being wholly absorbed in man was previously unable to be assayed satisfactorily (30).

CONCLUSION

In conclusion, experimental design guided the development of an optimal set of experimental conditions for PP (log *P* 3.04), where the apparent permeability was increased by approximately 10×10^{-6} cm/s. When these conditions were applied to octylparaben (log *P* 5.69) an apparent permeability more predictive of the *in vivo* situation was measured. The conditions (1.55% BSA in the receiver chamber, a stirring rate of 215 rpm and simulated intestinal fluid containing 3.02 mM sodium taurocholate: 1.51 mM lecithin in the donor chamber) can be employed in future Caco-2 transport studies when assaying very lipophilic compounds. The utility of using an experimental design such as Box-Behnken for the optimisation of cell culture assays was illustrated by this work.

ACKNOWLEDGEMENTS

Mark Lakeram was funded by a Biotechnology and Biological Sciences Research Council CASE award and Unilever Colworth, UK.

REFERENCES

1. P. Artursson, K. Palm, and K. Luthman. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Deliv. Rev.* **46**:27–43 (2001).
2. L. S. Gan, and D. R. Thakker. Applications of the Caco-2 model in the design and development of orally active drugs: elucidation of biochemical and physical barriers posed by the intestinal epithelium. *Adv. Drug Deliv. Rev.* **23**:77–98 (1997).
3. S. Yee. *In vitro* permeability across Caco-2 cells (colonic) can predict *in vivo* (small intestinal) absorption in man—fact or myth. *Pharm. Res.* **14**:763–766 (1997).
4. V. Pade, and S. Stavchansky. Link between drug absorption solubility and permeability measurements in Caco-2 cells. *J. Pharm. Sci.* **87**:1604–1607 (1998).
5. B. Angelova, and H-P. Schmauder. Lipophilic compounds in biotechnology—interactions with cells and technological problems. *J. Biotechnol.* **67**:13–32 (1999).
6. P. Augustijns, P. Annaert, P. Hevlen, G. Van der Mooter, and R. Kinget. Drug absorption studies of prodrug esters using the Caco-2 model: evaluation of ester hydrolysis and transepithelial transport. *Int. J. Pharm.* **166**:45–53 (1998).
7. G. A. Sawada, N. F. H. Ho, L. R. Williams, C. L. Barsuhn, and T. J. Raub. Transcellular permeability of chlorpromazine demonstrating the roles of protein binding and membrane partitioning. *Pharm. Res.* **11**:665–673 (1994).
8. A. Avdeef, P. E. Nielsen, and O. Tsinman. PAMPA—a drug absorption *in vitro* model 11. Matching the *in vivo* unstirred water layer thickness by individual-well stirring in microtitre plates. *Eur. J. Pharm. Sci.* **22**:365–374 (2004).
9. C. M. Meaney, and C. M. O'Driscoll. A comparison of the permeation enhancement potential of simple bile salt and mixed bile salt:fatty acid micellar systems using the CaCo-2 cell culture model. *Int. J. Pharm.* **207**:21–30 (2000).
10. F. Ingels, S. Deferme, E. Destexhe, M. Oth, G. Van den Mooter, and P. Augustijns. Simulated intestinal fluid as transport medium in the Caco-2 cell culture model. *Int. J. Pharm.* **232**:183–192 (2002).
11. F. Ingels, B. Beck, M. Oth, and P. Augustijns. Effect of simulated intestinal fluid on drug permeability estimation across Caco-2 monolayers. *Int. J. Pharm.* **232**:221–232 (2004).
12. N. Patel, S. Eskola, J. Murray, and B. Forbes. Use of simulated intestinal fluids with Caco-2 cells and rat ileum in Ussing chambers. *Drug Dev. Ind. Pharm.* **32**:151–161 (2006).
13. J. Karlsson, and P. Artursson. A method for the determination of cellular permeability coefficients and aqueous boundary layer thickness in monolayers of intestinal epithelial (Caco-2) cells grown in permeable filter chambers. *Int. J. Pharm.* **71**:55–64 (1991).
14. B. J. Aungst, N. H. Nguyen, J. P. Bulgarelli, and K. Oates-Lenz. The influence of donor and reservoir additives on Caco-2 permeability and secretory transport of HIV protease inhibitors and other lipophilic compounds. *Pharm. Res.* **17**:1175–1180 (2000).
15. G. Krishna, K-J. Chen, C-C. Lin, and A. A. Nomeir. Permeability of lipophilic compounds in drug discovery using *in-vitro* human absorption model, Caco-2. *Int. J. Pharm.* **222**:77–89 (2001).
16. P. Saha, and J. H. Kou. Effect of bovine serum albumin on drug permeability estimation across Caco-2 monolayers. *Eur. J. Pharm. Biopharm.* **54**:319–324 (2002).
17. Y. Nakagawa, and P. Moldeus. Mechanism of p-hydroxybenzoate ester-induced mitochondrial dysfunction and cytotoxicity in isolated rat hepatocytes. *Biochem. Pharmacol.* **55**:1907– 1914 (1998).
18. E. J. Routledge, J. Parker, J. Odum, J. Ashby, and J. P. Sumpter. Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic. *Toxicol. Appl. Pharmacol.* **153**:12–19 (1998).
19. P. D. Darbre, A. Aljarrah, W. R. Miller, N. G. Coldham, M. J. Sauer, and G. S. Pope. Concentrations of parabens in human breast tumours. *J. Appl. Toxicol.* **24**:5–13 (2004).
20. R. Golden, J. Gandy, and G. Vollmer. A review of the endocrine activity of parabens and implications for potential risks to human health. *Crit. Rev. Toxicol.* **35**:435–458 (2005).
21. R. Ragonese, M. Macka, J. Hughes, and P. Petocz. The use of Box-Behnken experimental design in the optimisation and robustness testing of a capillary electrophoresis method for the analysis of ethambutol hydrochloride in pharmaceutical formulation. *J. Pharm. Biomed. Anal.* **27**:995–1007 (2002).
22. M. Lakeram, D. J. Lockley, D. J. Sanders, R. Pendlington, and B. Forbes. Paraben transport and metabolism in the biomimetic artificial membrane permeability assay (BAMPA) and 3-day and 21-day Caco-2 cell systems. *J. Biomol. Screen* **12**:84–91 (2007).
23. K. S. Singh, J. Dodge, M. J. Durrani, and M. A. Khan. Optimisation and characterisation of controlled release pellets coated with an experimental latex. I. Anionic drug. *Int. J. Pharm.* **125**:243–255 (1995).
24. A. A. Karnschi and M. A. Khan. Box-Behnken design for optimisation of formulation variables of indomethacin coprecipitates with polymer mixtures. *Int. J. Pharm.* **131**:9–17 (1996).
25. P. Artursson. Epithelial transport of drugs in cell culture. I. A model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells. *J. Pharm. Sci.* **79**:476–482 (1990).

26. P. Wils, A. Warnery, V. Phung-Ba, S. Legrain, and D. Scherman. High lipophilicity decreases drug transport across intestinal epithelial cells. *J. Pharmacol. Exp. Ther.* **269**:654–658 (1994).
27. H. Lennernas. Human intestinal permeability. *J. Pharm. Sci.* **87**:403–410 (1998).
28. I. J. Hidalgo, K. M. Hillgren, G. M. Grass, and R. T. Borchardt. Characterisation of the unstirred water layer in Caco-2 cell monolayers using novel diffusion apparatus. *Pharm. Res.* **8**:222–227 (1991).
29. K. Naruhashi, I. Tamai, Q. Li, Y. Sai, and A. Tsuji. Experimental demonstration of the unstirred water layer effect on drug transport in Caco-2 cells. *J. Pharm. Sci.* **92**:1502–1508 (2003).
30. C. K. Pease, J. Kino-Thompson, D. Sanders, R. U. Pendlington, M. York, A. McEwan, S. G. Wood, A. Peters, D. Griffiths, G. Ford, and K. Dummer. Biokinetic equivalence of propylparaben and octylparaben: *in vitro* metabolism data and single dose oral administration study in man. *Drug Metab. Rev.* **38**:S1 (2006).