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In vitro model for predicting the access and distribution of drugs in the brain using hCMEC/D3 cells

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ABSTRACT

The BBB is a protective entity that prevents external substances from reaching the CNS but it also hinders the delivery of drugs into the brain when they are needed. The main objective of this work was to improve a previously proposed *in vitro* cell-based model by using a more physiological cell line (hCMEC/D3) to predict the main pharmacokinetic parameters that describe the access and distribution of drugs in the CNS: $Kp_{uu,brain}$, $f_{u,plasma}$, $f_{u,brain}$ and $V_{u,brain}$. The hCMEC/D3 permeability of seven drugs was studied in transwell systems under different conditions (standard, modified with albumin and modified with brain homogenate). From the permeability coefficients of those experiments, the parameters mentioned above were calculated and four linear IVIVCs were established. The best ones were those that relate the *in vitro* and *in vivo* $V_{u,brain}$ and $f_{u,brain}$ (r^2 = 0.961 and r^2 = 0.940) which represent the binding rate of a substance to the brain tissue, evidencing the importance of using brain homogenate to mimic brain tissue when an *in vitro* brain permeability assay is done. This methodology could be a high-throughput screening tool in drug development to select the CNS promising drugs in three different *in vitro* BBB models (hCMEC/D3, MDCK and MDCK-MDR1).

1. Introduction

The blood–brain barrier (BBB) is a protective entity that acts preventing drugs or nutrients from reaching the central nervous system (CNS). This characteristic helps to maintain brain homeostasis and allows the brain to function properly. However, this protective mission of the BBB displays a huge drawback since it makes extremely difficult to deliver drugs into the CNS when they are needed [1–3].

There are several pathways that molecules could use to cross the BBB: paracellular diffusion, transcellular diffusion, carrier-mediated transport, receptor-mediated transport, adsorptive-mediated transport and cell-mediated transport [4–6]. Nevertheless, the physicochemical properties of those molecules limit the use of one pathway or another. For instance, paracellular diffusion and transcellular diffusion are limited to very small hydrophilic or lipophilic molecules; carrier-mediated and receptor-meditated transports can be used by essential molecules, such as, glucose, amino acids, insulin or lipoproteins, that need to specifically bind their carrier or receptor; and molecules using the adsorptive-mediated route or the cell-mediated route need to have positive charge or be able to be internalized by an immune cell [6]. Furthermore, if a molecule reaches the brain, it can be returned to the circulatory system by means of several efflux transporters (ATP-binding cassette transporters) [7]. Because of all that, permeability evaluation tools are needed for evaluating the ability of new drugs or new delivery

Abbreviations: BBB, Blood-brain barrier; CNS, central nervous system; IVIVC, *in vitro-in vivo* correlation; FBS, fetal bovine serum; TEER, transepithelial electrical resistance; HBSS, Hank's balanced salt solution; $Kp_{uu,brain}$, unbound plasma–brain partition coefficient; $f_{u,plasma}$, unbound fraction of drug in plasma; $f_{u,brain}$, unbound fraction of drug in brain; $V_{u,brain}$, apparent volume of distribution in brain; P_{eff}, permeability coefficient; PE%, Prediction error percentage; Cl_{in}, influx clearance; Cl_{out}, efflux clearance.

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Table 1
Physicochemical properties and *in vivo* data from rat for each drug tested [25–28]. $V_{u,brain}$ units are mL/g brain.

	MW (g/mol)	logP	Strongest acidic pKa	Strongest basic pKa	BCS	P-gp	$Kp_{uu,brain}$	$f_{u,plasma}$	$f_{u,brain}$	$V_{u,brain}$
Amitriptyline	277.411	4.81		9.76	I	Substrate	0.730	0.090	0.002	310.000
Atenolol	266.341	0.43	14.08	9.67	III	Substrate	0.030	1.000	0.261	2.500
Carbamazepine	236.274	2.77	15.96		II	Inductor	0.771	0.385	0.170	3.729
Fleroxacin	369.344	0.98	5.32	5.99	IV		0.250	0.793	0.555	1.281
Genistein	270.240	3.08	6.55		II	Inhibitor	0.181	0.010	0.053	11.499
Pefloxacin	333.363	0.75	5.5	6.44	I	Substrate	0.199	0.860	0.514	1.367
Zolpidem	307.397	3.02		5.39	I		0.447	0.267	0.265	2.464

systems to cross the BBB while they are developed.

Drug transport into brain can be measured by *in silico*, *in vitro*, *in situ* or *in vivo* methods [8]. *In vitro* methods can be considered the most interesting ones as **(a)** they normally give better predictions than the *in silico* methods (they can evaluate other properties besides permeability, as cell toxicity) and **(b)** they are faster, cheaper and easier to handle than the *in vivo* ones [9]. During the last years, different cell-based *in vitro* models have been tested to evaluate drug penetration across BBB, such as primary cell cultures or immortalized cell lines from different origins (RBE4 from rat, MBEC4 from mouse, MDCK from dog or hCMEC/D3 from human, among others) [10–13].

Physiologically, BBB is constituted by endothelial cells of brain capillaries which enter deeply into the brain structure and allow brain cells to exchange oxygen, nutrients and waste substances with the circulatory system [14,15]. An ideal cell-based BBB model should meet the following characteristics (a) expressing tight junctions to form a selective barrier and maintain a high electrical resistance, (b) exhibiting functional efflux and influx transporters and a polarized structure, (c) being able to classify substances in accordance to their permeability, (d) being able to response to aggressions as *in vivo* BBB does and (e) simulating the differentiation pattern provoked by the shear stress from blood flow [10,16].

A lot of *in vitro* methods have been tested to reproduce the characteristics mentioned above [17,18]. Except for the latter characteristic, which can only be reached when dynamic *in vitro* BBB models are used, the hCMEC/D3 cell line when properly culture, possesses all the other mentioned properties. This cell line is one of the best known and most applied as BBB model cell line until the moment [19].

From a pharmacokinetic point of view, a good *in vitro* BBB model should be able to predict the rate and extent in which a substance will access to the brain [20–22]. Several factors can determine rate and extent of access to CNS, namely, the plasma levels of the substance, its binding to plasma protein (as only the free fraction will diffuse through the BBB), its effective permeability through the endothelial membrane, the contribution of influx and/or efflux transporters, the metabolic modifications occurred in the barrier itself and its binding to the brain tissue [15,23].

In 2013, Mangas-Sanjuan et al. developed a new *in vitro* method, using MDCKII and MDCKII-MDR1 cell lines, able to predict the main pharmacokinetic parameters that describe the entrance and distribution of different drugs in the CNS ($Kp_{uu,brain}$, $f_{u,plasma}$, $f_{u,brain}$ and $V_{u,brain}$) from the apparent permeability values (P_{app}) of those drugs [24]. The $Kp_{uu,brain}$ is the ratio between the free drug concentration in plasma and the free drug concentration in brain once the steady state has been reached, the $f_{u,plasma}$ is the free fraction of drug in plasma, the $f_{u,brain}$ is the free fraction of drug in the brain and the $V_{u,brain}$ represents the apparent volume of distribution in this organ.

As the MDCKII and MDCKII-MDR1 cell lines, despite having extremely tighten junctions, which has made them a good model for assessing BBB permeability, they have any (MDCKII) or just one (MDCKII-MDR1) BBB transporter [9]. The purpose of this research was to improve the previously mentioned *in vitro* model by using a more physiological cell line, hCMEC/D3 cell line, which, coming from human temporal lobe microvessels, has much more BBB transporters in its surface and should be able to predict the BBB permeability for not just passives drugs, but also those substrates of transporters [10,19]. For assessing this objective, the permeability of seven drugs (some present in the other model and some new ones) was studied in hCMEC/D3 cells under different conditions and the pharmacokinetic parameters mentioned above were calculated. Finally, *in vitro-in vivo* correlations (IVIVCs) between the predicted parameters and experimental parameters obtained in rat [25,26] were established.

2. Materials and methods

2.1. Drug and products

The drugs chosen because of their different properties, amitriptyline, atenolol, carbamazepine, fleroxacin, genistein, pefloxacin and zolpidem, were purchased from Sigma-Aldrich (Spain). Molecular properties and the *in vivo* $Kp_{uu,brain}$, $f_{u,plasma}$, $f_{u,brain}$ and $V_{u,brain}$ values of the studied drugs are shown in Table 1 [25–28].

Hydrocortisone, ascorbic acid, HEPES and bFGF (basic fibroblast growth factor) and HPLC grade chemicals as Methanol, water or Acetonitrile were purchased from Sigma-Aldrich. Fetal bovine serum (FBS), penicillin–streptomycin, chemically defined lipid concentrate, Hank's balanced salt solution (HBSS), collagen I rat protein and trypsin-EDTA were purchased from Gibco. EBM-2 medium was purchased from Lonza and Triton X-100 from Spi-Chem. Immortalized Human Cerebral Microvascular Endothelial Cell Line (hCMEC/D3 cell line) was purchased from Cedarlane (Canada).

2.2. Cell culture

hCMEC/D3 cells were maintained in EBM-2 culture medium adding 5% (v/v) FBS, 1% (v/v) penicillin–streptomycin, hydrocortisone (0.5 μg/ml), ascorbic acid (5 μg/ml), 1% (v/v) lipid concentrate, 1% (v/v) HEPES and bFGF (1 ng/ml - added directly into the flasks when cells were cultured).

Cells were maintained in an incubator at 37 °C, 5% CO_2 and 90% humidity in 75 cm^2 flasks at a cell density of 2.5×10^4 cells/cm^2.

2.3. Permeability studies

The BBB *in vitro* model for carrying out the permeability tests was obtained after seeding hCMEC/D3 cells at a density of 2.5×10^4 cells/cm^2 in the apical chamber, previously coated with 50 μg/mL collagen I rat protein in 0.02 M acetic acid in a 6-transwell plates (effective area: 4.2 cm^2, pore size: 0.4 μm and pore density: $100 \pm 10 \times 10^6$/cm^2) and incubating them until confluence (8 days) replacing the culture medium each two days.

The transepithelial electrical resistance (TEER) was measured all the days that the culture medium was changed and, additionally, at the beginning and at the end of the permeability studies to check that the cell monolayers maintained their integrity. The cell monolayers were considered properly formed when their TEER value, corrected by the value of an empty transwell, reached 30–50 Ω•cm^2 [29].

After 8 days of cell seeding, permeability tests were performed in non-sterile conditions in an orbital shaker at 37 °C and 100 rpm. The culture medium was replaced by HBSS, as isotonic buffer solution. Four

Table 2
Chromatographic methods used in HPLC. Acid water had 0.5% (v/v) trifluoroacetic acid.

	C (μM)	Wavelength	Mobile phase	Retention time (min)
Amitriptyline	250	240 nm	40% Acid water 60% Acetonitrile	1.020
Atenolol	150	231 nm	20% Methanol 60% Acid water 20% Acetonitrile	1.330
Carbamazepine	18	280 nm	65% Acid water 35% Acetonitrile	1.926
Fleroxacin	1.39	285 nm	70% Acid water 30% Acetonitrile	1.348
Genistein	3.81	254 nm	60% Methanol 15% Acid water 25% Acetonitrile	1.334
Pefloxacin	8.91	285 nm	65% Acid water 35% Acetonitrile	0.721
Zolpidem	158	231 nm	60% Water 20% Methanol 20% Acetonitrile	4.624

types of experiments were carried out, in which the apical chamber (2 mL) of the transwell plates represents the plasma and the basolateral chamber (3 mL) of the transwell plates represents the brain [24]. Drug solutions were placed in one chamber and HBSS was placed in the other one. The volumes used in the apical chamber and the basolateral chamber correspond to those specified by the transwell manufacturer and allow liquids to reach the same height on both sides of it.

- Standard experiment (A-B) - This experiment was performed from apical-to-basolateral direction. Drug dissolved in HBSS (2 mL) was placed at time 0 in the apical chamber.
- Standard experiment (B-A) - In this case, the experiment was carried out from basolateral-to-apical direction. The drug dissolved in HBSS (3 mL) was placed at time 0 in the basolateral chamber.
- Albumin experiment (A-B) - In this case, the content of the apical compartment was modified adding albumin 4% (w/v), similar concentration that on human blood, with the aim of mimicking better the plasma compartment and implementing the protein binding of each drug. Transports experiments were done from apical-to-basolateral direction. Drug dissolved in 4% albumin HBSS (2 mL) was placed at time 0 in the apical chamber.
- Brain homogenate experiment (B-A) - For improving the simulation of the brain compartment, in this type of experiment, drug solution in the basolateral compartment (3 mL) was prepared in 1:3 pig brain homogenate:phosphate buffer (180 mM, pH 7.4) solution. Pig brain was selected as surrogate for human brain to mimic the lipid and protein composition of this organ. They were obtained from a local slaughterhouse and were kept frozen until their use. Previous to the experiment brain homogenate was prepared by using a hand blender and adding 3 parts of phosphate buffer to get a texture liquid enough to be able to take samples.

In all conditions, drug solutions were prepared 30 min before the beginning of the experiments and they were left in the orbital shaker at 37 °C during that time. As some of the drugs showed a very low water solubility, all the drugs studied were firstly dissolved in dimethyl sulfoxide (DMSO) and then diluted in HBSS, being the final concentration of DMSO 0.9% (v/v) for amitryptiline, 0.32% (v/v) for zolpidem and 0.09% (v/v) for the rest of the drugs. Final concentrations of drug solutions are shown in Table 2.

During permeability study, aliquots of 200 μL were taken after 15, 30, 60, 90, 120 and 180 min from acceptor compartment and the same volume was replaced with 200 μL of HBSS at 37 °C. Additionally, four extra samples, used for checking the mass balance of the permeability tests, were taken: a sample from the donor compartment at the final point, both samples from apical and basolateral chambers after washing the plates for measuring TEER values after the experiment and a sample from the cell monolayer disrupted by a Triton X-100 (1%) solution at the end of the experiment.

2.4. HPLC analysis of the samples

Samples were evaluated using an ultraviolet (UV) HPLC set (Waters 2695 separation module and Waters 2487 UV detector) and a XBridge C18 column (3.5 μM, 4.6 × 100 mm). Run temperature was established at 30 °C, injection volume was 90 μL and flow rate was 1 mL/min. Other chromatographic conditions are summarized in Table 2.

All analytical methods were validated and demonstrated to be adequate regarding linearity, accuracy, precision, selectivity and specificity. Samples from albumin and brain homogenate experiments were diluted (50:50) with cold methanol to precipitate proteins. Then, all the samples, from all the experiments, were centrifuged at 10000 rpm for 10 min and supernatant was analyzed by HPLC. Acid water had 0.5% (v/v) trifluoroacetic acid.

2.5. Data analysis

All the calculations and plots shown in this paper were obtained with Excel®.

Four different methodologies [30] were used for calculating the permeability coefficient (P_{eff}, cm/s) for each drug and each experimental condition:

- The Sink equation (eq.1), in which dQ/dt is the apparent arrival of drug in the acceptor compartment, S is the surface area of the monolayer and C_0 is the initial concentration of drug administered in the donor compartment. This equation assumes sink conditions during all the experiment which means that the acceptor concentration is always lower than the 10% of the concentration administered in donor.

$$P_{eff} = \frac{\left(\frac{dQ}{dt}\right)}{S \cdot C_0} \quad (1)$$

- The Sink Corrected equation (eq. (2)) which, although assuming sink conditions, considers the change in donor concentration during the experiment. In this equation all the terms are the same as in the Sink one but C_D that is the concentration in the donor compartment at each sample time.

$$P_{eff} = \frac{\left(\frac{dQ}{dt}\right)}{S \cdot C_D} \quad (2)$$

- The Non-Sink equation (eq. (3)) which was developed with the aim of being able to calculate the permeability coefficient when sink conditions are, both, fulfilled or not fulfilled. $C_{receiver,t}$ is the concentration of the drug in receptor chamber at time t, Q_{total} is the total amount of compound in both chambers, $V_{receiver}$ and V_{donor} are the volumes of each compartment, $C_{receiver,t-1}$ is the drug concentration in receptor compartment at previous time, f is the sample replacement dilution factor, S is the area of the monolayer and Δt is the time interval.

$$C_{receiver,t} = \frac{Q_{total}}{V_{receiver} + V_{donor}} + \left(\left(C_{receiver,t-1} \cdot f \right) - \frac{Q_{total}}{V_{receiver} + V_{donor}} \right) \cdot e^{-P_{eff} \cdot S \cdot \left(\frac{1}{V_{receiver}} + \frac{1}{V_{donor}} \right) \cdot \Delta t} \quad (3)$$

- The Modified Non-Sink Equation (eq. (4)) which has the advantage of giving the opportunity of defining two different P_{eff} depending on time when the permeation rate is different at the beginning of the experiment. The terms of this equation are the same as in the Non-Sink one but the permeability coefficient can take two values $P_{eff,0}$ or $P_{eff,1}$. This methodology has demonstrated to be the best tool for obtaining the permeability values, in both sink and no sink conditions, when the initial permeation rate is altered in with regard to the rest of the transport profile [30].

$$C_{receiver,t} = \frac{Q_{total}}{V_{receiver} + V_{donor}} + \left(\left(C_{receiver,t-1} \cdot f \right) - \frac{Q_{total}}{V_{receiver} + V_{donor}} \right) \cdot e^{-P_{eff0,1} \cdot S \cdot \left(\frac{1}{V_{receiver}} + \frac{1}{V_{donor}} \right) \cdot \Delta t} \quad (4)$$

Finally, the permeability values obtained with the method that best suited each case were chosen for calculating the $Kp_{uu,brain}$, $f_{u,plasma}$, $f_{u,brain}$ and $V_{u,brain}$ parameters. The deduction of the equations used for obtaining the main pharmacokinetic parameters that describe the entrance and distribution of drugs in the CNS ($Kp_{uu,brain}$, $f_{u,plasma}$, $f_{u,brain}$ and $V_{u,brain}$) was previously explained in Mangas-Sanjuan *et al.* work [24]. Briefly:

- Kpuu,brain (eq. (5)), defined as the ratio between the unbound concentration in plasma and the unbound concentration in brain once the steady state has been reached, is estimated from the combination of the permeability values obtained in both standard experiments, apical-to-basolateral (Papp A → B) and basolateral-to-apical (Papp B → A). It is because Kpuu,brain can be also expressed as the ratio between the influx clearance (Clin) and the efflux clearance (Clout) through the BBB and, assuming that a clearance can be expressed as the product of a permeability and a surface area, Kpuu,brain calculation can be simplified to a relation between permeabilities [24].

$$Kp_{uu,brain} = \frac{Cl_{in}}{Cl_{out}} = \frac{P_{appA\to B} \cdot S}{P_{appB\to A} \cdot S} = \frac{P_{appA\to B}}{P_{appB\to A}} \quad (5)$$

- The ratio between the permeability coefficients obtained in both apical-to-basolateral experiments, the one modified with albumin (Papp ALB) and the standard one (Papp A → B), gives the fu,plasma (eq.6). This parameter represents the unbound fraction of drug present in plasma and can be obtained from the experiments mentioned above because, in both cases, the transport from the donor to the receiver chamber depends on the free concentration in the donor one (Cu,D). In the standard experiment, all the concentration in donor is unbound as HBSS has not proteins to which the drug can bind, but in the modified with albumin one a concentration of albumin (the most abundant plasma protein [31]) equal to that present in human blood has been added and drugs can bind to it. As in the permeability equations (eq. 1–4), the total concentration in donor (CD) is used (because the unbound fraction is not known), the permeability obtained in the modified experiment is an apparent one, that would be equal to the standard one if the fu,plasma were known when starting the calculations [24].

$$P_{appALB} \cdot C_D = P_{appA\to B} \cdot f_{u,plasma} \cdot C_D \to f_{u,plasma} = \frac{P_{appALB}}{P_{appA\to B}} \quad (6)$$

- Following the same argumentation that in fu,plasma, the unbound fraction of drug in brain, fu,brain (eq. (7)), can be obtained combining the permeability values got from both basolateral-to-apical experiments, the modified with brain homogenate one (Papp HOM) and the standard one (Papp B → A). Furthermore, the fu,brain parameter can be translated to the apparent distribution volume in brain, Vu,brain, one by means of the equation (8) where VECF is the volume of the brain extracellular fluid (0.2 mL/g brain) and VICF is the volume of the brain intracellular fluid (0.6 mL/g brain).

$$P_{appHOM} \cdot C_D = P_{appB\to A} \cdot f_{u,brain} \cdot C_D \to f_{u,brain} = \frac{P_{appHOM}}{P_{appB\to A}} \quad (7)$$

$$V_{u,brain} = V_{ECF} + \left(\frac{1}{f_{u,brain}} \right) \cdot V_{ICF} \quad (8)$$

In vitro-in vivo correlations were developed between the *in vivo* parameters obtained in rat by Friden *et al.* [25] and Kodaira *et al.* [26] (Table 1) and the *in vitro* parameters calculated with the equations above. Linear IVIVCs are shown in different graphs with their coefficient of determination (r^2) and their 95% confidence interval. The r^2 values were used for comparing the IVIVCs developed with this approach and the ones obtained by Mangas-Sanjuan *et al.* with the MDCKII and MDCKII-MDR1 cell lines [24].

2.6. Statistical tests

Differences between groups were evaluated with a t-student test. $P < 0.05$ was established as a significance level. The statistical analysis was made with the software SPSS, V.20.00.

3. Results and discussion

One of the most important problems that industries find when a new drug is developed for CNS treatment is the lack of crossing the BBB and, therefore, to reach its target. This fact has boosted the study of new *in vitro* tools able to predict which drugs are most promising to reach the brain with the aim of avoiding the big losses of investment that the withdrawal of a drug in an advanced phase of its development causes.

In this work, an *in vitro* model for calculating the main pharmacokinetic parameters that describe the entrance and distribution of drugs in the CNS ($Kp_{uu,brain}$, $f_{u,plasma}$, $f_{u,brain}$ and $V_{u,brain}$) has been improved in the hCMEC/D3 cell line. This model would be especially relevant in the future establishment of new therapeutic strategies targeted to the treatment of CNS pathologies (epilepsies, brain tumours, meningitis, multiple sclerosis, encephalitis or dementias among others).

In 2013, Mangas Sanjuan *et al.* proposed this model using two epithelial cell lines, the Madin-Darby canine kidney II (MDCKII) cell line and the wild cell line transfected with P-glycoprotein (MDCKII-MDR1) as, due to their strong tight junctions, they are considered good models for mimicking the BBB [24]. Currently, the endothelial hCMEC/D3 cell line is the best characterized and most used BBB cell model [19] which, despite its relatively lack of tightness (its TEER values are around 30–50 $\Omega \bullet cm^2$) [29] is able to overcome some of the main disadvantages of both MDCKII and MDCKII-MDR1 cell lines, as their differences in morphology, growth, metabolism and transporters with human BBB [9].

Although not measured, it is globally accepted that human brain microvessels have TEER values above 1000 $\Omega \bullet cm^2$ [29], which would be extremely far from the values detected in hCMEC/D3 monolayers. Nonetheless, previous studies have demonstrated that hCMEC/D3 cells monolayers express several proteins that are responsible of tight junctions' formation, such as: claudins, occludins or junction adhesion molecules, and they are able to restrict the permeability of lucifer yellow, a low molecular weight paracellular diffusion marker [19,29].

Table 3
Permeability values obtained for each drug and each different experimental condition (standards, modified with albumin and modified with brain homogenate).

	$P_{app\ A\rightarrow B}$ ($x10^{-6}$ cm/s)	$P_{app\ B\rightarrow A}$ ($x10^{-6}$ cm/s)	$P_{app\ ALB}$ ($x10^{-6}$ cm/s)	$P_{app\ HOM}$ ($x10^{-6}$ cm/s)
Amitriptyline	124.24	66.21	3.00	16.72
Atenolol	19.01	26.89	18.33	10.19
Carbamazepine	70.14	51.93	8.62	20.04
Fleroxacin	29.96	25.73	24.40	19.12
Genistein	38.38	116.16	5.74	20.60
Pefloxacin	24.95	33.14	4.27	21.29
Zolpidem	106.16	80.76	26.83	32.93

3.1. Permeability values and in vitro BBB parameters

The permeability coefficients obtained for each drug and each experimental condition are summarized in Table 3. Additionally, Fig. 1 shows a comparison between these permeability values with their standard deviation obtained in each experimental setting for each drug.

In Fig. 1, it is shown how the presence of albumin affects the permeability of those drugs that have some plasma protein binding, as amitriptyline, carbamazepine, genistein and zolpidem; in these drugs, the Peff values from apical-to-basolateral are considerably reduced when albumin is added to the experiment, but this fact does not happen in those drugs in which there is not *in vivo* protein binding, atenolol, fleroxacin and pefloxacin. On the other hand, the same figure shows the effect that brain homogenate provokes in the basolateral-to-apical permeability when the drug has a high *in vivo* brain binding (amitriptyline, atenolol, carbamazepine, genistein and zolpidem), in which case the Peff values get reduced when the basolateral-to-apical experiment is modified with brain homogenate.

The pharmacokinetic parameters estimated from the *in vitro* permeability coefficients with the equations described previously are shown in Table 4.

3.2. In vitro-in vivo correlations

In this investigation, four different linear IVIVCs have been obtained (Fig. 2). Fig. 2 shows the linear IVIVCs obtained between the *in vitro* $Kp_{uu,brain}$, $f_{u,plasma}$, $f_{u,brain}$ and $V_{u,brain}$ values and the *in vivo* $Kp_{uu,brain}$, $f_{u,plasma}$, $f_{u,brain}$ and $V_{u,brain}$ values with their coefficients of determination (r^2) and their 95% confidence intervals.

The best IVIVCs have been those that relate the *in vitro* $V_{u,brain}$ with the *in vivo* $V_{u,brain}$ and the *in vitro* $f_{u,brain}$ with the *in vivo* $f_{u,brain}$ with an r^2 of 0.961 and 0.940, respectively. These two parameters represent the binding rate of a substance to the brain tissue and, thus, they were obtained combining the permeability values from the studies performed in basolateral-to-apical direction, the standard one and the one modified with brain homogenate. The obtained results evidence the great utility of using brain homogenate to mimic brain tissue when an *in vitro* permeability test is developed. Nevertheless, the r^2 of 0.961 for the correlation between the *in vitro* $V_{u,brain}$ with the *in vivo* $V_{u,brain}$ was obtained after removing the amitriptyline data whose *in vivo* $V_{u,brain}$ value was 310.00 mL/g brain, a huge value in comparison with the rest of *in vivo* data (Table 4). It was not necessary to remove this point when the correlation of $f_{u,brain}$ was obtained, fact that reveals that the use of this system and equation (8), that relates both parameters $f_{u,brain}$ and $V_{u,brain}$, it is not accurate when the binding of a drug to the tissue is extremely high. According to table 4 and the mentioned results, it can be said that, right now, the superior limit for the prediction of $V_{u,brain}$ with this methodology would be an *in vivo* $V_{u,brain}$ value of 11.5 mL/g brain (genistein *in vivo* $V_{u,brain}$).

For the other parameters, the unbound fraction of drug in plasma ($f_{u,plasma}$) and the unbound plasma – brain partition coefficient ($Kp_{uu,brain}$), the correlation is not as good as the other ones, although a clear

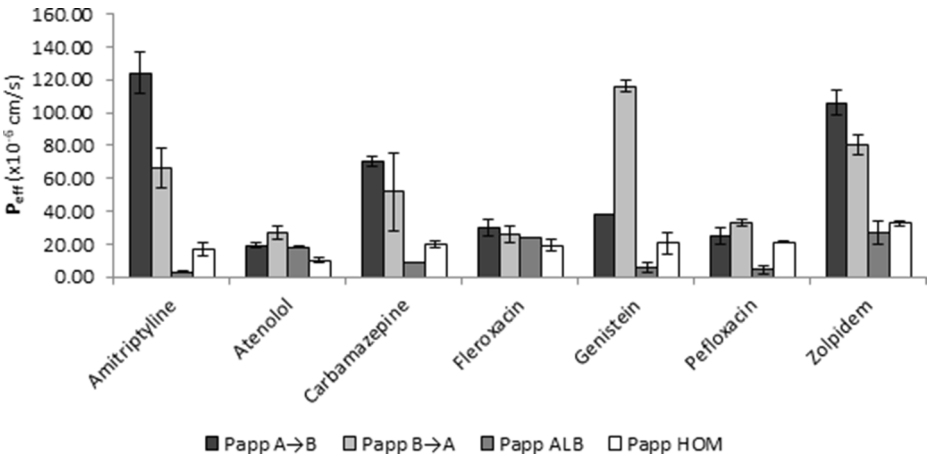

Fig. 1. Comparison of the different permeability values with their standard deviation obtained in each experimental setting for each drug.

Table 4
In vitro pharmacokinetic parameters calculated with the equations (5), 6, 7 and 8 from the permeability coefficients obtained in the different experimental settings and *in vivo* parameters published in Friden *et al* and Kodaira *et al.* [25,26]

	$Kp_{uu,brain}$		$f_{u,plasma}$		$f_{u,brain}$		$V_{u,brain}$ (mL/g brain)	
	In vitro	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*
Amitriptyline	1.876	0.730	0.024	0.090	0.252	0.002	2.577	310.00
Atenolol	0.707	0.030	0.964	1.000	0.379	0.261	1.784	2.500
Carbamazepine	1.351	0.771	0.123	0.385	0.386	0.170	1.755	3.729
Fleroxacin	1.164	0.250	0.814	0.793	0.743	0.555	1.007	1.281
Genistein	0.330	0.181	0.150	0.010	0.177	0.053	3.584	11.499
Pefloxacin	0.753	0.199	0.171	0.860	0.642	0.514	1.134	1.367
Zolpidem	1.314	0.447	0.253	0.267	0.408	0.265	1.671	2.464

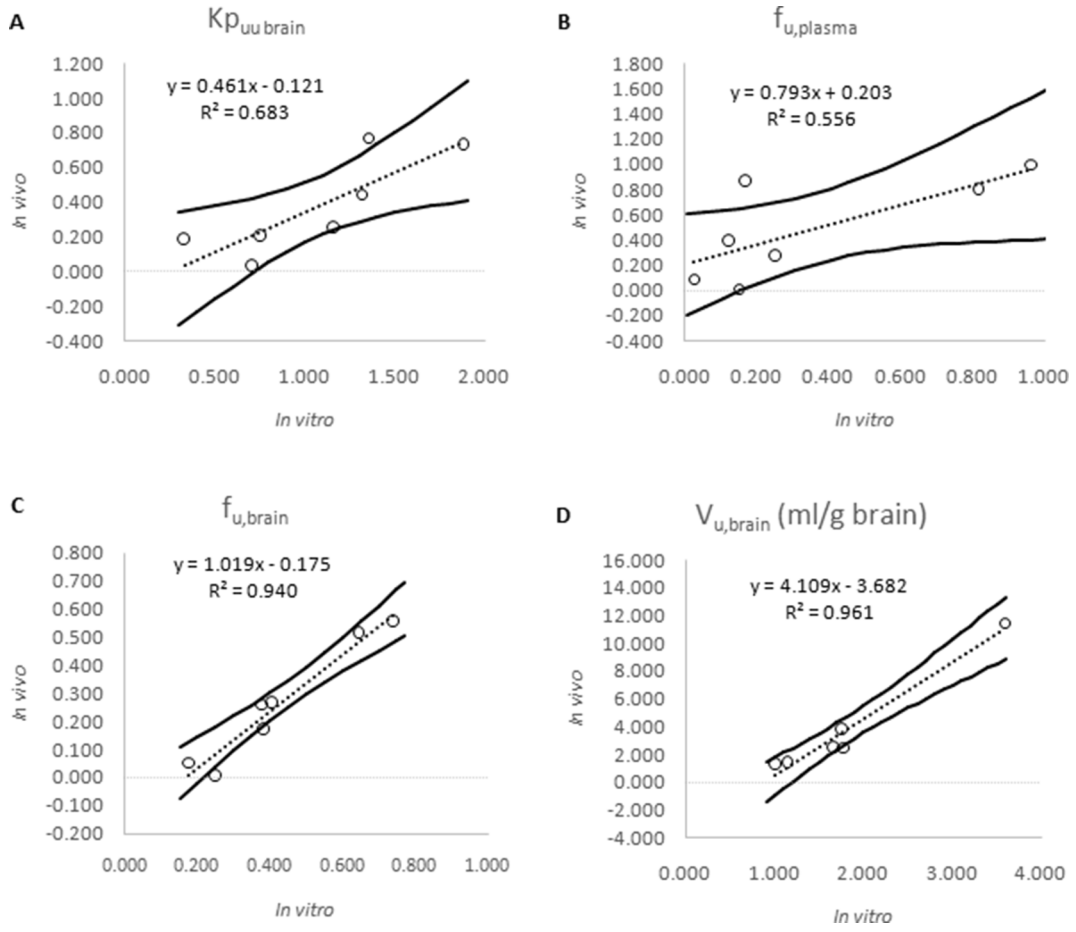

Fig. 2. Linear *in vitro-in vivo* correlations (dotted line) with their coefficient of determination (r^2) and their 95% confidence interval (solid line). IVIVCs obtained between: A. the *in vitro* $Kp_{uu,brain}$ values and the *in vivo* $Kp_{uu,brain}$ values; B. the *in vitro* $f_{u,plasma}$ values and the *in vivo* $f_{u,plasma}$ values; C. the *in vitro* $f_{u,brain}$ values and the *in vivo* $f_{u,brain}$ values and D. the *in vitro* $V_{u,brain}$ values and the *in vivo* $V_{u,brain}$ values.

Table 5
Coefficient of determination (r^2) values for the correlations obtained in hCMEC/D3 cell line in this work and in MDCKII and MDCKII-MDR1 cell lines by Mangas-Sanjuan *et al.* [24]

	MDCKII	MDCKII-MDR1	hCMEC/D3
$Kp_{uu,brain}$ IVIVC	0.063	0.401	0.683
$f_{u,plasma}$ IVIVC	0.846	0.452	0.556
$f_{u,brain}$ IVIVC	0.616	0.624	0.940
$V_{u,brain}$ IVIVC	0.985	0.839	0.961

tendency between *in vitro* data and *in vivo* data can be seen (Fig. 2). *In vitro* $Kp_{uu,brain}$ and *in vivo* $Kp_{uu,brain}$ correlation was developed with an r^2 of 0.683 and the correlation between the *in vitro* $f_{u,plasma}$ and the *in vivo* $f_{u,plasma}$ had an r^2 of 0.556.

In Table 5 the r^2 values for the IVIVCs obtained in this work and the ones obtained by Mangas-Sanjuan *et al.* with the MDCKII and MDCKII-MDR1 cell lines are summarized, for the comparison of the correlations from the different cell lines. The correlation between the *in vitro* $f_{u,brain}$ and the *in vivo* $f_{u,brain}$ for the MDCKII and the MDCKII-MDR1 was not published in Mangas-Sanjuan *et al.* and it was obtained after transforming the published $V_{u,brain}$ values into $f_{u,brain}$ values with equation (8).

In Table 5, it can be seen that for the $Kp_{uu,brain}$ and $f_{u,brain}$ IVIVCs, the highest r^2 values are reached with the hCMEC/D3 cell line. Additionally, the dissimilarity between the $f_{u,brain}$ and $V_{u,brain}$ r^2 values for both MDCKII ($f_{u,brain}$ $r^2 = 0.616$ and $V_{u,brain}$ $r^2 = 0.985$) and MDCKII-MDR1 ($f_{u,brain}$ $r^2 = 0.624$ and $V_{u,brain}$ $r^2 = 0.839$) cell lines confirms that the system and equation (8) are not completely accurate for relating both parameters. Otherwise, according to the r^2 values, the best cell line for predicting the $f_{u,plasma}$ parameter would be the MDCKII cell line ($r^2 = 0.846$) [24].

As results differ from one parameter to other, it cannot be argued that hCMEC/D3 cell line is the best cell model for predicting all the pharmacokinetic parameters $Kp_{uu,brain}$, $f_{u,plasma}$, $f_{u,brain}$ and $V_{u,brain}$ and, thus, the three tested cells lines could be used for making predictions. Nonetheless, as hCMEC/D3 monolayers are a more physiological BBB model, their use will be more appropriate when the transport of new drugs or new delivery formulations want to be tested, especially, if these new therapeutic agents are substrates of several transporters.

Due to the lack of human *in vivo* data of the parameters employed in this work, a limitation of this study could be that in all the correlations the predicted parameters from the *in vitro* experiments were related with rat *in vivo* data [25,26], so parameters obtained with a BBB cell line of human origin are mixed with data that came from an animal [32]. Nevertheless, in 2011 Avdeef compared the permeabilities values obtained *in vitro* with several brain microcapillary endothelial cell models from different species (porcine, bovine, rodent and human) with the *in vivo* permeabilities obtained in rodents and he saw that there was not an evident difference in the correlations for the different species [33]. Therefore, this methodology is considered appropriate for the early stages of drug development, even before starting the preclinical *in vivo* studies, as it promotes the fulfilment of the 3Rs principles (reduction, refinement and replacement) [34].

4. Conclusion

A previous *in vitro* method developed by Mangas-Sanjuan *et al.* [30] has been tested in an alternative cell line (hCMEC/D3). This study confirms that the four proposed experimental settings (apical-to-basolateral standard experiment, basolateral-to-apical standard experiment, apical-to-basolateral with albumin experiment and basolateral-to-apical with brain homogenate experiment) can be used to predict the main pharmacokinetic parameters that describe the entrance and distribution of substances in the CNS ($Kp_{uu,brain}$, $f_{u,plasma}$, $f_{u,brain}$ and $V_{u,brain.}$). Therefore, this methodology can be further adapted to be a high-throughput screening tool to select the most promising drugs to reach the brain in early stages of drug development in, at least, three different *in vitro* BBB cell models (hCMEC/D3, MDCK and MDCK-MDR1 cell lines).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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