Mimicking brain tissue binding in an in vitro model of the blood-brain barrier illustrates differences between in vitro and in vivo methods for assessing the rate of brain penetration

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Mimicking brain tissue binding in an *in vitro* model of the blood-brain barrier illustrates differences between *in vitro* and *in vivo* methods for assessing the rate of brain penetration

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II. Abstract

Assessing the rate of drug delivery to the central nervous system (CNS) *in vitro* has been used for decades to predict whether CNS drug candidates are likely to attain their pharmacological targets, located within the brain parenchyma, at an effective dose. The predictive value of *in vitro* blood-brain barrier (BBB) models is therefore frequently assessed by comparing *in vitro* BBB permeability, usually quoted as the endothelial permeability coefficient (P_e) or apparent permeability (P_{app}), to their rate of BBB permeation measured *in vivo*, the latter being commonly assessed in rodents.

In collaboration with AstraZeneca (DMPK department, Södertälje, Sweden), the *in vitro* BBB permeability (P_{app} and P_e) of 27 marketed CNS drugs has been determined using a bovine *in vitro* BBB model and compared to their *in vivo* permeability (P_{vivo}), obtained by rat *in-situ* brain perfusion. The latter was taken from published data from Summerfield *et al*. (2007).

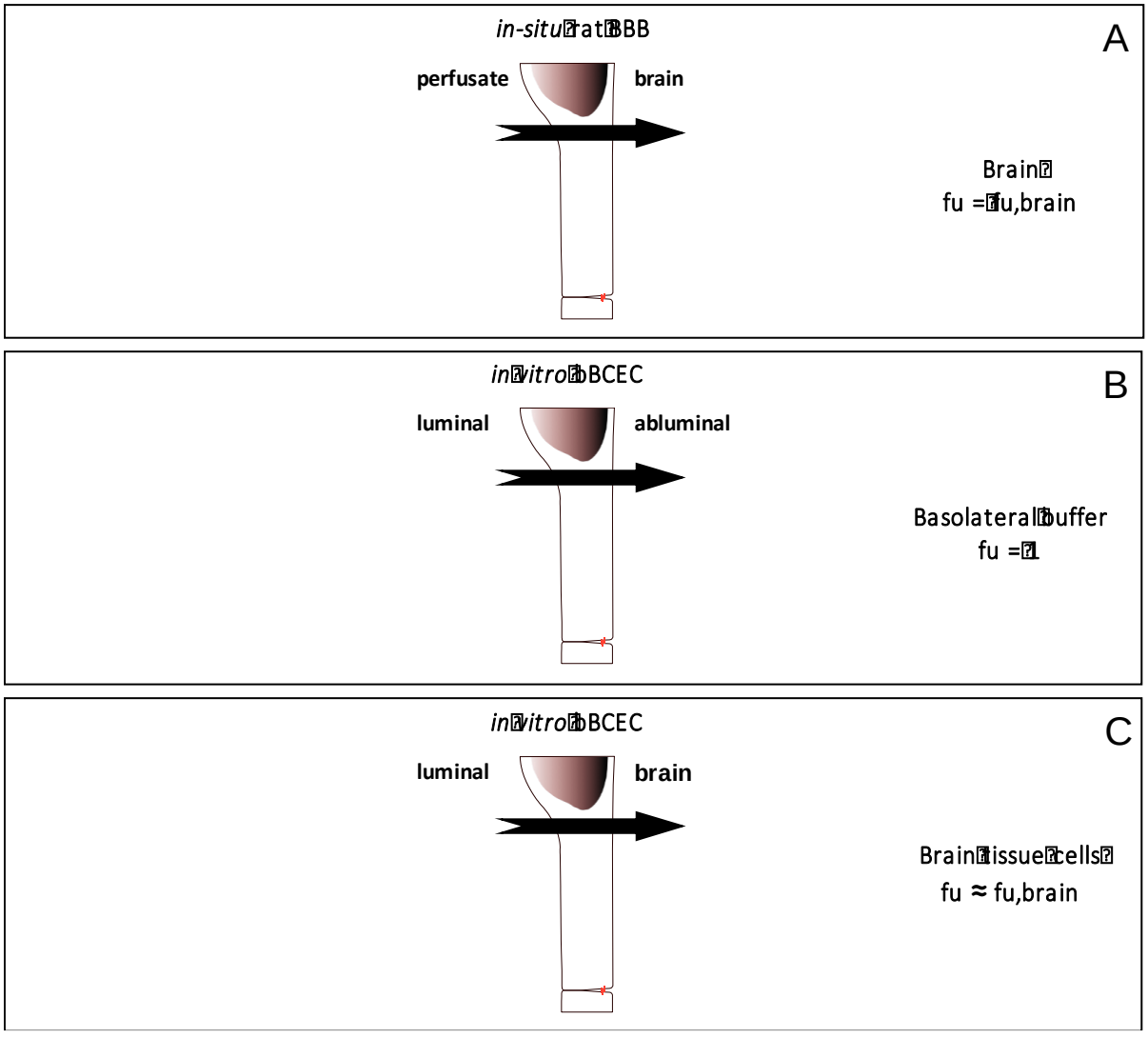
This comparison confirmed previous reports, showing a strong *in vitro*/*in vivo* correlation for hydrophilic compounds, characterized by low brain tissue binding and a weak correlation for lipophilic compounds, characterized by high brain tissue binding. This observation can be explained by the influence of brain tissue binding on the uptake of drugs into the CNS *in vivo* and the absence of possible brain tissue binding *in vitro*.

The use of glial cells (GC) in the *in vitro* BBB model to mimic brain tissue binding and the introduction of a new calculation method for *in vitro* BBB permeability (P_{vitro}) resulted in a strong correlation between the *in vitro* and *in vivo* rate of BBB permeation for the whole set of compounds. These findings might facilitate further *in vitro* to *in vivo* extrapolation for CNS drug candidates.

III. Keywords

blood-brain barrier, endothelial cells, brain exposure, drug discovery, BBB permeability, brain tissue binding, *in vitro* model, *in-situ* brain perfusion.

IV. Graphical Abstract



V. Abbreviations

BBB, blood-brain barrier

bBCEC, bovine brain capillary endothelial cells

EC, endothelial cells

CNS, central nervous system

$f_{u,br}$, fraction unbound brain

GC, glial cells

MB, mass balance/compound recovery

P_{app}, apparent permeability value

P_e, endothelial permeability coefficient

PS, permeability surface area product

PS_e, total PS

PS_f, filter PS

PS_t, endothelial PS

P_{vivo}, *in-situ* brain permeability value

P_{vitro}, *in vitro* permeability value

1. Introduction

The development of drugs for treating central nervous system (CNS) disorders is a medically challenging and commercially risky field, which is known to have a high attrition rate [1]. The blood–brain barrier (BBB), which is a major interface between the circulatory system and the brain parenchyma, can hinder a drug candidate's access to pharmacological targets located within the brain parenchyma. As brain capillaries are the main entry route for the CNS, they can therefore also prevent neuropharmaceuticals from reaching the brain parenchyma at an effective dose. Consequently, the therapeutic potential of a drug candidate is not only related to its activity at the target, but also to its ability to attain an effective dose at the target site. Ideally, this issue should be addressed as early as possible in the drug discovery process [2].

Since animal-based assays tend to be time-consuming and require bioanalytical input or access to radiolabelled compounds, they are generally performed at a relatively late stage of development and are not particularly suitable for dealing with the flow of compounds generated by combinatorial chemistry and high-throughput screening. To overcome the limitations of such *in vivo* studies, several animal-free techniques have-been developed since the early 90's for determining the BBB permeability of potential drug candidates [3]. *In vitro* BBB permeability is usually quoted as the endothelial permeability coefficient (P_e) or apparent permeability (P_{app}) and converted to the permeability surface area product that represents the clearance across the BBB into the brain.

In many cases, the intended use of *in vitro* BBB permeability in the drug discovery process is to predict whether investigational drugs are likely to achieve a relevant CNS concentration to elicit the desired pharmacological effect. However, BBB permeability is a measurement of the rate of drug delivery to the CNS (i.e. speed at which compounds cross the BBB) which must be considered separately from the extent (e.g. the ratio of drug concentration in the brain and plasma) of equilibration of the drug across the BBB and the intra-brain distribution data in order to fully understand brain drug delivery and its consequences for central drug action [4].

Therefore, care should be taken when interpreting results of correlations between *in vitro* and *in vivo* models and when comparing different *in vitro* or *in vivo* approaches as there can be many reasons for divergent results [5]. For instance, in the past, the extent and the rate of transport (i.e. BBB permeability) have sometimes been compared and used indiscriminately [6]. Furthermore, since 2002, methods have been developed to estimate drug binding to brain

tissue, usually expressed as the fraction unbound brain ($f_{u,br}$, i.e. the ratio of the amount of free drug in the interstitial fluid divided by its total amount in brain tissue). The availability of high-throughput equilibrium dialysis [7,8] or brain slice methods [9] to estimate a drug's $f_{u,br}$ has highlighted that the *in vivo* BBB permeability (i.e. the rate of drug delivery to the CNS) is influenced by their ability to bind to brain tissue [6,10,11]. Consequently, as *in vitro* experimental designs can fail to take into account binding to brain tissue [6,12], this might confound the interpretation of data and generate misleading correlations and conclusions when comparisons between *in vitro* and *in vivo* models are made.

The well-tested rodent *in-situ* brain perfusion method [13] enables an accurate assessment of BBB permeability *in vivo* and could therefore be considered as an ideal validation tool for assessing *in vitro* BBB models.

In collaboration with AstraZeneca (DMPK department, Södertälje, Sweden) the *in vitro* permeability (P_{app} and P_e) of 27 marketed CNS drugs has been determined using a bovine *in vitro* BBB model, adapted to high-throughput screening [14], and compared to their *in vivo* permeability (P_{vivo}), obtained by rat *in-situ* brain perfusion. The latter was taken from published data by Summerfield *et al.* (2007) [6]. The selected CNS compounds are covering a wide range of physico-chemical properties and the relationship between their BBB permeability *in vitro* and their binding to brain tissue (based on published $f_{u,br}$) was examined in this study. For this, a new method to assess *in vitro* permeability (P_{vitro}) was used, as it implements brain tissue binding in the assay.

2. Material and Methods

2.1. Test compounds

The marketed CNS compounds were obtained from Sigma-Aldrich (Stockholm, Sweden), except for risperidone, olanzapine, saquinavir, donepezil, venlafaxine and ziprasidone, which were obtained from Sequoia Research Products (Pangbourne, UK). [U-^{14}C]-Sucrose was obtained from Perkin-Elmer (Waltham, MA, USA). All other compounds were provided by AstraZeneca R&D, Södertälje, Sweden from in-house sources. Reagents were obtained from Sigma-Aldrich (Stockholm, Sweden).

147 All compounds were dissolved at a concentration of 2 µM in Krebs-Ringer HEPES buffer (NaCl
148 150 mM, KCl 5.2 mM, $CaCl_2$ 2.2 mM, $MgCl_2$ 0.2 mM, $NaHCO_3$ 6 mM, Glucose 2.8 mM, HEPES 5
149 mM, water for injection).
150
151 *2.2. Traditional method to assess in vitro BBB permeability (apparent permeability, P_{app} and*
152 *endothelial permeability, P_e)*
153 The traditional method for assessing the *in vitro* BBB permeability is taken from the co-culture
154 model of Dehouck *et al*. (1990) [15], which was slightly modified as described by Culot *et al.*
155 (2008)[14] to adapt it to industrial high throughput screening requirements. In brief, primary
156 bovine brain capillary endothelial cells (bBCECs) were seeded at 6 x 10^5 cells.mL^{-1} on a semi-
157 permeable membrane (0.4 µm, 24-well system, Millicell-24 cell culture insert plate, Millipore
158 Corporation, MA, USA) coated with rat tail collagen (prepared by using the method of Bornstein
159 (1958) [16], and placed in a single-well feeding cell culture plate (Millipore Corporation, MA,
160 USA). After 3 days, medium was changed to BBB inducing medium (containing 1% of
161 conditioned medium from the traditional glial and EC co-culture) (Cellial Technologies, Lens,
162 France).
163
164 Drug *in vitro* permeability assessment was carried out 1 day later at pH = 7.4 and T = 37°C. At
165 the initiation of the permeability experiments, the receiver wells of a cell-free 24-well plate
166 were filled with 0.8 mL buffered Ringer's solution and filter inserts containing confluent
167 monolayers of bBCEC, were placed in the 24-well plate. The test solution, containing the
168 compound at 2 µM, was added to the bBCEC monolayer at a volume of 0.4 mL and the plate
169 was placed on an orbital shaker (model PX-MIS 6-1, Polymix, Kinematica AG, Switzerland) with
170 low shaking velocity (60 rpm) for exactly 30 minutes. In parallel, the integrity of the bBCEC
171 monolayer was assessed by permeability measurements of either ^{14}C-sucrose (1.813 kBq.mL^{-1})
172 or lucifer yellow (50 µM). Aliquots were taken from the donor solution in the beginning of the
173 experiment (C_0) and from the donor and receiver compartment at the end of the experiment.
174 A total of 3 inserts with and 3 inserts without cells were assessed for each test compound.
175
176 The *in vitro* brain permeability was measured by either the calculation for P_{app} (cm.s^{-1}) (Equation
177 1) or by the calculation for P_e (cm.min^{-1}) (Equation 4).
178

The P_{app} was calculated as followed:

Eq. 1: $$Papp = \frac{J}{A.C_0}$$

With J representing the flux or rate of appearance of the compound in the receiver compartment (amount.s^{-1}); C_0, the concentration in the donor compartment at t_0 (amount.mL^{-1}); and S, the surface area of the filter insert, which is 0.7 cm^2 for the 24-well format. This results in cm.s^{-1} as the unit for P_{app}.

The P_e was calculated as described by Siflinger-Birnboim *et al.* [17]. By dividing the amount of compound in the receiver compartment by the drug concentration in the donor compartment (Eq. 2), the cleared volume (CL in μL) was obtained at each time point.

Eq. 2: $$CL = \frac{Areceiver}{Cdonor}$$

With $A_{receiver}$ representing the amount of the compound in the receiver compartment; and C_{donor}, the concentration of the compound in the donor compartment.

When the average cumulative CL is plotted over time, the slope equals the permeability surface area product (PS) of the filter. The PS of a filter coated with bBCECs is called the total PS (PS_t) and the PS of a filter without bBCECs is called the filter PS (PS_f). The PS value for the bBCEC monolayer (PS_e) can be computed out of PS_t and PS_f (Equation 3). Units of PS and surface area are μL.min^{-1} and cm^2, respectively.

Eq. 3: $$1/PSe = 1/PSt - 1/PSf$$

To generate P_e (cm.min^{-1}), the PS_e value was divided by the surface area of the filter.

Eq. 4: $$Pe = \frac{PSe}{S}$$

To assess possible adsorption to plastic or non-specific binding to cells, the mass balance (MB, %) was calculated from the amount of recovered compound in both compartments at the end

of the experiment divided by the total amount of compound added in the donor compartment at t_0.

2.3. New method to assess in vitro BBB permeability (P_{vitro})

The new *in vitro* permeability (P_{vitro}) was obtained by using the same protocol as the traditional method explained above, but with some modifications in experimental design and calculation method.

Primary cultures of mixed glial cells (GC) were prepared from the cerebral cortex of new-born rats. After removing the meninges, the brain tissue was forced gently through a nylon sieve. Glial cells were plated on 6-well dishes (Nunc, Roskilde, Denmark) at a concentration of 1.2 x 10^5 cells.mL^{-1} in 2 mL DMEM supplemented with 10% (v/v) fetal calf serum (Hyclone Laboratories). The medium was changed twice a week. Three weeks after seeding, cultures of GC were stabilized and composed of astrocytes (60%, characterized with glial fibrillary acidic protein), oligodendrocytes (30%, characterized with O4) and microglial cells (10%, characterized with ED-1).

As explained above, 1 x 10^6 cells of bBCECs were seeded on a gelatin-coated Petridish (100 mm diameter, Corning). After 2 days, the bBCECs are subcultured in Transwell inserts (0.4 μm pore size, 6-well system, Corning Incorporate) coated with rat tail collagen and placed in a, with stabilized primary rat GC seeded, 6-well. After 12 days in the incubator at 37°C and 5 % CO_2, drug *in vitro* permeability assessments were carried out at pH = 7.4 and T = 37°C.

At the initiation of the permeability experiment, the receiver wells of a 6-well plate containing the primary rat GC, were rinsed twice with 5 mL and filled with 2.5 mL buffered Ringer's solution and Transwell inserts containing confluent monolayers of bBCECs, were placed in the 6-well plate. The test solution, containing the compound at 2 μM, was added to the bBCEC monolayer at a volume of 1.5 mL and the plate was placed on an orbital shaker (model PX-MIS 6-1, Polymix, Kinematica AG, Switzerland) with low shaking velocity (60 rpm) for exactly 60 minutes. In parallel, the integrity of the bBCEC monolayer was assessed by permeability measurements of either ^{14}C-sucrose (1.813 kBq.mL) or lucifer yellow (50 μM). Aliquots were taken from the donor solution in the beginning of the experiment (C_0) and from the donor and receiver compartment at the end of the experiment. A total of 3 inserts with and 3 inserts without cells were assessed for each test compound.

All samples were analysed using tandem mass spectrometry (cfr. *Analytical procedures*) and raw data were computed using the blue-norna® calculator to generate P_{vitro} ($cm.min^{-1}$) according to the following equations (Equation 5 and 6):

Eq. 5: $$in\ vitro\ K_{in}, t\ = \frac{(C_{donor,0} - C_{donor,end}) \times V_{donor}}{C_{donor,end} \times T}$$

Eq. 6: $$P_{vitro} = \frac{in\ vitro\ K_{in}}{S}$$

Where C_{donor} (in mM) is the concentration of compound in the donor compartment at either the onset or end of the transport experiment, T (in min) is the duration of the permeability experiment, V_{donor} (in mL) is the volume of the donor compartment, which is 1.5 mL and S is the surface area of the Transwell insert (i.e. 4.67 cm^2).

2.3. Analytical procedures

The amount of the radiolabel (^{14}C-sucrose) and fluorescent label (lucifer yellow) were determined by liquid scintillation (Packard Instrument Company, Meriden, USA) and fluorescence spectrophotometry (Synergy H1, BioTek Instruments SAS, Colmar, France), respectively. For the measurement of lucifer yellow, an excitation wavelength of 432 and an emission wavelength of 538 was used. A blank value was subtracted from the measured values.

All CNS compounds were determined by the liquid chromatography – mass spectrometry (LC/MS) method. All samples were analysed using the following instruments: mass spectrometer, Quattro Premier XE (Waters); autosampler, acquity sample manager; UPLC pump, acquity binary solvent manager (Waters); robot for sample preparation, Biomek FX (Beckman- Coulter). The following chemicals and reagents were used: ammonium acetate (Merck), acetonitrile gradient grade (Merck), methanol gradient grade (Merck), laboratory deionised water, further purified with a Milli-Q water purifying system and ammonium acetate 1 mol/L in milli-Q water. Samples were stored in -20°C. Prior to analysis, samples were thawed and shaken. If reanalysis was necessary, samples were stored overnight at 10 °C (in an auto sampler).

For chromatography, the following system was used: analytical column, acquity UPLC BEH C18 1.7 µm, 2.1 x 30 mm (Waters). Eluent A was 10 mM ammonium acetate containing 2% acetonitrile and Eluent B was 10 mM ammonium acetate containing 80% acetonitrile. The flow rate was 0.6 $mL.min^{-1}$ (loop: 10 µL, injection volume: 5-10 µL). The gradient started at 0.2 min with 2% of Eluent B and increased to 100% of Eluent B within 0.3 min followed by a column washing step at 100% of Eluent B for 0.2 min.

Quantification of unknown samples was performed, using QuanLynx software. Response factors were constructed by plotting peak area of the analyte against concentration of each analyte using an average response factor of the donor (D_0/C_0) sample injections. The average RF function without weighting was used.

3. Results and Discussion

In this study, the *in vitro* BBB transport of 27 CNS compounds has been assessed and the relationship between either their P_{app} or P_e values *in vitro* and their *in vivo* permeability was compared. To evaluate the influence of brain tissue binding in BBB transport, the compounds were arbitrarily classified in four categories of $f_{u,br}$ (Table 1). Both *in vivo* permeability and $f_{u,br}$ data were obtained from one single study of Summerfield *et al.* (2007) [6] (Table 2).

Several authors tend to use the P_{app} calculation when assessing BBB permeability, while other used the P_e calculation which also accounts for the ability of the compound to cross the cell culture insert. The latter therefore required the assessment of the permeability of the compounds over inserts without cells (PSf).

Although the ability of the compounds to cross the filter is not accounted for in the P_{vitro} calculation, the distribution of the compounds across similar collagen coated inserts (i.e. 6 well-system) without cells has been assessed. All compounds were considered to not be limited by their ability to cross the coated insert. This was based on the sufficient amount of compounds found in the receiver compartment at the end of the one hour transport experiment (i.e. more than 20% of initial amount – data not shown).

Table 1 *– In vitro BBB permeability values for the 27 CNS drugs under study obtained by the traditional method (P_{app} and P_e). The permeability values are accompanied by their corresponding (average) mass balance value (MB). The MB was also measured in absence of bovine brain capillary endothelial cells (bBCEC or EC).*

	Compound name	P_{app} (+ EC, - GC) (10^{-6} cm.s^{-1})		P_e (+ EC, - GC) (10^{-3} cm.min^{-1})		Average MB - EC, - GC	Average MB + EC, - GC
		Mean	S.D.	Mean	S.D.	%	%
fu > 0.1	Bupropion	5.55	1.12	20.81	1.60	n.d.	87
	Carbamazepine	5.15	1.51	11.42	0.80	105	100
	Donepezil	5.43	2.28	18.01	1.70	86	77
	Gabapentin	0.93	0.09	0.79	0.10	112	103
	Sumatriptan	0.87	0.01	0.76	0.10	n.d.	89
	Venlafaxine	4.91	2.53	11.30	1.50	106	99
0.1 > fu > 0.05	Mirtazapine	5.09	3.16	25.12	2.10	n.d.	71
	Phenytoin	4.16	0.72	7.71	0.40	101	92
	Risperidone	3.91	1.28	11.12	1.00	90	83
	Selegiline	4.97	2.53	13.30	0.70	82	83
	Trazodone	4.60	0.49	23.15	2.50	n.d.	82
0.05 > fu > 0.01	Atomoxetine	3.17	2.59	5.43	0.70	n.d.	63
	Citalopram	4.10	0.81	5.99	1.10	108	87
	Diazepam	5.20	2.52	18.56	1.80	99	90
	Doxepin	4.73	2.54	7.89	0.30	104	77
	Loxapine	3.75	1.93	9.40	1.30	n.d.	60
	Mesoridazine	2.97	0.92	6.57	0.90	n.d.	59
	Olanzapine	5.38	1.06	16.01	1.60	111	97
0.01 > fu	Amitriptyline	3.14	2.28	5.49	0.20	73	66
	Chlorpromazine	2.01	2.62	2.51	0.30	51	34
	Fluoxetine	3.79	1.98	8.42	1.20	83	66
	Maprotiline	2.20	2.06	3.85	0.80	n.d.	44
	Perphenazine	0.95	1.32	0.98	0.10	49	33
	Sertraline	1.11	3.23	1.10	0.40	51	26
	Thiothixene	0.72	1.29	0.91	0.10	n.d.	34
	Thioridazine	1.03	1.33	1.30	0.10	41	23
	Ziprasidone	1.50	0.71	2.56	0.30	84	50

As exposure to CNS drugs might affect the tightness of the *in vitro* BBB during the BBB permeability assessment of the drug, the integrity of the bBCEC monolayer during the drug permeability experiment was assessed in parallel by the permeability assessment of either the radiolabel ^{14}C-sucrose or the fluorescent label lucifer yellow. All BBB permeability results presented here correspond to experiments where P_e for the marker molecule(s), in presence of the tested compounds, did not result in a significant increase compared to a control experiment without the tested compounds.

Table 2 *– Physico-chemical characteristics: fraction unbound brain ($f_{u,br}$) and lipophilicity (LogP) and the in vivo blood-brain barrier (BBB) permeability (P_{vivo}) for the 27 CNS drugs under study determined by Summerfield et al. (2007)* [6].

	Compound name	$f_{u,br}$		LogP	P_{vivo} (10^{-3} cm.min^{-1})	
		Mean	S.D.		Mean	S.D.
fu > 0.1	Bupropion	0.171	0.0270	3.27	10.10	1.00
	Carbamazepine	0.119	0.0070	2.10	6.40	1.40
	Donepezil	0.126	0.0150	4.14	10.54	2.53
	Gabapentin	0.782	0.1490	-1.90	1.10	0.07
	Sumatriptan	0.724	0.0690	1.17	0.01	0.01
	Venlafaxine	0.216	0.0230	2.69	3.89	2.90
0.1 > fu > 0.05	Mirtazapine	0.080	0.0030	2.90	12.80	5.00
	Phenytoin	0.082	0.0060	2.26	3.30	0.60
	Risperidone	0.099	0.0030	3.27	5.70	1.27
	Selegiline	0.074	0.0030	3.08	12.81	3.00
	Trazodone	0.055	0.0060	2.68	11.00	0.40
0.05 > fu > 0.01	Atomoxetine	0.019	0.0060	3.95	12.90	3.60
	Citalopram	0.049	0.0040	3.58	3.90	0.67
	Diazepam	0.036	0.0050	2.63	13.20	3.07
	Doxepin	0.025	0.0030	4.08	14.61	2.70
	Loxapine	0.011	0.0030	3.18	11.40	2.27
	Mesoridazine	0.016	0.0010	3.83	5.60	0.87
	Olanzapine	0.034	0.0090	3.61	15.20	0.93
0.01 > fu	Amitriptyline	0.009	0.0010	5.10	21.43	2.93
	Chlorpromazine	0.002	0.0004	5.18	17.40	4.10
	Fluoxetine	0.004	0.0010	4.09	18.00	2.27
	Maprotiline	0.006	0.0027	4.89	13.30	3.30
	Perphenazine	0.004	0.0002	4.15	15.70	1.60
	Sertraline	0.001	0.0001	5.06	29.30	8.27
	Thiothixene	0.003	0.0004	4.01	18.70	3.47
	Thioridazine	0.001	0.0040	5.93	13.34	2.07
	Ziprasidone	0.001	0.0001	4.64	6.65	0.73

3.1. Relationship between P_{app} and P_e with P_{vivo} for compounds with decreasing $f_{u,br}$

We evaluated the relationship between BBB permeability *in vitro* (i.e. P_e and P_{app}) and *in vivo* (i.e. P_{vivo}) for compounds with decreasing $f_{u,br}$. The latter data (i.e. P_{vivo}) was coming from literature [6].

Strong correlations between P_{vivo} and, either *in vitro* P_{app} (Figure 1A) or P_e (Figure 1B), are obtained for compounds characterized by an $f_{u,br} > 0.1$ ($n = 6$, $R^2 = 0.78$, $p = 0.0204$ for P_{app}; and $n = 6$, $R^2 = 0.93$, $p = 0.0017$ for P_e) and $f_{u,br} > 0.05$ (Figure 1C and D, $n = 11$, $R^2 = 0.60$, $p = 0.0052$ for P_{app}; and $n = 11$, $R^2 = 0.80$, $p = 0.0002$ for P_e).

The stronger correlation between P_{vivo} and P_e than between P_{vivo} and P_{app} confirms previous works and supports the use of the P_e calculation in such study [14,18]. The good correlation between *in vitro* and *in vivo* permeability confirms previous findings showing that this *in vitro*

BBB model is highly predictive for the *in vivo* rate of brain penetration for several CNS compounds [14].

However, the same correlation appears very weak after the inclusion of compounds into the dataset characterized by an $f_{u,br}$ between 0.05 and 0.01 (Figure 1E and 1F, n = 18, R^2 = 0.42, p = 0.0036 for P_{app}; and n = 18, R^2 = 0.38, p = 0.066 for P_e). No correlation is shown after the inclusion of compounds with an $f_{u,br}$ < 0.01 (Figure 4G and 4H, n = 27, R^2 = 0.02, p = 0.4900 for P_{app}; and n = 27, R^2 = 0.005, p = 0.7296 for P_e).

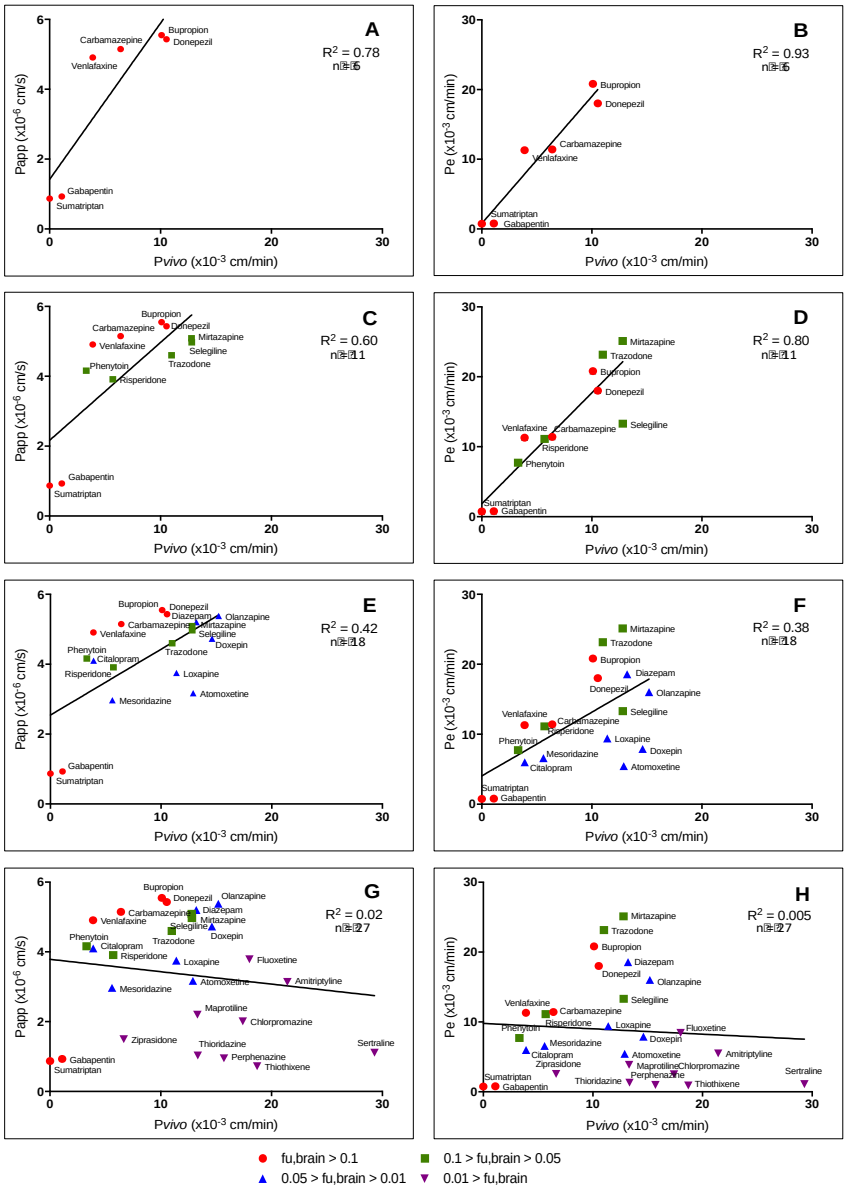


Figure 1 *– Relationship between in vivo brain permeability (P_{vivo}), obtained by brain perfusion in rodents, taken from Summerfield et al. (2007)* [6]*, and in vitro BBB permeability, either depicted by the apparent permeability (P_{app}) (Graph A, C, E and G), or by the endothelial permeability (P_e) (Graph B, D, F and H), and this for compounds of decreasing $f_{u,br}$.*

3.2. Relationship between P_{vivo}/P_{app} and P_{vivo}/P_e and $f_{u,br}$

The observed discrepancies between P_{vivo} and *in vitro* P_e or P_{app} for compounds with low $f_{u,br}$ corroborates what has been published by several other investigators i.e. that *in vivo* permeability is a composite function of both permeability across the BBB and brain tissue binding, whereas *in vitro* permeability uncouples non-specific binding (Figure 2) [6]. An important note here is that the perfusate used to determine the *in vivo* permeability is Krebs-Ringer HEPES buffer, and therefore, P_{vivo} does not account for plasma protein binding.

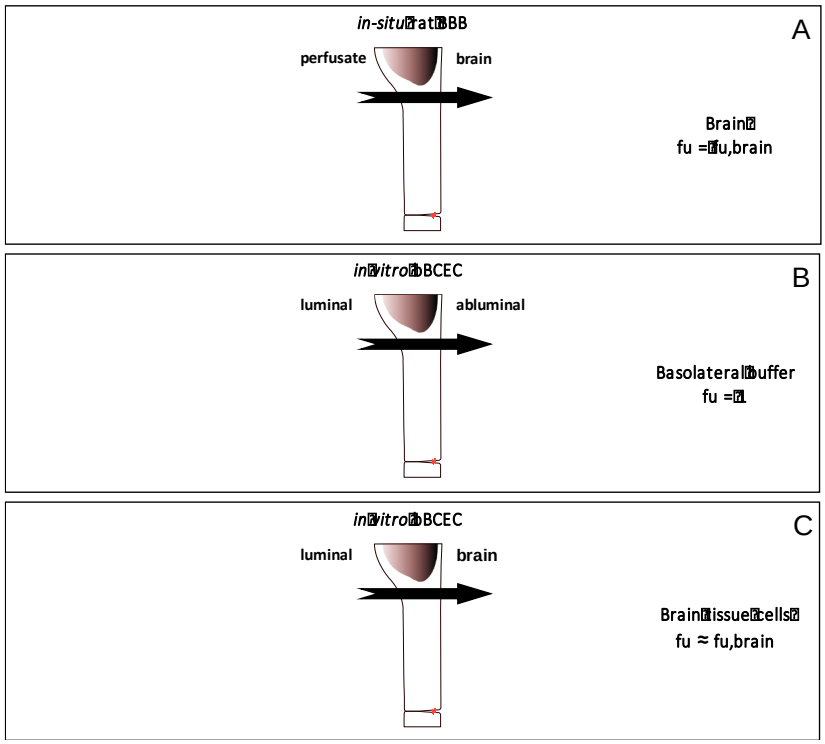


Figure 2 *– Differences in $f_{u,br}$ depending on the experimental design of the model: A) In-situ rat BBB model, B) In vitro bovine BBB model (consisting of bBCEC) without glial cells (GC) in the receiver compartment during the drug permeability experiment; and C) In vitro BBB model (consisting of bBCEC) with GC in the receiver compartment during the drug permeability experiment.*

Indeed, it can be observed that the lack of correlation is mainly due to several lipophilic compounds characterized by high tissue binding. This indicates that brain tissue binding may confound actual *in vivo* permeability *in-situ* and that the observed low *in vitro* permeability for these compounds may be a true reflection of their diffusion process across the BBB. This is supported by the relationship between $f_{u,br}$ and P_{vivo}/P_{app} (Figure 3A, n = 27, $R^2 = 0.64$, $p < 0.0001$) or by the relationship between $f_{u,br}$ and P_{vivo}/P_e (Figure 3B, n = 27, $R^2 = 0.70$, $p < 0.0001$).

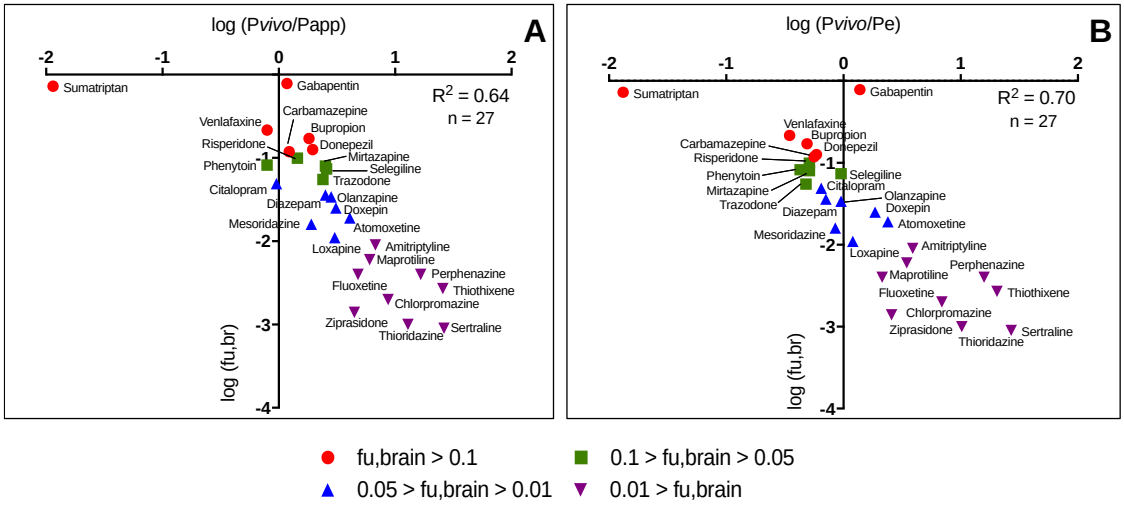


Figure 3 *– Relationship between $f_{u,br}$ and A) P_{vivo}/P_{app}; and B) P_{vivo}/P_e. Regression analysis included all compounds (n = 27). P_{vivo} values used for the different ratios were taken from Summerfield et al. (2007) [6].*

Based on previous findings i.e. that discrepancies between P_{vivo} and *in vitro* permeability is related to brain tissue binding, we have developed a new *in vitro* method to obtain the *in vitro* permeability which accounts for brain tissue binding, P_{vitro}. This new *in vitro* method, and the corresponding parameter, therefore improves the predictive power of the *in vitro* assay. This new method is based on the presence of glial cells (GC) in the receiver compartment to mimic the effect of brain tissue binding *in vivo* during the permeability experiment (Figure 4A).

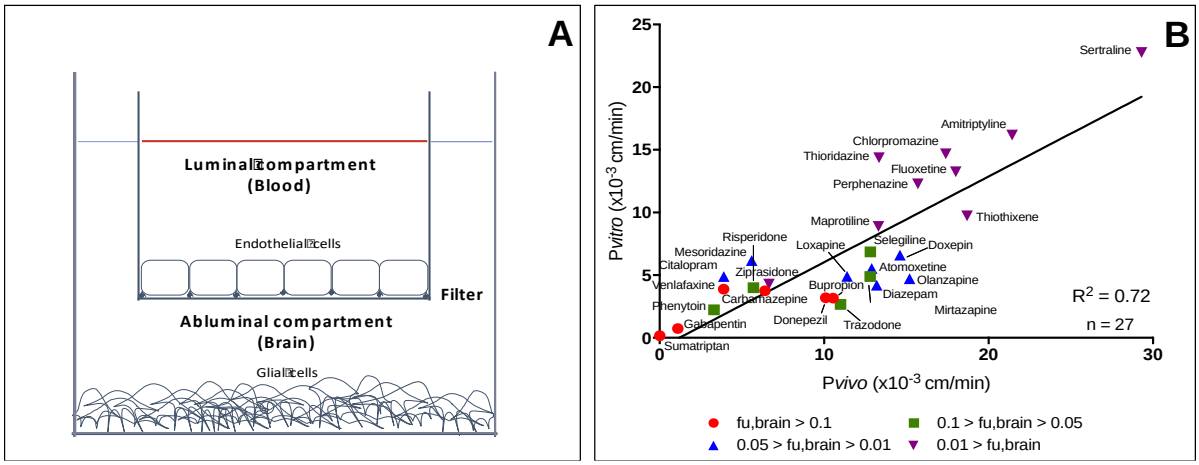


Figure 4 *– A) General scheme of the in vitro BBB model from Dehouck et al. (1990)* [15]*. Bovine brain capillary endothelial cells (bBCEC) are cultured on the upper side of a permeable support and primary rat glial cells (astrocytes, oligodendrocytes and microglia) are cultured at the bottom of the well. B) Relationship between in vivo brain permeability (P_{vivo}), taken from Summerfield et al. (2007)* [6]*, obtained by brain perfusion in rodents, and in vitro BBB permeability, obtained by the new in vitro method (P_{vitro}) for all 27 compounds under study.*

3.3. Relationship between P_{vivo} and P_{vitro}

In comparison to P_{app} and P_e, the correlation between P_{vitro} and P_{vivo} for all 27 compounds is significantly improved (Figure 4B, n = 27, R^2 = 0.72, $p < 0.0001$). This highlights the interest of the introduced new method as no correlation was found between P_{vivo} and *in vitro* P_{app} or P_e for the same dataset, consisting of compounds with different physico-chemical properties and $f_{u,br}$ (Table 3) (Figure 1G and 1H).

Table 3 *– In vitro BBB permeability values obtained for the 27 CNS drugs under study by the new method to assess BBB permeability (P_{vitro}). Permeability values are given for measurements in presence of glial cells and consist of a corresponding (average) mass balance value (MB).*

fu group	*Compound name*	*P_{vitro} (+ EC, + GC) (10^{-3} cm.min^{-1})*		*Average MB (+ EC, + GC)*
		Mean	*S.D.*	*%*
fu > 0.1	*Bupropion*	*3.19*	*0.53*	*87*
	Carbamazepine	*3.75*	*1.06*	*86*
	Donepezil	*3.16*	*0.53*	*79*
	Gabapentin	*0.74*	*0.53*	*98*
	Sumatriptan	*0.17*	*0.02*	*131*
	Venlafaxine	*3.88*	*1.06*	*83*
0.1 > fu > 0.05	*Mirtazapine*	*4.89*	*1.60*	*69*
	Phenytoin	*2.24*	*0.53*	*99*
	Risperidone	*4.00*	*1.06*	*73*
	Selegiline	*6.86*	*2.13*	*81*
	Trazodone	*2.66*	*0.53*	*92*
0.05 > fu > 0.01	*Atomoxetine*	*5.53*	*2.13*	*61*
	Citalopram	*4.89*	*1.06*	*64*
	Diazepam	*4.20*	*0.53*	*77*
	Doxepin	*6.60*	*1.06*	*58*
	Loxapine	*4.90*	*1.60*	*60*
	Mesoridazine	*6.17*	*1.60*	*62*
	Olanzapine	*4.72*	*0.53*	*68*
0.01 > fu	*Amitriptyline*	*16.17*	*3.72*	*33*
	Chlorpromazine	*14.68*	*3.19*	*28*
	Fluoxetine	*13.24*	*2.13*	*34*
	Maprotiline	*8.88*	*1.60*	*41*
	Perphenazine	*12.29*	*1.60*	*34*
	Sertraline	*22.77*	*2.66*	*19*
	Thiothixene	*9.73*	*2.13*	*38*
	Thioridazine	*14.36*	*1.06*	*44*
	Ziprasidone	*4.28*	*1.06*	*44*

The new calculation method is based on the compound's clearance from the donor compartment with the assumption that the cleared compound is entering the brain.

To further evaluate the impact of GC in the *in vitro* BBB model during the permeability experiment, and to rule out that the observed difference between P_{vitro} and P_e was solely

attributed to the different calculation method, P_{vitro} was calculated in presence and absence of GC for 3 different compounds (i.e. carbamazepine, diazepam and chlorpromazine) characterized by different $f_{u,br}$ (Table 4) (Figure 5).

Table 4 *– In vitro BBB permeability values obtained for 3 CNS drugs (i.e. carbamazepine, diazepam and chlorpromazine) by the new method to assess BBB permeability in vitro (P_{vitro}). The P_{vitro} values are given for measurements in presence and absence of glial cells (GC) and consist of a corresponding (average) mass balance value (MB). The MB for the condition without bBCEC (or EC) is also given.*

fu group	*Compound name*	*Average MB (- EC, - GC) %*	*P_{vitro} (10^{-3} cm.min^{-1}) (+ EC, - GC) Mean*	*S.D.*	*MB (+ EC, - GC) %*	*P_{vitro} (10^{-3} cm.min^{-1}) (+ EC, + GC) Mean*	*S.D.*	*Average MB (+ EC, + GC) %*
fu > 0.1	*Carbamazepine*	*92*	*2.01*	*1.11*	*89*	*3.75*	*1.06*	*86*
0.05 > fu > 0.01	*Diazepam*	*100*	*2.22*	*0.18*	*89*	*4.20*	*0.53*	*91*
0.01 > fu	*Chlorpromazine*	*95*	*7.30*	*0.24*	*53.3*	*14.68*	*3.19*	*32*

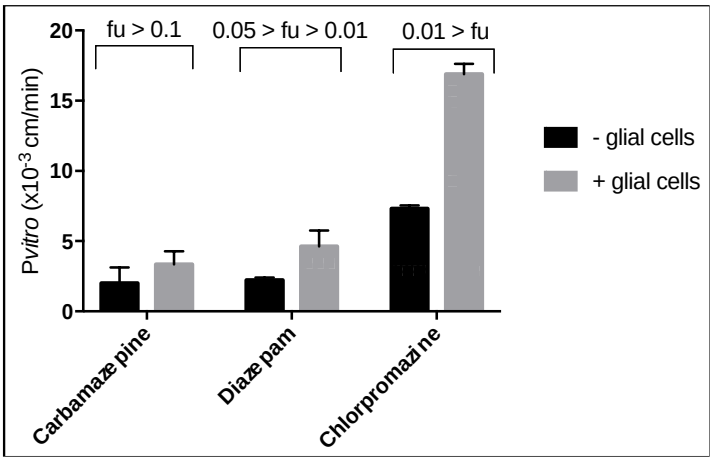


Figure 5 *– Comparison of P_{vitro} in presence and absence of glial cells for 3 compounds (i.e. carbamazepine, diazepam and chlorpromazine) characterized by a different $f_{u,br}$.*

As expected, the presence of GC during the *in vitro* permeability experiment did not resulted in a significant difference between P_{vitro} (with and without GC) for compounds with a relatively high $f_{u,br}$, such as carbamazepine. In contrast, the P_{vitro} of diazepam and chlorpromazine, two compounds with a relatively low $f_{u,br}$, is higher in presence of GC during the BBB permeability experiment than in their absence (Figure 5). This result illustrates that a difference between P_e or P_{app} and P_{vitro} is not an effect of the use of a different calculation method of BBB permeability, but rather reflects the effect of brain tissue binding on the distribution of the compound in the *in vitro* assay.

Indeed, the difference in brain tissue binding affinity for carbamazepine, diazepam, and chlorpromazine, indicated by their $f_{u,br}$, is also reflected *in vitro* by the mass balance (MB). In presence of GC, the MB is much lower for chlorpromazine than for diazepam and carbamazepine (Table 4). This poor recovery of chlorpromazine in the presence of GC (and to a lower extent of diazepam) reflects the sequestration of the compound within the bBCEC and GC, as the MB of the three compounds in the conditions without either bBCEC or GC does not suggest a strong binding to plastic.

Altogether, our results support that permeability *in vivo* is a composite function of both permeability across the BBB and tissue binding [4,6]. This can be illustrated by compounds such as chlorpromazine, which has a high permeability *in vivo* (i.e. P_{vivo} = 17.40 x 10^{-3} cm.min^{-1}) but one of the lowest permeability *in vitro* (i.e. P_e = 2.51 x 10^{-3} cm.min^{-1}). The reason for this discrepancy is not necessarily that the *in vitro* prediction based on the calculation of P_e is wrong, but simply that when chlorpromazine is assessed *in vivo*, a large portion may be associated with the endothelial cell wall. Therefore, when the whole brain is subsequently homogenized, some of the compound associated with the endothelial cell wall, will be erroneously interpreted as having permeated the BBB. In this *in vivo* dataset, the total brain concentration of a compound was corrected based on the apparent brain distribution volume of atenolol to compensate for drug contained in the capillary vascular space [6]. Atenolol is a drug that does not significantly accumulate in brain tissue during the time course of the experiment.

In fact, *in vitro* MB calculations indicate that a large portion of chlorpromazine is associated with the bBCEC, as shown by the low recovery in presence of bBCEC and this even in absence of GC. In complete absence of cells (i.e. bBCEC and GC), the recovery of chlorpromazine in this format was close to 100 % and demonstrated the absence of non-specific binding to plastic. Consequently, it may very well be the case that chlorpromazine is slowly diffusing across the BBB, as the *in vitro* permeability data suggests.

By combining *in vitro* permeation across the brain endothelium and binding to brain tissue, this new method (i.e. P_{vitro}) offers an improved correlation with *in vivo* data, that we obtained from literature [6]. Although this can be seen as an advantage, it should be noted that the use of the traditional *in vitro* BBB permeability assay could still be valuable as it assesses the BBB permeability without accounting for non-specific binding to brain tissue. This is important

information to consider while trying to integrate pharmacokinetic parameters into a coherent model of brain penetration and distribution.

4. Conclusion

This study provides a better understanding of the use of *in vitro* BBB models as a tool to predict brain exposure *in vivo*. In this study, 27 CNS compounds were assessed for their BBB permeability *in vitro,* the latter being compared with *in vivo* permeability values obtained from literature [6]. A lack of correlation for highly lipophilic compounds (and a very low $f_{u,br}$) is observed when using the traditional method to assess BBB permeability *in vitro*, which suggests a rather poor predictive power of the traditional permeability assay. As a remark it should be stated that this bovine BBB model has been used both at University of Artois and in house at AstraZeneca (former local DMPK group, Södertälje, Sweden) for more than a decade to predict BBB permeability of compounds [19]. For this, it is likely that numerous compounds having the same characteristics as chropromazine (i.e. highly lipophilic, very low MB) have been screened in this assay. However, as a rule of control, bBCEC integrity and MB were and are always assessed prior the use of the obtained experimental data in permeability calculations. No permeability calculation was performed when the MB was having a value lower than 80 %, as a poor MB could be an indication of several factors e.g. intracellular accumulation of the compound, metabolization of the compound within the endothelium or a lack of accuracy of the used quantification method. Consequently, the resulting poor predictive value obtained by the *in vitro* BBB model compared to the *in vivo* permeability could not be identified.

It is only by categorizing compounds based on their $f_{u,br}$ that the observed discrepancy could be identified. This shows and confirms what was already published by several authors, that brain tissue binding is influencing the rate of brain penetration for highly lipophilic compound [6,10,11]. This suggests that the lack of correlation between *in vitro* and *in vivo* permeability values is not necessarily due to a poor *in vitro* permeability assay, but rather to the difference in experimental design. The traditional *in vitro* permeability assay lacks brain tissue and so, provides no possibility for compounds to bind to brain tissue. The contrary is true in case of the *in-situ* brain perfusion method, where compounds face the brain parenchyma after passing the BBB.

By making use of GC to mimic brain tissue binding *in vitro*, the method that is introduced here provides *in vitro* BBB permeability values that better correlate with *in vivo* permeability values. These results highlight the influence of brain tissue binding, which is sometimes neglected in similar studies and might therefore facilitate *in vitro* to *in vivo* extrapolation, as well as the integration of *in vitro* data into physiologically based pharmacokinetic models of CNS drug distribution.

The recent development of *in vitro* BBB models in which bBCEC are cultivated in close proximity with other brain cells such as brain pericytes [20] or neuronal progenitors [21] might also benefit of the introduction of the P_{vitro} calculation method, as the same cells (e.g. brain pericytes, neuronal progenitors, etc.) used to induce BBB characteristics could be used to mimic possible brain tissue binding. Finally, the calculation of P_{vitro} in presence and absence (i.e. uncoupling with non-specific binding) of the brain cells in these assays might facilitate the clarification of the influence of brain tissue binding on the distribution of different compounds across the BBB.

5. Acknowledgements

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6. Table Legends

Table 1 *– In vitro BBB permeability values for the 27 CNS drugs under study obtained by the traditional method (P_{app} and P_e). The permeability values are accompanied by their corresponding (average) mass balance value (MB). The MB was also measured in absence of bovine brain capillary endothelial cells (bBCEC or EC).*

Table 2 *– Physico-chemical characteristics: fraction unbound brain ($f_{u,br}$) and lipophilicity (LogP) and the in vivo blood-brain barrier (BBB) permeability (P_{vivo}) for the 27 CNS drugs under study determined by Summerfield et al. (2007)* [6].

Table 3 *– In vitro BBB permeability values obtained for the 27 CNS drugs under study by the new method to assess BBB permeability (P_{vitro}). Permeability values are given for measurements in presence of glial cells and consist of a corresponding (average) mass balance value (MB).*

Table 4 – In vitro BBB permeability values obtained for 3 CNS drugs (i.e. carbamazepine, diazepam and chlorpromazine) by the new method to assess BBB permeability in vitro (P_{vitro}). The P_{vitro} values are given for measurements in presence and absence of glial cells (GC) and consist of a corresponding (average) mass balance value (MB). The MB for the condition without bBCEC (or EC) is also given.

7. Figure Legends

Figure 1 – Relationship between in vivo brain permeability (P_{vivo}), obtained by brain perfusion in rodents, taken from Summerfield et al. (2007) [6]*, and in vitro BBB permeability, either depicted by the apparent permeability (P_{app}) (Graph A, C, E and G), or by the endothelial permeability (P_e) (Graph B, D, F and H), and this for compounds of decreasing $f_{u,br}$.*

Figure 2 – Differences in $f_{u,br}$ depending on the experimental design of the model: A) In-situ rat BBB model, B) In vitro bovine BBB model (consisting of bBCEC) without glial cells (GC) in the receiver compartment during the drug permeability experiment; and C) In vitro BBB model (consisting of bBCEC) with GC in the receiver compartment during the drug permeability experiment.

Figure 3 – Relationship between $f_{u,br}$ and A) P_{vivo}/P_{app}; and B) P_{vivo}/P_e. Regression analysis included all compounds (n = 27). P_{vivo} values used for the different ratios were taken from Summerfield et al. (2007) [6].

Figure 4 – A) General scheme of the in vitro BBB model from Dehouck et al. (1990) [15]*. Bovine brain capillary endothelial cells (bBCEC) are cultured on the upper side of a permeable support and primary rat glial cells (astrocytes, oligodendrocytes and microglia) are cultured at the bottom of the well. B) Relationship between in vivo brain permeability (P_{vivo}), taken from Summerfield et al. (2007)* [6]*, obtained by brain perfusion in rodents, and in vitro BBB permeability, obtained by the new in vitro method (P_{vitro}) for all 27 compounds under study.*

Figure 5 – Comparison of P_{vitro} in presence and absence of glial cells for 3 compounds (i.e. carbamazepine, diazepam and chlorpromazine) characterized by a different $f_{u,br}$.

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