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Evaluation of *in vitro* brain penetration: Optimized PAMPA and MDCKII-MDR1 assay comparison

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Abstract

Parallel artificial membrane permeability assay (PAMPA) is arising in ADMET screening as a powerful tool to determine the passive permeability of new potential chemical entities. In an attempt to set up a sensitive high throughput method to assess passive blood–brain barrier (BBB) penetration we focused our attention on the effect of solvent and the influence of phospholipids on the permeability in PAMPA. Moreover, the high throughput nature of the assay was maximized by decreasing the incubation time and performing the assay in a cassette mode. UPLC system coupled with a mass spectrometer enormously reduces the analytical time, contemporaneously increasing the sensitivity of the method.

P_{app} values obtained from PAMPA were compared to permeability values from MDCKII-MDR1 assay. Evaluation of the two *in vitro* models with *in vivo* data was performed to test the predicting capacity of the two methods. Their contemporary assessment was shown to be an helpful tool in understanding the prevalent mechanism of penetration through the BBB.

Keywords: BBB permeability screening; PAMPA; MDCKII-MDR1; High throughput screening; UPLC/MS

1. Introduction

The blood–brain barrier (BBB) is one of the key issues in the pharmaceutical industry since central nervous system (CNS) drugs must penetrate the barrier while drugs targeting peripheral tissues should be impaired in the passage. The BBB is a complex endothelium formed by capillary endothelium cells with tight junctions and is rich in active transporters that facilitate or impair the passage. Several methods have been recently explored for the prediction of *in vivo* results: computational methods, physical measurements such as log *P*/log *D* and cell culture systems (Garberg et al., 2005).

Among cell cultures, prediction with primary bovine brain endothelial cells gives the best scoring to the *in vivo* system (Gumbleton and Audus, 2001) but difficulties in establishing and maintaining primary culture, as well the tediousness of the method, make the assay unfeasible as a high throughput screening assay. Among cell lines, MDCKII-MDR1 are the most widely used and promising (Garberg et al., 2005) but, in spite of their cultivation time reduced to 3 days, the assay still results in a higher cost than the test with artificial membranes.

Parallel artificial membrane permeability assay (PAMPA) was originally reported with 10% (w/v) egg lecithin in dodecane (Kansy et al., 1996) but variation in the phospholipid composition have been studied (Sugano et al., 2001; Seo et al., 2006). A comparison of the three most used PAMPA models, HDM, DOPC and DS-PAMPA were recently carried out by Avdeef and Tsinman (2006) explaining permeability's differences reported in literature for some standards. Because of the nature of the assay, PAMPA was used mainly for the prediction of the gastrointestinal absorption (Kerns et al., 2004). Attempts to modify the monolayer to improve the prediction of BBB penetration were done using porcine polar brain lipids (Di et al., 2003). While phospholipid composition was studied in depth, solvent's influence was not sufficiently investigated to evaluate if the use of porcine brain lipid is significantly affecting the permeability of the model. Aim of the paper was to evaluate the effective influence of phospholipids in PAMPA, by changing various solvent conditions. P_{app} values obtained from PAMPA were compared to permeability values from MDCKII-MDR1 assay, evaluation of the two *in vitro* models with *in vivo* data was performed to

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test the predicting capacity of the two methods. Simultaneous evaluation was shown to be a helpful tool in understanding the prevalent mechanism of penetration through the BBB. A significant improvement of the throughput of PAMPA was reached by performing the assay in cassette mode and the analysis by UPLC/MS.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

All chemicals were purchased from Sigma–Aldrich (Italy), except for amprenavir (Toronto Research Chemicals Inc.), RP60180 (Rhone Poulenc) and haloperidol (RBI) and the in-house synthesized compounds: NiK-13509, NiK-15019, NiK-21273, NiK-19735, NiK-20906. PAMPA were conducted in phosphate buffer (PBS, 28 mM KH_2PO_4 and 41 mM Na_2HPO_4, pH 7.4) in Multiscreen Millipore^{TM} plate MAIPN45 and MSSACCEPTOR acceptor plate (Millipore Corporation, Bedford, MA, USA). Dodecane was purchased from Sigma–Aldrich, hexane from Merck, Dimethylsulfoxide (DMSO) from Riedel-de-Haën; all the solvents were reagent grade. Polar Brain Lipids (PBL) were acquired from AVANTI Polar Lipids Inc. (Alabaster, AL, USA) and phosphatidylcholine from Sigma–Aldrich.

The MDCKII-MDR1 cell line was obtained from The Netherlands Cancer Institute (Amsterdam, NL) at passage 20. Cell culture medium and supplies for MDCKII-MDR1 cells were obtained from GIBCO. Transport medium used for the permeability studies was Hank's balanced salt solution (8.1 μM Na_2HPO_4, 138 μM NaCl, 0.5 μM $MgCl_2$, 1.47 μM KH_2PO_4, 2.67 μM KCl, 0.9 μM $CaCl_2$, 5.6 μM glucose and 0.33 μM sodium pyruvate, pH 7.4). HTS-Transwell polycarbonate filter membrane inserts (0.7 cm^2 surface area, 0.4 μm pore size) used for the permeabilities studies were purchased from Costar (Cambridge, MA, USA).

2.2. Methods

2.2.1. PAMPA

Test solutions 500 μM were obtained by serial dilutions in PBS pH 7.4 from 10 mM stock solutions in DMSO (final concentration 5%). A test at lower concentration 50 μM (0.5% DMSO final concentration), was similarly performed on *cimetidine*, *propranolol* and *caffeine* without differences in the results. Acidic pH were not taken into account for the set up of the assay because the brain penetration occurs *in vivo* at neutral pH. Phospholipid membranes were constituted by adding upon the porous filter of each well 5 μL of PBL 20 mg/mL in a dodecane/hexane solution. Test solutions (200 μL) were added to each donor well, while the acceptor wells were filled with 270 μL of PBS.

Donor and acceptor plates were assembled and incubated at room temperature under gentle shaking (200 rpm) for 16 h (literature method; Di et al., 2003) or 2 h. After incubation the sandwich was disassembled and the solutions transferred in a 96-well plate for analysis.

Equilibrium solutions were obtained by adding 100 μL of donor solution to 135 μL of PBS.

2.2.2. MDCKII-MDR1 cell culture

The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 0.1 mg/mL streptomycin–penicillin 100 U/mL, in an atmosphere of 95% air and 5% CO_2 at 37 °C. Stock cells were passaged at 90% confluency by tripsinization. For transport studies, cells were seeded onto 12-well HTS-Transwell inserts at a cell density of 300,000 cells/cm^2 and were grown for 3 days in an atmosphere of 95% air and 5% CO_2 at 37 °C.

2.2.3. MDCKII-MDR1 assay

Stock solutions of drugs (20 mM) were prepared in 100% DMSO and then diluted to the final concentration of 10 μM, in Dulbecco's PBS. Drugs were tested in both directions, apical-to-basolateral (A → B) and basolateral-to-apical (B → A), in duplicate. The ratio BA/AB >2 indicates an efflux phenomena. Permeability studies were conducted at 37 °C in incubator for 60 min. The monolayer integrity was evaluated by measuring the TransEpithelial Electrical Resistance (TEER) by using the Millicell-ERS system (Millipore Corporation) and it was considered integer if the resistance was between 200 and 300 Ω cm^2. After the transport study the monolayer integrity was measured in each well by adding a 0.02 mg/mL solution of lucifer yellow (LY); the test was conducted at 37 °C for 60 min, and the fluorescence (RFU) was measured at 485/535 nm. The percent of LY passed across the cell monolayer into the basolateral solution was calculated using the equation (Watanabe et al., 1999):

$$\text{rejection}\,(\%) = 100 \times \left(\frac{\text{RFU}\,[\text{LY\_acceptor}]}{\text{RFU}\,[\text{LY\_starting\_solution}]} \right)$$

Wells with rejection over 5% were discarded.

2.2.4. PAMPA and MDCKII-MDR1 analysis

Samples were analysed in duplicate by an UPLC/MS system.

Chromatographic runs were performed on an Acquity BEH C18 2.1 mm × 50 mm column (1.7 μm particle) maintained at 35 °C, with a flow of 600 μL/min and with an injection volume of 5 μL. Mobile phases were: (A) 95% water, 5% ACN + 0.1% trifluoroacetic acid, (B) 5% water, 95% ACN + 0.1% trifluoroacetic acid. Initial mobile phase composition was 98% A and 2% B for PAMPA, changing to 100% B in 0.3 min. The system was maintained at 100% B up to 1 min followed by 0.5 min of reconditioning. For MDCKII-MDR1 the analytical run gradient was performed in 2 min and injection volume was 10 μL.

The acquisitions were performed in single ion recording (SIR) mode on a ZQ single quadrupole mass spectrometer (Waters), in positive ESI with the following conditions: capillary 3.25 kV, cone 20 V, source temperature 115 °C, desolvation temperature 350 °C. Calibration curve were performed on *cimetidine*, *caffeine* and *propranolol* to evaluate the linearity range. Peak area were considered linear up an intensity of 10^7 cps otherwise samples are diluted within the linearity range. LOQ for all the standards were between 500 nM and 1 μM.

2.2.5. Data analysis

Apparent permeability (P_{app}) for PAMPA, was calculated according to the following equation, obtained from the Sugano et al. (2001) equation with some modifications in order to obtain permeability values in nm/s, comparable with MDCKII-MDR1 assay permeability:

$$P_{app} = \left\{ (-C) \times \ln \left(1 - \frac{[\text{drug}]_{acceptor}}{[\text{drug}]_{equilibrium}} \right) \right\} \times 10^7$$

where

$$\frac{[\text{drug}]_{acceptor}}{[\text{drug}]_{equilibrium}} = \frac{\text{Peak area}_{acceptor}}{\text{Peak area}_{equilibrium}} \quad \text{and}$$

$$C\,(\text{cm/s}) = \left\{ \frac{V_D \times V_A}{(V_D + V_A) \times \text{area} \times \text{time}} \right\}$$

and V_D is the donor solution volume (cm^3), V_A the acceptor solution volume (cm^3), *area* the filter area (cm^2) and *time* is the incubation time (s). Drug concentration is estimated by using the peak area integration. This is acceptable because P_{app} is calculated as ratio of two area peaks from the same type of matrix. Goodness of the result is supported by an in-range mass balance value. Same considerations are valid for the MDCKII-MDR1 assay too.

The P_{app} in MDCKII-MDR1 assay was calculated according to the equation (Artursson and Karlsson, 1991):

$$P_{app} = \frac{J}{C_0} = \frac{V_r \times C_r}{A \times t \times C_0}$$

where J is the permeability flux, C_0 the donor concentration at t_0 evaluated by peak area integration, V_r the acceptor volume, C_r the acceptor concentration at time t evaluated by peak area integration and A is the membrane surface area.

Mass balance was calculated with the following equation.

$$\text{MB}\,(\%) = \frac{(C_{At} \times V_A) + (C_{Bt} \times V_B)}{C_0 \times V_D} \times 100$$

where C_{At} and C_{Bt} are the drug concentrations in the apical (A, 'donor' in PAMPA) and basolateral (B, 'acceptor' in PAMPA) chambers at time t, C_0 is the concentration of the donor solution (MDCKII-MDR1 assay) at time 0, or the equilibrium solution concentration (PAMPA), V_A and V_B the volumes of the apical and basolateral chambers and V_D is the volume of the donor solution (MDCKII-MDR1) at time 0, or the equilibrium solution volume (PAMPA). Mass balance >70% was accepted. A loss of material in solution could be due to aspecific absorption to the transwell walls or membrane uptake, metabolism or compounds degradation.

Data are presented as the average P_{app} (nm/s) ± S.D. A ratio of the B → A/A → B P_{app} values are calculated in the MDCKII-MDR1 assay. Involvement of a Pgp-mediated efflux mechanism is indicated if the B → A/A → B ratio is >2.

Data are expressed as mean of six replicates for each compound ± standard deviation.

2.2.6. Cassette PAMPA

A set of nine standards was chosen with various permeabilities and different pK_a's in order to have, in solution at pH 7.4, differently charged drugs. Four different mixtures were prepared with nine standards (*naloxone*, *cimetidine*, *sulfasalazine*, *verapamil*, *atenolol*, *scopolamine*, *naltrexone*, *deprenyl*, *indomethacin*) choosing, for each of the mixture, differently charged compounds with low and high permeability.

Initially, compounds mixtures at different concentrations (from 500 to 10 μM) were injected into the UPLC/MS to verify if the contemporary acquisition in SIR of six compounds could produce a loss of intensity in the signal. Signal loss was not more than 0.2% even at low concentration. With UPLC the analytical run time was reduced to 1.5 min.

3. Results and discussion

3.1. Studies on solvent variation in the phospholipid solutions

Influence of the solvent on the permeability of the PAMPA monolayer was tested on a set of 19 standards from available commercial drugs. Care was taken to ensure that these compounds had broadest structural diversity, differing in physical chemical properties and *in vivo* brain penetration.

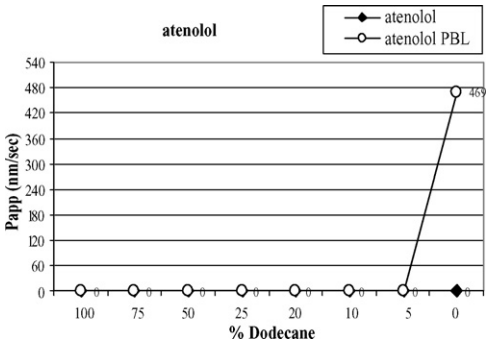
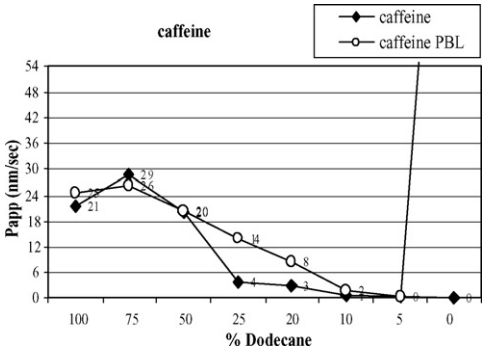
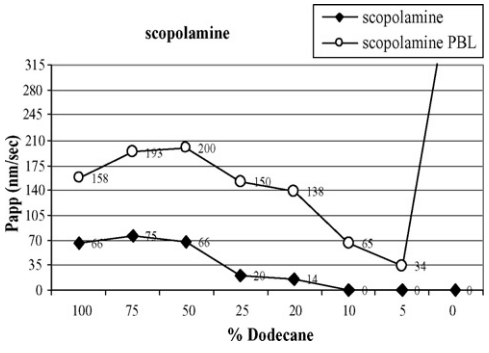
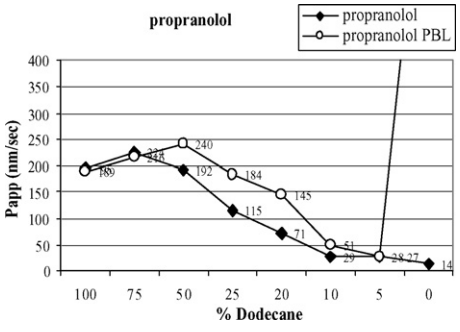
A permeability profile was built by plotting the permeability values obtained varying the percentage of dodecane in the monolayer constitution (Table 1). Percentage of dodecane in the PBL solution ranged from 100 to 5% as indicated in the charts of Table 1 while the concentration of the phospholipids in the preparation was maintained constant by the addition of *n*-hexane. After the deposition of the monolayer, hexane was evaporated leaving only phospholipids and dodecane. A second assay was performed for each standard adding the same percentage of dodecane but in absence of PBL to better understand the role of dodecane in the passive permeability assay. Compounds were divided into four categories by homology of their profiles. Results are shown in Table 1 with an exemplificative chart of one compound for each category.

For low, medium-low and high permeability compounds (first, second and fourth categories) differences in the permeability with or without the phospholipids were not significant. Profiles were almost overlapping for low and medium-low permeability compounds. Profiles with and without PBL, for medium-high permeability compounds (third category), have a similar pattern but P_{app} values with PBL are at least three times those obtained without phospholipids.

For all the standards belonging to the third category the ratio between P_{app} values with and without PBL was ranging from 2 (*quinidine*) to 30 (*amprenavir)*. For these compounds, permeability in presence of PBL, is influenced by the percentage of dodecane, increasing progressively with the decrease of dodecane concentration (till 50%).

From the above data we could hypothesize that, in the commonly used PAMPA (Di et al., 2003, 20 mg/mL of PBL in 100% dodecane) permeability is determined by dodecane itself and not by phospholipids, at least for compounds with either low

Table 1
The tested compounds could be grouped into four categories on the base of each permeability profile chart

Standards	pK_a	Permeability profile chart	Category and permeability
Atenolol	9.6[b]		First
Sulfasalazine	2.4[c]		
Indomethacin	4.5[a]		Low or null
Cimetidine	6.8[b]		
Antipyrine	1.4[c]		Second
Caffeine	1.5[c]		Medium-low
Scopolamine	7.5[c]		Third
Naltrexone	8.1[c]		
Naloxone	7.9[b]		
Quinidine	8.4[b]		Medium-high
Amprenavir	1.9[c]		
Nalbuphine	8.7[c]		
Verapamil	9.0[a]		
Propranolol	9.5[b]		Fourth
Desipramine	10.1[a]		
Fluoxetine	8.7[c]		
Haloperidol	8.6[a]		
Deprenyl	7.4[c]		High
Lidocaine	7.9[a]		

[a] From Avdeef and Tsinman (2006).
[b] From ACD pK_a DB.
[c] From others.

or high permeability even though P_{app} values with BPL were generally higher than those with dodecane only. This is in agreement with the results reported by Avdeef and Tsinman (2006) where the enhancement in permeability was attributed to an increase in the negative charges of the monolayer. Similarly, permeability values for all compounds increased enormously using PBL dissolved only in 100% hexane. This evidence strengthen the hypothesis that the "dodecane–porous membrane" system creates the primary permeability filter while phospholipids are able only to slightly influence the permeability of the assay. However, for borderline compounds (medium permeability) the dodecane–lipid ratio could determine the permeability value, shifting the compounds from CNS− (not able to cross the BBB) to CNS+ (able to cross the BBB). In particular the maximum differences among P_{app} values were measured with a PBL monolayer made in a 1:1 dodecane:hexane solution. In these conditions, the addition of phospholipids allows the correct classification of some standards, such as *amprenavir* and *nalbuphine*, as CNS+ compounds. These compounds are otherwise classified as CNS− in the standard conditions.

The influence of the phospholipid composition was studied comparing the permeability obtained in PAMPA with a monolayer formed by phosphatidylcholine (PC) (Di et al., 2003) 20 mg/mL in dodecane/hexane 1:1 to the one with PBL 20 mg/mL (data not shown). The only appreciable difference was found for *indomethacine* which showed a higher P_{app}, therefore classified as CNS+ using the first system, whereas with PBL was correctly classified as CNS−. Other permeability values obtained with PC were either equal or higher (third category compounds) than using PBL but with no changes in the classification of the compounds with respect to the brain penetration.

PAMPA results were evaluated comparing the permeability to the assay with MDCKII-MDR1 cells, a frequently used *in vitro* model for the prediction of brain penetration.

3.2. PAMPA versus MDCKII-MDR1

The set of 19 standards ('training set') previously used was assayed on MDCKII–MDR1 cells as described in Section 2. Results of both tests, PAMPA and MDCKII-MDR1 assays, are compared to literature data in Table 2.

A comparison of the permeability by plotting P_{app} values from both tests (Fig. 1) allows some considerations about the mechanism involved in the transport and therefore the forecast capacity of the assays.

As already reported by Kerns et al. (2004), in an analogous comparison among PAMPA and Caco2 assays, Fig. 1 could be divided into three areas. Compounds with an active absorption mechanism or with a paracellular flux (high MDCKII-MDR1 permeability versus low PAMPA permeability) are found in the 'A' area; standards with passive permeability are placed in the 'B' area (P_{app} similar for both assays). Compounds subjected to efflux phenomena (low MDCKII-MDR1 permeability versus high PAMPA permeability) are usually found in the 'C' area.

Since the correlation of the P_{app} values of the two assays is not always linear, the division of the graph in Fig. 1 allows to correct classify high permeable compounds which otherwise could be categorized incorrectly. The classification was obtained considering a wider passive area with respect to what reported by Kerns et al. (2004). Standards falling in the 'A' area of the graph are *antipyrine*, *caffeine* and *indomethacin*. *Antipyrine*, from literature data (Sakurada et al., 1978), shows an *in vivo* low brain permeability more in agreement with the PAMPA P_{app} value (70 nm/s) than the MDCKII-MDR1 P_{app} value (658 nm/s). A higher passage due to paracellular mechanism in the MDCKII-MDR1 assay, where tight-junctions are less close than those in brain, could be hypothesized. *Caffeine* is known to have an active transport and a paracellular transport (McCall et al.,

Table 2
'Training set' of 19 compounds used in the validation of PAMPA as described in Section 2

Compound	P_{app} MDCKII-MDR1 A → B	P_{app} MDCKII-MDR1 B → A	Ratio BA/AB	P_{app} PAMPA dodecane/hexane 1:1	CNS classification *in vivo*
Cimetidine	11 ± 2	39 ± 4	3.55	1 ± 1	−
Propranolol	379 ± 35	194 ± 25	0.51	307 ± 78	+
Verapamil	335 ± 45	323 ± 54	0.96	343 ± 71	+
Caffeine	482 ± 37	244 ± 25	0.51	30 ± 6	+
Nalbuphine	130 ± 15	150 ± 23	1.15	425 ± 74	+
Fluoxetine	179 ± 17	121 ± 15	0.68	382 ± 18	+
Haloperidol	323 ± 62	162 ± 37	0.5	547 ± 114	+
Lidocaine	716 ± 23	391 ± 14	0.55	638 ± 38	+
Atenolol	10 ± 5	8 ± 2	0.8	1 ± 1	−
Antipyrin	658 ± 7	478 ± 15	0.73	70 ± 9	+/−
Sulfasalazine	6 ± 1	3 ± 1	0.5	3 ± 1	−
Scopolamine	156 ± 34	151 ± 28	0.97	140 ± 56	+
Naltrexone	369 ± 59	278 ± 15	0.75	168 ± 34	+
Chlorpheniramine	303 ± 48	232 ± 87	0.77	602 ± 25	+
Naloxone	410 ± 18	169 ± 60	0.41	202 ± 15	+
Indomethacin	291 ± 5	160 ± 5	0.46	10 ± 10	−
Deprenyl	325 ± 142	249 ± 24	0.77	338 ± 73	+
Desipramine	342 ± 9	202 ± 47	0.59	345 ± 70	+
Quinidine	31 ± 3	354 ± 93	11.4	288 ± 77	−

Compounds were tested with MDCKII-MDR1 in both directions. Ratio BA/AB > 2 indicates an efflux phenomena. *In vivo* CNS classification is also reported. The permeability values are expressed in nm/s.

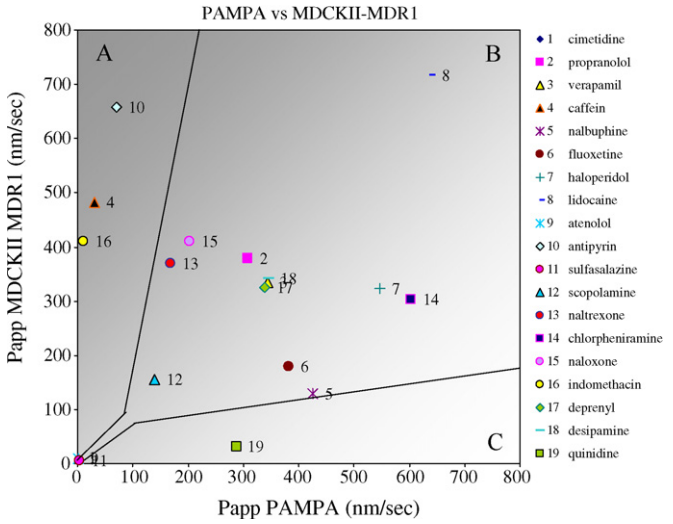


Fig. 1. Plot of PAMPA and MDCKII-MDR1 permeability values of the 'training set'. Compounds with active absorption mechanism or with a paracellular flux are found in the 'A' area; standards with passive permeability are placed in the 'B' area. Compounds subjected to efflux phenomena are found in the 'C' area.

1982) but low passive permeability into the brain. It is therefore correctly classified by both assays. *Indomethacin* penetrates peripheral tissues *in vivo* but it is excluded from brain (Gamache and Ellis, 1986); the PAMPA method correctly predicts the *in vivo* situation, contrary to the MDCKII-MDR1 P_{app} prediction. *Quinidine* was the only standard found in the 'C' area. This standard is known in the literature as a Pgp substrate confirmed by the high BA/AB ratio. The permeability values of the two assays permit the classification of the compounds in the correct area.

As expected, most of the standards were classified in the 'B' area of passive permeability. *Cimetidine*, *sulfasalazine* and *atenolol* have very low permeability in both assays and they are correctly predicted as *in vivo* CNS− compounds (Mahar Doan et al., 2002). *Propranolol* and *lidocaine* are correctly classified as CNS+ (Pardridge et al., 1983). *Verapamil* and *quinidine* have both a high passive permeability but *quinidine* is subjected to Pgp efflux (Polli et al., 2001) and in fact *verapamil* is found in the brain whereas *quinidine* is not. *Naloxone*, *naltrexone*, *desipramine*, *fluoxetine*, *chlorpheniramine*, *scopolamine*, *nalbuphine* and *deprenyl* (Mahar Doan et al., 2002) are correctly predicted by both methods as CNS positive compounds with a passive permeability area.

3.2.1. The validation set

An additional set of 15 compounds either CNS+ or CNS− was used as "validation set" and screened with both permeability assays to estimate the predictive capacity of the assays. Results are summarized in Table 3.

In the 'A' area of Fig. 2, we can find two in-house compounds; these are CNS positive compounds and are able to cross the BBB. *NiK-15019* and *NiK-13509* have a small enough MW to permit a paracellular route. *Astemizole* falls in the lower corner of 'A' area (PAMPA 17 nm/s, MDCKII-MDR1 50 nm/s) and it does not cross the BBB *in vivo* (Mahar Doan et al., 2002), PAMPA prediction is in this case more accurate than with MDCKII-MDR1.

In the 'B' area we find *buspirone*, *SCH23390*, *RP60180*, *methysergide* (Maurer et al., 2005; Iorio et al., 1983); correctly predicted as BBB high permeability compounds; *enoxacin* and *dopamine* correctly classified as CNS negative compounds.

Amprenavir, *domperidone* and *vinblastine* do not cross the BBB *in vivo*, because they are substrate for Pgp (Polli et al., 2001; Mahar Doan et al., 2002) and are correctly classified in the 'C' area. *NiK-19735*, *NiK-21273* and *NiK-20906* are *in vivo* CNS+ compounds, but just the first of them is correctly classified by MDCKII-MDR1, whereas PAMPA right classify the compounds as able to cross the BBB.

By plotting the P_{app} values of each tested standard compared to respective *in vivo* behaviour we can evaluate the predictive capacity of the PAMPA and MDCKII-MDR1 assay (Fig. 3).

On a set of 34 tested compounds we can affirm that the PAMPA test correctly predicts 26 compounds; MDCKII-MDR1

Table 3
Permeability values of the 15 compounds of the 'validation set' with MDCKII-MDR1 assay and PAMPA

Compound	P_{app} MDCKII-MDR1 A → B	P_{app} MDCKII-MDR1 B → A	Ratio BA/AB	P_{app} PAMPA dodecane/hexane 1:1	CNS classification *in vivo*
Vinblastine	16 ± 4	282 ± 42	17.63	170 ± 53	−
Amprenavir	18 ± 1	377 ± 28	20.9	110 ± 13	−
Astemizole	50 ± 21	108 ± 37	2.16	17 ± 12	−
Methysergide	200 ± 28	362 ± 19	1.81	372 ± 13	+
NiK-13509	357 ± 23	199 ± 14	0.56	7 ± 3	+
NiK-15019	276 ± 81	177 ± 16	0.64	10 ± 3	+
Dopamine	4 ± 1	1 ± 1	0.25	0 ± 0	−
Domperidone	5 ± 1	314 ± 51	62.8	233 ± 18	−
Enoxacin	23 ± 1	21 ± 1	0.91	20 ± 8	−
SCH23390	356 ± 31	289 ± 31	0.81	448 ± 75	+
RP60180	339 ± 18	247 ± 73	0.73	347 ± 70	+
Buspirone	689 ± 22	331 ± 14	0.48	538 ± 49	+
NiK-19735	75 ± 15	475 ± 101	6.3	237 ± 9	+
NiK-21273	27 ± 3	587 ± 33	21	333 ± 14	+
NiK-20906	6 ± 1	215 ± 27	35.8	291 ± 15	+

Compounds were tested with MDCKII-MDR1 in both directions. Ratio BA/AB > 2 indicates an efflux phenomena. The permeability values are expressed in nm/s.

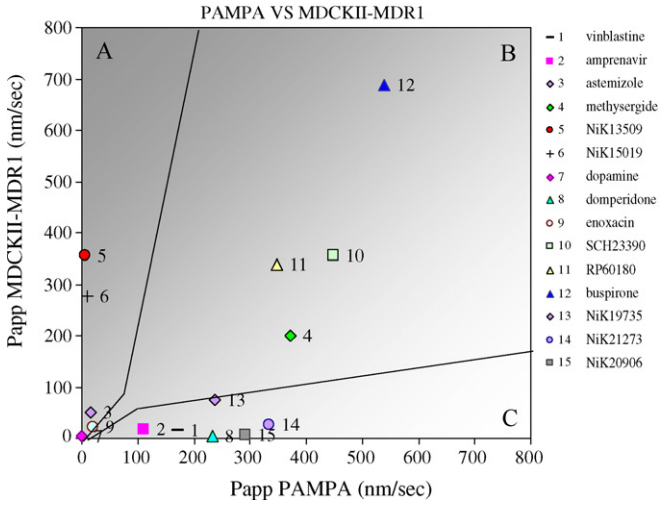


Fig. 2. Plot of PAMPA and MDCKII-MDR1 permeability values of the 'validation set'.

29 out of 34 whereas the combination of the two assays give a 100% prediction score.

3.3. PAMPA in high throughput

In order to maximize the throughput of the assay various approaches have been tested:

(1) Reduction in the incubation time. Incubation time with PBL previously reported was 18 h (Di et al., 2003) but its decrease has been set up for other lipidic monolayers down to 4 h (Balimane et al., 2005) and to 45 min using the individual well magnetic stirrers (Avdeef and Tsinman, 2006).
(2) Cassette incubation mode coupled with a cassette analysis.

Both approaches were set up using the modified monolayer PBL in dodecane:hexane 1:1.

3.3.1. Shorter incubation time

The incubation time was reduced from 16 to 2 h keeping the plate under constant agitation at 200 rpm to reduce the "unstirred water layer" and facilitate the passage of the drugs. P_{app} values determined using both conditions are reported in Fig. 4. Compounds are similarly classified; a general tendency to higher P_{app} values is noted for highly permeable compounds whereas no significant differences were reported for compounds with medium or low permeability. The reason of this difference in P_{app} values could be attributed to different concentration of the acceptor solution which is similar to the equilibrium for the 16 h experiment and is lower in the other assay. The P_{app} values obtain with the PAMPA 2 h are more comparable with the MDCKII-MDR1 values because same incubation time is applied and the experiment is more similar to an *in vivo* situation where is not reached an equilibrium steady-state. Since the results of the assay are usually ranked according to their P_{app} low permeability (P_{app} less than 20 nm/s), medium permeability (20 nm/s $< P_{app} <$ 60 nm/s) and high permeability compounds ($P_{app} >$ 60 nm/s) the differences reported shortening the period of incubation were not significant.

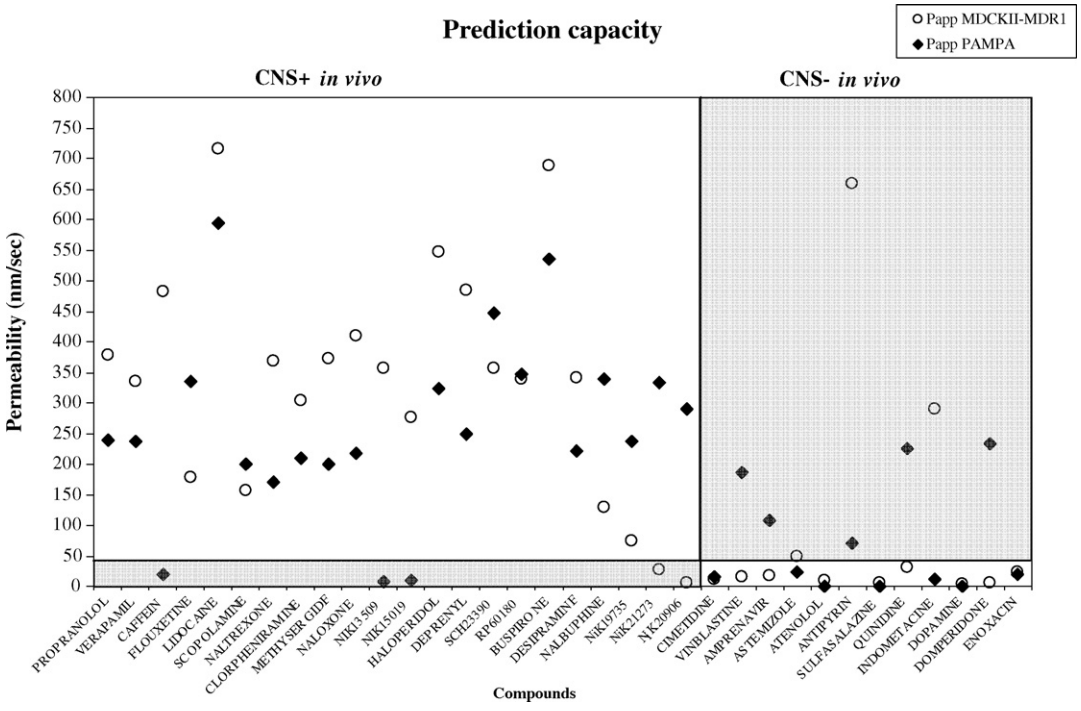


Fig. 3. Predictive capacity of PAMPA and MDCKII-MDR1 assay, evaluated on the tested compounds by comparison with *in vivo* brain penetration. The PAMPA P_{app} values are represented with a circle, while MDCK-MDR1 P_{app} values with a filled square. The chart is divided in two areas according to CNS+ and CNS− *in vivo* results. P_{app} = 40 nm/s is the filter used to classify the compounds as able or not able to cross the BBB in the *in vitro* experiments. Compounds in white background zone are correctly predicted whereas in the gray background zone are not.

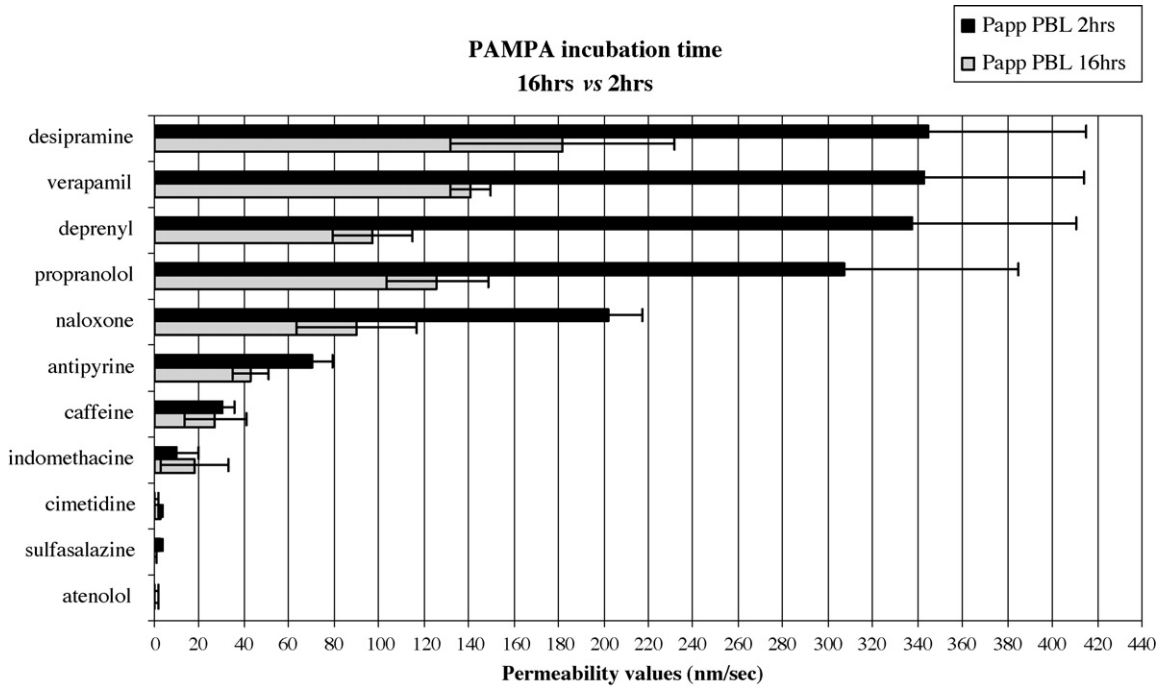


Fig. 4. Comparison of PAMPA permeability values obtained by incubating a set of standards either for 16 h or 2 h. Data are average of three replicates.

Table 4
PAMPA P_{app} values obtained either by single incubation or by multiple incubation performed dividing the standards in four set of six compounds each

Mixture number	Standards	P_{app} cassette incubation	P_{app} single incubation
1	Naloxone	203 ± 1	202 ± 15
	Cimetidine	3 ± 5	1 ± 1
	Sulfasalazine	3 ± 3	3 ± 1
	Verapamil	404 ± 20	343 ± 71
	Atenolol	8 ± 1	1 ± 1
	Scopolamine	108 ± 6	140 ± 56
2	Naltrexone	185 ± 10	168 ± 34
	Deprenyl	456 ± 69	338 ± 73
	Indomethacin	4 ± 1	10 ± 10
	Sulfasalazine	5 ± 1	3 ± 1
	Naloxone	167 ± 10	202 ± 15
	Scopolamine	108 ± 7	140 ± 56
3	Verapamil	283 ± 19	343 ± 71
	Indomethacin	3 ± 0	10 ± 10
	Cimetidine	0 ± 0	1 ± 1
	Deprenyl	384 ± 8	338 ± 73
	Sulfasalazine	4 ± 0	3 ± 1
	Naltrexone	181 ± 1	168 ± 34
4	Naltrexone	220 ± 38	168 ± 34
	Cimetidine	0 ± 0	1 ± 1
	Indomethacin	2 ± 1	10 ± 10
	Atenolol	0 ± 0	1 ± 1
	Scopolamine	126 ± 25	140 ± 56
	Deprenyl	306 ± 9	338 ± 73

The cassette permeability values are in agreement with those from the traditional 'single well–single compound' method.

3.3.2. Cassette incubation

PAMPA performed by incubation of three standards in a cassette was reported by Balimane et al. (2005) and we investigated the instrument suitability to further increase the number of standards in the incubation. In Table 4 are reported PAMPA P_{app} values obtained either by single incubation or by multiple incubation performed on set of six compounds each, chosen within a pool of nine standards. The cassette permeability values are in agreement with those from the traditional 'single well–single compound' method.

4. Conclusions

Studies on the monolayer constitution to improve the prediction capacities for brain penetration of PAMPA showed that permeability is mostly dependent on the percentage of dodecane and the effect of phospholipids is relevant only for compounds with a medium permeability value (50–100 nm/s). The choice of dodecane:hexane 1:1 allows to obtain the greatest differences between the permeabilities with and without PBL improving the classification of the standards with medium P_{app} values.

An attempt to set up a high throughput PAMPA for brain penetration was fulfilled by performing a cassette assay incubating for 2 h up to six drugs contemporarily. The analysis with UPLC/MS permits a further increase of the assay's speed, allowing us to obtain the P_{app} data for six compounds in 4.5 min (donor, acceptor and equilibrium wells) and contemporarily increasing the sensitivity with respect to the UV detection method.

Although PAMPA cannot be considered as a substitute for a cellular permeability assay, optimized PAMPA for brain penetration is an adequate model for high throughput screening permeability prediction, with a score on the tested standards of 77% versus 85% of MDCK-MDR1 model. Comparison of

the two set of data allows a better classification of the compounds and gives some insight into the transport mechanism involved in the passage through the blood–brain barrier giving a full prediction score (100%) based on the *in vivo* results.

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