Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Medicinal Chemistry 38 (2003) 223–232
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
www.elsevier.com/locate/ejmech

Original article

High throughput artificial membrane permeability assay for blood–brain barrier

Li Di [a,*], Edward H. Kerns [a], Kristi Fan [b], Oliver J. McConnell [a], Guy T. Carter [c]

[a] *Discovery Analytical Chemistry, Chemical Sciences, Wyeth Research, P.O. Box CN 8000, Princeton, NJ 08543-8000, USA*
[b] *Biological Chemistry, Wyeth Research, P.O. Box CN 8000, Princeton, NJ 08543-8000, USA*
[c] *Discovery Analytical Chemistry, Chemical Sciences, Wyeth Research, 401 North Middletown Road, Pearl River, NY 10965, USA*

Received 30 July 2002; received in revised form 12 December 2002; accepted 16 December 2002

Abstract

The recent advances in high throughput screening for biological activities and combinatorial chemistry have greatly expanded the number of drug candidates. Rapid screening for BBB penetration potential early in drug discovery programs provides important information for compound selection and guidance of synthesis for desirable CNS properties. In this paper, we discuss a modification of the parallel artificial membrane permeation assay (PAMPA) for the prediction of blood–brain barrier penetration (PAMPA-BBB). The assay was developed with 30 structurally diverse commercial drugs and validated with 14 Wyeth Research compounds. The PAMPA-BBB assay has the advantages of: predicting passive blood–brain barrier penetration with high success, high throughput, low cost, and reproducibility.

Keywords: Artificial membrane; Permeability; PAMPA; Blood–brain barrier; Brain penetration; CNS

1. Introduction

One major hurdle for successful CNS drugs is to penetrate the blood–brain barrier (BBB) and reach the therapeutic targets. On the other hand, for drugs acting in peripheral tissues, penetration through the BBB might cause unwanted side effects. In today's drug discovery research, screening for BBB penetration potential early in the program is of great importance [1–4]. The purpose of this study was to investigate and validate a predictive high throughput passive BBB assay for early drug discovery research.

The blood–brain barrier is made of brain endothelial cells with tight junctions (Fig. 1) [5,6]. Most CNS drugs enter the brain by transcellular passive diffusion, due to the tight junction structure and limited transport pathways. There are also two active processes in the BBB that influence penetration [7,8]: active influx transporters (e.g. amino acid, peptide), and active efflux transporters (e.g. P-glycoprotein, multi-drug resistant proteins). In addition, partial or complete metabolism of a compound will limit its penetration into the brain.

There are several in vitro methods and computational models that have been used in drug discovery to predict the BBB penetration potential of test compounds. In vitro methods include log P/log D [9], Δ log P [10], immobilized artificial membrane (IAM) [11], polar surface area (PSA) [12], linear free energy equations [13,14], surface tension [15,16], and membrane permeability across cell culture systems [17]. These methods are useful, but the reliability of prediction and resources required may be limiting. For example, we recently tested 30 compounds using the surface tension method and found that the BBB penetration of 70% of the compounds was predicted correctly. Making multiple measurements of log D, log P, HPLC retention time, or partition coefficients for each compound can be time consuming. Cell culture systems have the advantage that

Abbreviations: PAMPA, parallel artificial membrane permeation assay; BBB, blood–brain barrier; CNS, central nervous system; CNS+, high brain penetration; CNS−, low brain penetration; PML, pION membrane lipid (20 mg mL^{-1} phosphatidylcholine in dodecane); PBL, porcine polar brain lipid; P_e, effective permeability.

* Corresponding author.
E-mail address: dil@wyeth.com (L. Di).

0223-5234/03/$ - see front matter
doi:10.1016/S0223-5234(03)00012-6

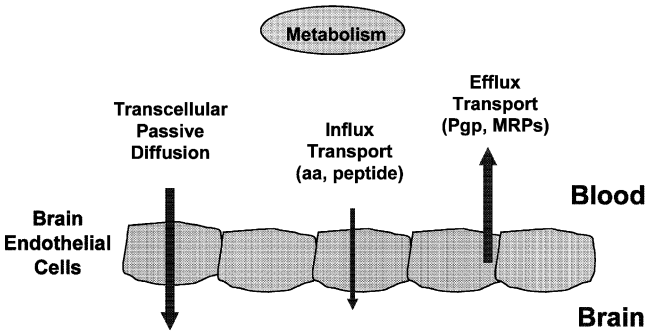

Fig. 1. BBB transport pathways.

they incorporate both passive transport and active transporters. However, their application, as a high-throughput screening tool, is limited by the elaborate membrane preparation and cost of resources. In silico methods have been described [18–20]. In our experience these have a similar throughput to in vitro methods, require significant computer time and the method is conformation dependent. The parallel artificial membrane permeability assay (PAMPA), first introduced by Kansy, et al. [21], has been widely used in the pharmaceutical industry as a high throughput permeability assay to predict oral absorption [3,22,23]. Here we describe the development of a modified PAMPA assay, using porcine brain lipids, to improve the prediction of BBB penetration. The assay was developed using 30 structurally diverse commercial drugs and was validated with 14 Wyeth Research compounds. Investigations of the assay conditions and the effect of the lipid composition (porcine brain lipid vs. lipid typically used in PAMPA studies for intestinal absorption prediction) on the specificity of BBB penetration are described.

2. Experimental

2.1. Materials

Thirty commercial drugs for method development were obtained from Aldrich (Milwaukee, WI), Sigma (St. Louis, MO) and Fluka (Ronkonkoma, NY). Fourteen compounds for method validation were obtained from Wyeth Research (Princeton, NJ). The porcine polar brain lipid (PBL) (catalog no. 141101) was from Avanti Polar Lipids, Inc. (Alabaster, AL). The composition is shown in Table 1. Universal buffer and pION membrane lipid (PML, 20 mg mL^{-1} phosphatidylcholine in dodecane) was obtained from pION Inc. (Woburn, MA). DMSO was reagent grade from Aldrich. Dodecane was from EM Science (Gibbstown, NJ). The acceptor plate was a 96-well filter plate (Multiscreen®, catalog no. MAIPN4550, PVDF membrane, pore size is 0.45 μM) from Millipore (Bedford, MA). The donor plate was an indented 96-well plate from pION. The 96 well UV plate (COSTAR®) was from pION. The 96 well, V-bottom, sample plate (COSTAR®) was from VWR (So. Plainfield, NJ). The 2-mL 96 deep-well plate (MATRIX) for dilution was from Matrix (Hudson, NH).

Table 1
Porcine polar brain lipid (PBL) composition (from Avanti Polar Lipids, Inc.)

Component	% Weight
Phosphatidylethanolamine	33.1
Phosphatidylserine	18.5
Phosphatidylcholine	12.6
Phosphatidic acid	0.8
Phosphatidylinositol	4.1
Other [a]	30.9

[a] Other = cerebrosides, sulfatides, pigments.

2.2. Instrumentation

A PSR4 instrument from pION was used for liquid handling, instrument control, and calculations. The instrument included a four-probe liquid handling robot (Tecan Genesis model RSP100, Durham, NC) and a 96-well UV plate reader (Molecular Devices, model Spectra Max 190, Sunnyvale, CA).

2.3. PAMPA-BBB procedure

Test compounds were dissolved in DMSO at 5 mg mL^{-1}. Ten microlitres of this compound stock solution were diluted 200-fold in universal buffer at pH 7.4 and mixed by the robot to make secondary stock solution (final concentration 25 μg mL^{-1}). Two hundred microlitres of the secondary stock solution were added to the donor wells. The filter membrane was coated with PBL in dodecane (selected empirically as 4 μL volume of 20 mg mL^{-1} PBL in dodecane) and the acceptor well was filled with 200 μL of pH 7.4 buffer. The acceptor filter plate was carefully put on the donor plate to form a 'sandwich' (consisting of the aqueous donor with test compound on the bottom, artificial lipid membrane in the middle, and aqueous acceptor on the top). The test compound diffused from the donor well through the lipid membrane and into the acceptor well. The 'sandwich' was left undisturbed for 18 hrs while the permeation occurred. The concentration of drug in the acceptor, the donor, and the reference wells was determined using the UV plate reader. Effective permeability (P_e) of the compounds was calculated by using the pION PSR4p software.

When the method is applied for drug discovery research, samples are analyzed in triplicate and the average of the three runs is reported. Quality control standards are run with each sample set to monitor the

consistency of the analysis set. Verapamil is used as high permeability standard ($P_e = 16 \times 10^{-6}$ cm s^{-1}) and theophylline is used as low permeability standard ($P_e = 0.12 \times 10^{-6}$ cm s^{-1}).

2.4. Method development

2.4.1. Selection of method development compounds

A set of structurally diverse compounds was selected for method development (Table 2). They were 30 commercial drugs [24]. Fourteen of the compounds were classified in the literature as 'CNS+' (high brain penetration) and 16 of the compounds were classified as 'CNS−' (low brain penetration). These compounds were shown by principal component analysis (PCA) with structural descriptors to represent a broad chemical space. A literature classification may result from one of several approaches: (a) kinetic method that measures the rate by which the drugs enter the brain [25]; (b) blood–brain distribution under steady state [10]; (c) pharmacological activity that is directly linked to brain penetration; or (d) total brain to plasma in vivo ratio (B/P) calculated from area under the curve. The method by which the compounds were classified as CNS+ or CNS− is listed in Table 2 along with the reference. Selection of this set of compounds for method development assumes that these different methods classify compounds the same. This has not been proven, but for method validation a separate set of compounds with one method (in vivo B/P) of classification was used.

The predicted 'log BB' from an in silico method [19] is shown in Table 2. Conformational analysis was performed on all 30 compounds. The lowest energy conformer of the solutes were generated by carrying out Monte Carlo search using MMFF94 force field with GB/SA continuum solvation model. Full geometry optimization was then performed using AM1 Hamiltonian in the gas phase. The solvation free energies in

Table 2
PAMPA-BBB results for 30 commercial drugs used in method development

Compounds	Literature CNS penetration classification [a] [24]	In silico prediction of BBB permeation log BB [19]	PAMPA-BBB permeability P_e (10^{-6} cm s^{-1})	PAMPA-BBB assay classification [b]
Alprazolam	CNS+(P/B, A [26])	−0.077	5.4	CNS+
Caffeine	CNS+(P/B [27])	0.21	1.3	CNS−
Chlorpromazine	CNS+(C [28])	0.47	6.5	CNS+
Clobazam	CNS+(P/B, A [26])	0.22	17	CNS+
Clonidine	CNS+(C [29])	0.092	5.3	CNS+
Desipramine	CNS+(C [28])	0.33	12	CNS+
Diazepam	CNS+(P/B, D [26])	0.18	16	CNS+
beta-Estradiol	CNS+(P/B, A, S [30])	0.037	12	CNS+
Imipramine	CNS+(C [28])	0.53	13	CNS+
Oxazepam	CNS+(P/B, A [26])	−0.17	10	CNS+
Progesterone	CNS+(P/B, A, S [30])	−0.17	9.3	CNS+
Promazine	CNS+(C [28])	0.43	8.8	CNS+
Testosterone	CNS+(P/B, A, S [30])	0.26	17	CNS+
Thiopental	CNS+(C [28])	0.60	18	CNS+
Aldosterone	CNS−(P/B, A, S [30])	0.24	1.2	CNS−
Astemizole	CNS−(C [31])	– [c]	11	CNS+
Atenolol	CNS−(C [32])	−0.25	0.8	CNS−
Hydrocortisone	CNS−(P/B, A, S [30])	– [c]	1.9	CNS−
Dopamine	CNS−(C [33])	−0.15	0.2	CNS−
Enoxacin	CNS−(D [34])	−0.15	0.9	CNS−
Isoxicam	CNS−(P/B, A, S [35])	−0.21	0.3	CNS−
Lomefloxacin	CNS−(D [34])	−0.11	1.1	CNS−
Loperamide	CNS−(D [36])	– [c]	0.0	CNS−
Corticosterone	CNS−(P/B, A, S [30])	0.68	5.1	CNS+
Norfloxacin	CNS−(D [34])	−0.49	0.1	CNS−
Ofloxacin	CNS−(D [34])	−0.45	0.8	CNS−
Piroxicam	CNS−(P/B, A, S [35])	−0.23	2.5	CNS+/−
Terfenadine	CNS−(C [37])	– [c]	0.0	CNS−
Tenoxicam	CNS−(P/B, A, S[35])	−0.50	0.1	CNS−
Cimetidine	CNS−(D [38])	−0.41	0.0	CNS−

[a] Method of classification in the literature and reference are indicated in parentheses. Method of CNS penetration: P, perfusion; D, distribution; C, comment in literature with unspecified method. Solution used for perfusion experiments: B, buffer; A, various plasma proteins (such as HSA, BSA, AAG); S, serum.

[b] See Section 2.6 for a description of the PAMPA-BBB classification ranges.

[c] Data is not available due to uncertainty involved in the calculation.

water were computed using the polarized continuum model of Tomasi et al. [46,47] at HF/321G* level. Finally, the log BB was computed using Eq. (1) in Ref. [19]. The quality of the calculated ΔG value could be improved with the increase of basis set to HF/6-31G**, but the computational time would have increased substantially. The approach of reference [19] uses AM1-SM2.1 in the AMSOL 5.0 program to calculate free energy of solvation, while we used PCM model [46,47] in Gaussian, because we don't have direct access to the AMSOL program. Our current ab initio approach with PCM model should be a good alternative to AM1-SM2.1 model to evaluate the classification model reported here.

2.4.2. Evaluation of PBL for PAMPA-BBB

PAMPA was selected to investigate as a high-throughput assay for BBB permeation prediction. Preliminary experiments indicated that the PML, used for PAMPA to predict gastrointestinal permeation, did not provide a strong differentiation between compounds with high and low BBB permeation. Thus, PBL was investigated as an artificial membrane for predicting BBB permeation. PBL was prepared at the same concentration (20 mg mL^{-1}) in dodecane and was applied to the filter membrane in the same volume (4 μL) as for the standard PAMPA method. Thirty commercial drugs (above), with BBB permeation properties reported in the literature, were tested with PBL.

2.4.3. Study of the effect of lipid composition for PAMPA-BBB

The effects on the prediction of BBB permeation of: (a) variation in the volume of lipid applied to the filter matrix; and (b) variation in PBL concentration in dodecane, were investigated. Experiments were designed using the statistical software MODDE™ from Umetrics (Kinnelon, NJ), in order to observe the maximum effect with the minimum experiments that were necessary. The design type was CCF, with the volume varied from 4 to 20 μL and the concentration of the PBL varied from 0 to 100 mg mL^{-1}. Eleven experiments were generated by the software with three center points at 9 μL and 22 mg mL^{-1}. The center points were created to monitor the reproducibility of the assay. The experiments were conducted the same way as described in Section 2.3.

2.4.4. Comparison of PBL and PML for PAMPA-BBB

After optimum PBL conditions were determined, the predictivity of the method using PBL and PML was investigated. The set of 30 commercial drugs was run using each method and was compared. In addition, 33 Wyeth Research compounds having known CNS penetration were run with the two methods. Finally, data was extracted from the corporate database for 2286 compounds that had been run using the regular PAMPA method (using PML) and the PAMPA-BBB method (using PBL).

2.5. Validation

2.5.1. Selection of validation compounds

In-house (Wyeth Research) compounds used for method validation were compounds for which a brain/plasma ratio had been determined in rats as part of ongoing discovery research projects or from pharmacological responses. For in vivo brain penetration study, test compounds had been dosed p.o., i.p. or i.v. and blood and brain were taken at selected time points. The blood was purged from the brain with saline using the beating heart, and the whole brain tissue was homogenized. The samples were extracted with acetonitrile and assayed by LC-MS-MS. The brain/plasma ratio of test compounds was calculated based on AUC_{brain}/AUC_{plasma}. These compounds were demonstrated by PCA of structural descriptors to represent a broad chemical space that is consistent with the method development compounds.

2.5.2. Validation experiment

Validation with 14 in-house compounds used the final PAMPA-BBB procedure in Section 2.3. The compounds were classified as in Section 2.6 and compared to classification from the in vivo B/P measurements described in Section 2.5.1 or pharmacological responses.

2.6. Classification of compounds based on PAMPA-BBB Results

Following the pattern established in the literature for BBB permeation prediction [24], it is useful to classify compounds according to ranges to assist in communicating the data to collaborators in drug discovery. Here are ranges that have been useful in our laboratory:

a) 'CNS+' (high BBB permeation predicted); P_e (10^{-6} cm s^{-1}) >4.0
b) 'CNS−' (low BBB permeation predicted); P_e (10^{-6} cm s^{-1}) <2.0
c) 'CNS+/−' (BBB permeation uncertain); P_e (10^{-6} cm s^{-1}) from 4.0 to 2.0

These ranges were derived empirically from experiments in Section 2.4.2 by correlation of previously reported BBB permeation ranges [24] with observed PAMPA-BBB P_e values. (Some researchers may prefer to set ranges which meet their own criteria and others prefer to work only with the data. As with any classification of compounds using high-throughput pharmaceutical profiling screens, it is unwise to terminate the study of a compound based on PAMPA-BBB classification alone.)

3. Results and discussion

3.1. Selection of PBL for PAMPA-BBB

Selection of PBL for the PAMPA-BBB method was based on two reasons. First the lipid is commercially available from Avanti Polar Lipids, Inc. for a consistent supply. Second, a lipid material obtained from brain has a higher likelihood of approximating the lipid composition of brain endothelial cells of the BBB. The composition of brain endothelial cells membranes has been reported [39,40]. Due to the complexity of the endothelial lipid composition and changes with age, species and other factors, it is hard to recreate exactly the same membrane material. PBL used in these studies contains the major phospholipid components in approximately the same ratios as in vivo. A high throughput PAMPA assay will never be an exact mimic of the brain endothelial cell membrane, because of the dodecane diluent and the non-bilayer lipid structure of the PAMPA artificial membrane. The purpose was to come up with a lipid formulation that is practical, reproducible, and provides reliable predictions.

3.2. Evaluation of PBL for PAMPA-BBB

The permeability (P_e) of test compounds was determined at pH 7.4 in the PAMPA-BBB assay. For accurate prediction of oral absorption, permeability in the PAMPA assay is typically measured at several different pH values to mimic the physiological pH of the GI track [21–23]. Only pH 7.4 is necessary for PAMPA-BBB assay, due to the constant pH in the blood compartment.

The results for the 30 commercial drugs using PBL with the PAMPA assay are shown in Table 2 and Fig. 2. The P_e values for most of the compounds, previously reported in the literature as CNS+ and CNS−, were separated into two P_e ranges. CNS+ compounds tended to have a P_e value of greater than 4.0×10^{-6} cm s^{-1} and CNS− compounds tended to have a P_e value of less than 2×10^{-6} cm/ s^{-1}. The separation furthermore suggests that the assay with PBL was able to capture fundamental properties of BBB permeation. A close examination revealed that the outliers, which did not fit within these ranges, are known to be affected by active transport processes (influx, efflux) or by metabolism. The PAMPA-BBB membrane is a physicochemical barrier. Drugs that are actively transported are likely to be mis-classified. The only false negative outlier, caffeine, is known to enter the brain by both passive diffusion and carrier mediated transport [27]. PAMPA-BBB underestimated the in vivo permeability of caffeine, because of the active influx mechanism. The four false positive outliers are corticosterone, terfenadine, loperamide and astemizole. Corticosterone and loperamide

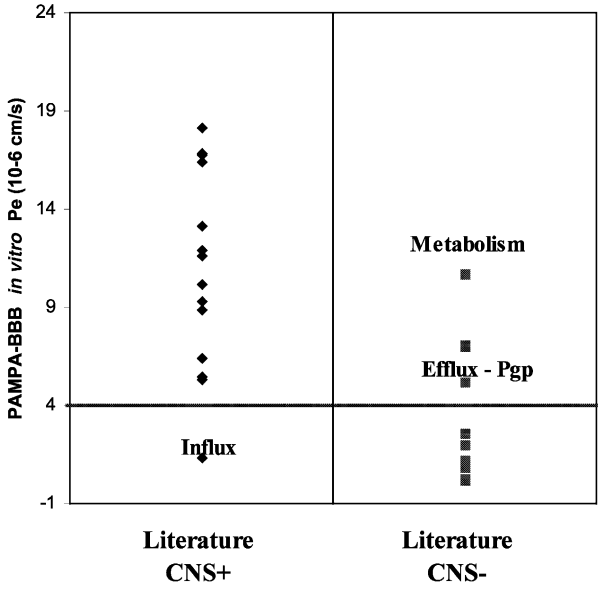

Fig. 2. Results for evaluation of PBL for PAMPA-BBB using 30 method development compounds (see Section 3.2). Of the 30 compounds, 14 were previously classified in the literature as CNS+, 16 were previously classified in the literature as CNS− (some compounds overlap in the diagram). Compounds were mis-classified if they experience significant active processes (caffeine has been reported to be transported into the brain both by both passive diffusion and active transport; corticosterone and loperamide have been reported to be effluxed from the brain by P-gp; astemizole is known to be metabolized rapidly during systemic circulation; terfenadine is a Pgp substrate and is metabolized rapidly).

are Pgp substrates [41,42]. PAMPA-BBB overestimated the in vivo brain penetration of these compounds, because Pgp efflux in vivo opposes BBB penetration by pumping the drugs out of the brain. Astemizole is metabolically unstable [43]. It metabolizes rapidly during systemic circulation, before it has a chance to enter the brain. Terfenadine is a Pgp substrate and is metabolically unstable. PAMPA-BBB overestimated the permeability of terfenadine, due to active processes [44,45]. For passive diffusion, the assay predicted the test compounds correctly. The results for outliers were all affected by active processes. The results also corresponded to the groupings of the in silico calculations.

3.3. Study of the effect of lipid composition for PAMPA-BBB

To test the effect of lipid composition, 11 experiments were conducted with different lipid concentrations and volumes for the 30 commercial drugs. The results are shown in Table 3 and Fig. 3. Compounds that are actively transported were excluded from the graph.

The experiment at a concentration of 22 mg mL^{-1} and a volume of 4 μL gives the optimal separation for the CNS+ and CNS− compounds. A concentration of

20 mg mL^{-1} was used in subsequent experiments for the sake of simplicity.

3.4. Comparison of PBL and PML for PAMPA-BBB

A comparison was made for the use of PBL and PML (20 mg mL^{-1} phosphatidylcholine in dodecane) for the 30 commercial drugs. The results are shown in Fig. 4. For most compounds, the trends are similar. However, the assay using PML underestimated the BBB permeability of three CNS+ compounds (Alprazolam, Clonidine and Oxazepam). The comparison for the 33 Wyeth Research compounds is shown in Fig. 5. The PML gave 9 false negative predictions, while the PBL gave only 2 false negative predictions. The PML lipid composition underestimated the BBB penetration of the CNS+ compounds.

A comparison of the PAMPA and PAMPA-BBB results for 2286 compounds is shown in Fig. 6. The results indicate that the two membrane composition have unique selectivity.

3.5. Validation

The results for the 14 Wyeth validation compounds are shown in Table 4 and Fig. 7. The assay predicted all the compounds correctly. This also suggests that the compounds were transported primarily by passive diffusion.

3.6. Application of PAMPA-BBB assay in drug discovery

The PAMPA-BBB assay is being used routinely at Wyeth Research to support discovery projects. Project

Table 3
Effect of lipid composition on CNS classification

Assay	1	2	3	4	5	6	7	8	9	10	11
Run order	11	4	5	1	9	3	6	10	2	7	8
Volume (μL)	4	4	9	4	20	9	9	9	4	9	20
Conc. (mg/mL)	0	5	5	22	5	22	22	22	100	100	100
Total brain lipid (μg)	0	20	45	88	100	198	198	198	400	900	2000
Compounds	Permeability (10^{-6} cm s^{-1})										
Alprazolam	4.4	4.5	5.3	7.9	5.7	7.6	7.7	7.6	11.6	12.5	18.2
Caffeine	1.4	1.1	1.4	1.5	1.6	1.5	1.7	1.9	1.6	2.1	2.7
Chlorpromazine	6.1	4.9	2.9	6.1	2.9	4.7	5.1	5.1	3.6	7.2	23.1
Clobazam	17.8	19.5	17.8	20.8	15.6	15.2	18.1	14.8	13.3	17.9	18.4
Clonidine	2.6	3.5	2.8	8.5	4.4	6.9	8.2	8.4	11.8	10.3	eq
Desipramine	16.2	15.9	11.7	15.3	10.2	7.5	9.9	8.8	6.0	12.0	eq
Diazepam	19.3	19.0	13.5	13.3	11.6	15.2	13.5	9.7	13.3	16.5	17.2
beta-Estradiol	22.9	19.2	16.3	18.4	24.0	16.5	15.0	15.0	18.3	15.0	15.0
Imipramine	10.4	11.6	10.8	12.2	10.2	8.7	9.2	7.3	6.5	15.9	eq
Oxazepam	1.8	5.8	2.8	8.6	6.5	7.6	eq	eq	9.6	23.0	eq
Progesterone	12.9	10.3	6.8	10.8	3.0	6.1	5.5	3.7	8.4	8.0	17.9
Promazine	11.6	8.5	4.5	8.2	3.6	2.8	3.7	5.1	3.9	5.3	6.2
Testosterone	16.6	18.4	18.7	17.4	15.3	14.7	10.8	10.6	14.6	13.1	11.8
Thiopental	20.1	17.3	17.4	16.3	13.2	12.9	13.1	13.5	13.0	17.8	19.4
Aldosterone	0.1	0.1	0.1	1.3	0.2	1.2	1.5	1.7	3.8	4.6	5.1
Astemzole	14.4	13.4	10.4	11.7	8.3	6.9	10.9	8.9	8.2	13.1	22.3
Atenolol	0.8	0.5	0.9	0.4	2.0	1.4	2.3	2.9	1.8	2.8	5.7
Hydrocortisone	0.0	0.7	0.1	2.0	0.4	2.2	2.4	2.5	4.3	5.4	6.5
Dopamine	0.0	0.1	0.1	0.1	0.1	0.2	0.3	0.5	0.1	0.2	1.7
Enoxacin	0.1	0.1	0.1	1.0	0.2	1.0	1.2	1.8	1.6	1.8	1.8
Isoxicam	0.1	0.1	0.1	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.6
Lomefloxacin	0.1	0.0	0.0	1.1	0.2	1.1	1.1	1.3	1.9	2.7	2.7
Loperamide	9.3	11.0	3.4	eq	9.3	7.8	11.9	24.0	7.0	16.3	eq
Corticosterone	2.8	2.7	3.0	6.9	3.5	7.6	7.6	8.1	14.2	13.4	16.3
Norfloxacin	0.0	0.4	0.1	0.2	0.2	0.2	0.3	0.4	0.3	0.3	0.4
Ofloxacin	0.1	0.1	0.1	0.7	0.2	0.7	1.0	1.0	1.9	2.6	2.4
Piroxicam	1.4	1.3	1.3	1.3	1.2	1.3	1.3	1.4	1.2	1.3	1.3
Terfenadine	5.4	5.6	7.6	8.2	13.1	10.6	13.6	eq	7.2	12.5	eq
Tenoxicam	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1
Cimetidine	1.6	1.5	0.9	2.1	5.0	3.6	5.5	6.4	3.2	11.7	eq

The data in this table are plotted in Fig. 3 and are presented here for reference purposes.

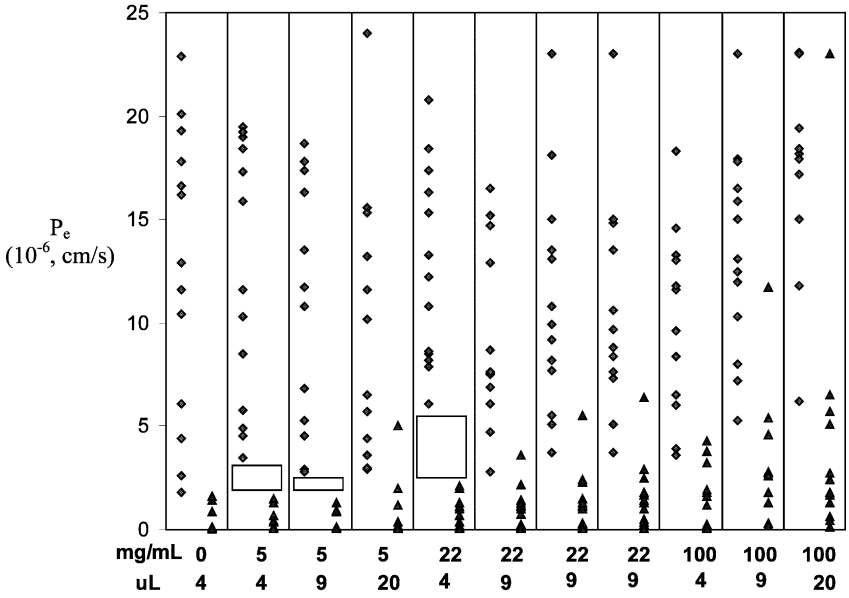

Fig. 3. Plot of the data from Table 3 indicating the effect of lipid composition on CNS classification. Eleven experiments were performed with varying lipid composition. For each experiment the CNS+ compounds are on the left (diamond-shaped) and the CNS− compounds are on the right (triangle-shaped). Compounds affected by active processes were not considered. The goal was to find conditions under which the CNS+ and CNS− compounds separated into defined classes. The conditions of the 22 mg mL^{-1} and 4 μL volume provided the best separation of compounds.

teams use the assay in conjunction with in vitro potency and structure diversity to prioritize compounds for in vivo animal studies. The assay also is being used for diagnostic purposes to study transport mechanisms. The current screening paradigm is that a front-end high throughput, low cost, PAMPA-BBB assay gives important information about passive diffusion through BBB. A lower throughput Pgp assay, using the Caco-2 model, gives efflux mechanism information for discovery project compounds. The combination of these two assays gives a more complete understanding of properties of discovery compounds that may affect their BBB penetration. In vivo data continues to be important to support and validate in vitro results, especially for new

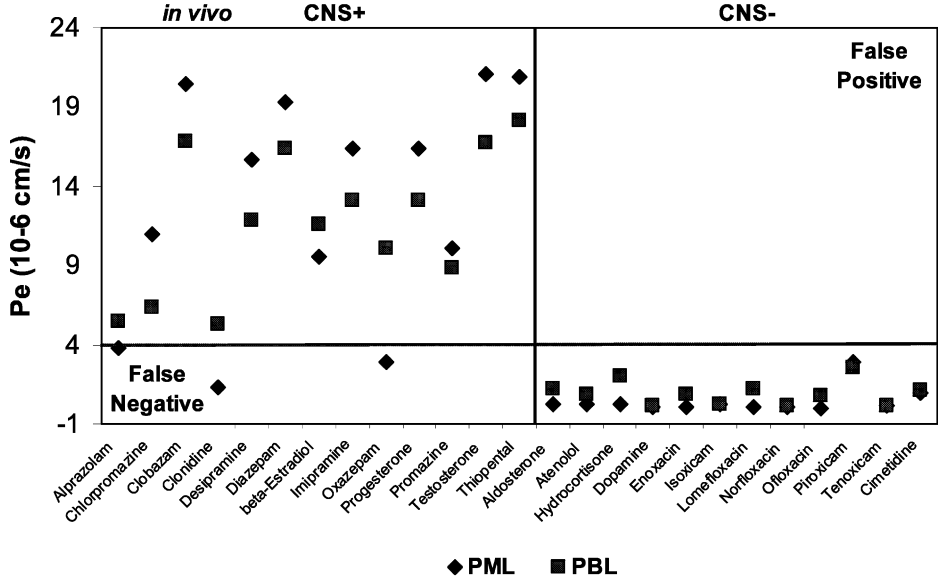

Fig. 4. Comparison of PAMPA with PBL and PML for 30 commercial drugs (excluding the actively transported compounds).

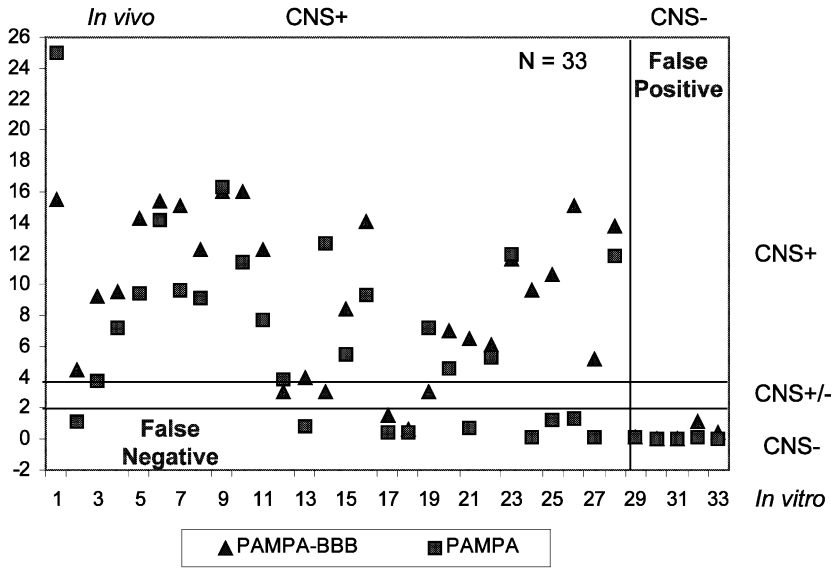

Fig. 5. Comparison of PAMPA-BBB with PBL and PAMPA with PML for 33 Wyeth compounds.

chemical series and new projects. In vivo data is also useful to help diagnose active processes and support late-stage discovery projects.

4. Conclusions

A PAMPA-BBB assay has been successfully developed. The assay predicts passive diffusion through the BBB with high success. The assay is high throughput, low cost, reproducible and consumes very small amount of sample (<0.5 mg).

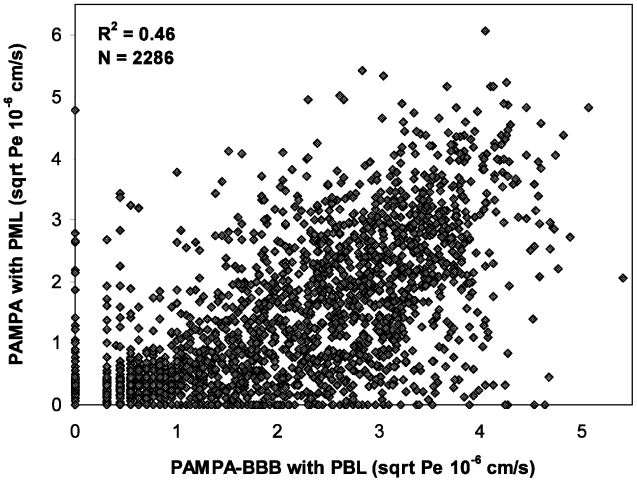

Fig. 6. Comparison of PAMPA-BBB with PBL and PAMPA with PML results for compounds from on-going Wyeth Research programs.

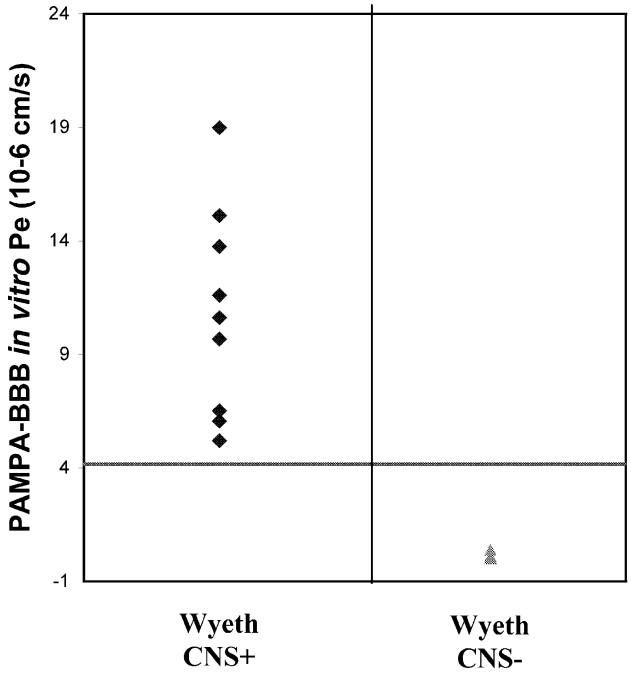

Fig. 7. Results for set of 14 validation compounds with PAMPA-BBB (see Section 3.4). Of these compounds: nine were CNS+ and five were CNS− from in vivo B/P studies and pharmacological responses. Some of the compounds overlap in the diagram.

Acknowledgements

The authors wish to thank Magid Abou-Gharbia for his leadership and support. Thanks to John Butera, Ron Bernotas and Terry Andree for providing sample information and support. Thanks to Susan Petusky,

Table 4
PAMPA-BBB results for 14 Wyeth Research compounds used for validation

Compounds	In-house in vivo studies classification	BBB permeability P_e (10^{-6} cm s^{-1})	PAMPA-BBB assay classification [a]
Wyeth 1	CNS+	6.5	CNS+
Wyeth 2	CNS+	6.1	CNS+
Wyeth 3	CNS+	19	CNS+
Wyeth 4	CNS+	12	CNS+
Wyeth 5	CNS+	9.7	CNS+
Wyeth 6	CNS+	11	CNS+
Wyeth 7	CNS+	15	CNS+
Wyeth 8	CNS+	5.2	CNS+
Wyeth 9	CNS+	14	CNS+
Wyeth 10	CNS−	0.4	CNS−
Wyeth 11	CNS−	0.1	CNS−
Wyeth 12	CNS−	0.1	CNS−
Wyeth 13	CNS−	0.0	CNS−
Wyeth 14	CNS−	0.1	CNS−

[a] See Section 2.6 for a description of the PAMPA-BBB classification ranges.

Mark Tischler, Chantel Sabus, Larry Mallis for applying the assay to support discovery projects and providing feedback. Thanks to Mei-yi Zhang, June Sonenberg-Reines, Margaret Zaleska, and Suzanne Aschmies for the in vivo B/P data. Thanks to Barbara Lences for useful discussion.

References

[1] D.E. Clark, S. Pickett, Drug Discov. Today 5 (2000) 49–58.
[2] A. George, Current Opin. Drug Discov. Dev. 2 (1999) 286–292.
[3] E. Kerns, J. Pharm. Sci. 90 (2001) 1838–1858.
[4] E. Kerns, L. Di, Current Opin. Med. Chem. 2 (2002) 87–98.
[5] J. Van Asperen, U. Mayer, O. Van Tellingen, J.H. Beijnen, J. Pharm. Sci. 86 (1997) 881–884.
[6] K. Audus, R. Borchardt, Handbook Exp. Pharmacol. 100 (1991) 43–70.
[7] W.M. Pardridge, J. Neurochemistry 7 (1998) 1781–1792.
[8] I. Tamai, A. Tsuji, J. Pharm. Sci. 89 (2000) 1371–1388.
[9] V.A. Levin, J. Med. Chem. 23 (1980) 682–684.
[10] R.C. Young, R.C. Mitchell, T.H. Brown, C.R. Ganellin, R. Griffiths, M. Jones, K.K. Rana, D. Saunders, I.R. Smith, N.E. Sore, T.J. Wilks, J. Med. Chem. 31 (1988) 656–671.
[11] A. Reichel, D.J. Begley, Pharm. Res. 15 (1998) 1270–1274.
[12] D.E. Clark, J. Pharm. Sci. 88 (1999) 815–821.
[13] J.A. Platts, M.H. Abraham, Y.H. Zhao, A. Hersey, L. Ijaz, D. Butina, Eur. J. Med. Chem. 36 (2001) 719–730.
[14] J.A. Gratton, M.H. Abraham, M.W. Bradbury, H.S. Chadha, J. Pharm. Pharmacol. 49 (1997) 1211–1216.
[15] H. Fischer, R. Gottschlich, A. Seelig, J. Membr. Biol. 165 (1998) 201–211.
[16] A. Seelig, R. Gottschlich, R.M. Devant, Proc. Natl. Acad. Sci. USA 91 (1994) 68–72.
[17] K. Audus, L. Ng, W. Wang, R.T. Borchardt, Pharm. Biotechnol. 8 (1996) 239–258.
[18] M.H. Abraham, H.S. Chadha, R.C. Mitchell, J. Pharm. Sci. 83 (1994) 1257–1268.
[19] F. Lombardo, J.F. Blake, W.J. Curatolo, J. Med. Chem. 39 (1996) 4750–4755.
[20] U. Norinder, P. Sjöberg, T. Österberg, J. Pharm. Sci. 87 (1998) 952–959.
[21] M. Kansy, F. Senner, K. Gubernator, J. Med. Chem. 41 (1998) 1007–1010.
[22] F. Wohnsland, B. Faller, J. Med. Chem. 44 (2001) 923–930.
[23] C. Zhu, L. Jiang, T. Chen, K. Hwang, Eur. J. Med. Chem. 37 (2002) 399–407.
[24] P. Crivori, G. Cruciani, P. Carrupt, B. Testa, J. Med. Chem. 43 (2000) 2204–2216.
[25] J.A. Gratton, S.L. Lightman, M.W. Bradbury, J. Physiol. 470 (1993) 651–663.
[26] D.R. Jones, S.D. Hall, E.K. Jackson, R.A. Branch, G.R. Wilkinson, J. Pharmacol. Exp. Ther. 245 (1988) 816–822.
[27] A.L. McCall, W.R. Millington, R.J. Wurtman, Life Sci. 31 (1982) 2709–2715.
[28] N.O. Martindale, in: J.E.F. Reynolds (Ed.), The Extra Pharmacopoeia, Pharmaceutical Press, London, 1989.
[29] S.K. Kulkarni, A.K. Mehta, J. Kunchandy, Drugs Today 20 (1984) 497–507.
[30] W.M. Pardrigde, Endocr. Rev. 2 (1981) 103–123.
[31] F.H.L. Awouters, C.J.E. Niemegeers, P.A.J. Janssen, Arzneim. Forsch. 33 (1983) 381–388.
[32] P.F.C. Bayliss, A.S.M. Duncan, Br. J. Clin. Pharmacol. 2 (1975) 527–531.
[33] B.B. Hofman, R.J. Lefkowitz, in: J.G. Hardman, L.E. Limbird, P.B. Molinoff, R.W. Ruddon, A.G. Gilman (Eds.), Goodman and Gillmans. The Pharmacological Basis of Therapeutics, 9th ed., McGraw-Hill, New York, 1996, pp. 199–248.
[34] W.M. Scheld, Rev. Infect. Dis. 11 (Suppl. 5) (1989) S1194–S1202.
[35] P. Jolliet, N. Simon, F. BrJe, S. Urien, A. Pagliara, P.A. Carrupt, B. Testa, J.P. Tillement, Pharm. Res. 14 (1997) 650–656.
[36] J. Heykants, M. Michiels, A. Knaeps, J. Brugmans, Arzneim. Forsch 24 (1974) 1649–1653.
[37] M. Weiner, Arzneim. Forsch. 32 (1982) 1193–1195.
[38] C. Young, R.C. Mitchell, T.H. Brown, C.R. Ganellin, R. Griffiths, M. Jones, K.K. Rana, D. Saunders, I.R. Smith, N.E. Sore, T.J. Wilks, J. Med. Chem. 31 (1998) 656–671.
[39] D.P. Selivonchick, B.I. Roots, Lipids 12 (1976) 165–169.
[40] C. Bénistant, M. Dehouck, J. Fruchart, R. Cecchelli, M. Lagarde, J. Lipid Res. 36 (1995) 2311–2319.

[41] C.K. Van Kalken, H.J. Broxterman, H.M. Pinedo, N. Feller, H. Dekker, J. Lankelma, G. Giaccone, Br. J. Cancer 67 (1993) 284–289.
[42] A.H. Schinkel, E. Wagenaar, A.A.M. Carla, L. van Deemter, J. Clin. Invest. 97 (1996) 2517–2524.
[43] A. Brown, R. Griffiths, C.A. Harvey, D.A.A. Owen, J. Pharmacol. 87 (1986) 569–578.
[44] A.J. Davies, V. Harinda, A. McEwan, R.R. Ghose, Br. Med. J. 298 (1989) 325.
[45] S.D. Raeissi, I.J. Hidalgo, J. Segura-Aguilar, P. Artursson, Pharma. Res. 16 (1999) 625–632.
[46] S. Miertus, E. Scrocco, J. Tomasi, Chem. Phys. 55 (1981) 117–129.
[47] V. Barone, M. Cossi, J. Phys. Chem. A 102 (1998) 1995–2001.