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Critical review

Pharmacology

In silico, *in vitro* and *in vivo* methods to analyse drug permeation across the blood-brain barrier: A critical review

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Abstract

Introduction

The existence of the blood–brain barrier in the human body leads to the insufficiency in delivering therapeutic compounds into the brain for the effective treatment of various neurological disorders. In order to determine the possibility of such agents to penetrate through the blood–brain barrier, different *in silico, in vitro* and *in vivo* methods may be implemented. Some of them are often provided with unreliable results while others are not feasible in high-throughput screening environment. The goal of this review was to characterise the latest state-of-the-art methods that have been developed and used in the pharmaceutical research in the last few decades to assess the permeation of novel therapeutic entities across the blood–brain barrier.

We carried out a literature research and study selection by searching for published biomedical articles in the PubMed archive.

Conclusion

Overall, the combination of *in silico, in vitro* and *in vivo* methods in the blood–brain barrier research may lead to the discovery of promising drug compounds and more accurate information of brain uptake mechanisms.

Introduction

In the drug discovery process, drug permeation across the blood–brain barrier (BBB) is fundamental for neuropharmaceuticals to reach their site of action within the central nervous system (CNS). This BBB consists of highly specialised microvascular endothelial cells together with pericytes, astrocytes, microglia, neurons and basement membrane[1]. The capillary endothelial cells are connected by proteins (occludin, claudins and junctional adhesion molecules) forming tight junctions (TJs), which seal the intercellular space, thereby restricting the permeability for the CNS-active substances[2,3]. In addition, these cells contain numerous active membrane transporters to regulate transcellular transport of drug-like molecules and their metabolites between the blood–brain interface.

Over 98% of all known therapeutics are unable to penetrate the BBB due to their molecular properties and physicochemical factors, including hydrophilicity, hydrophobicity, polar surface area, molecular size and charge (Figure 1). On the contrary, the permeation of the CNS-inactive compounds would generate various undesired side effects. Receptor-mediated and non-specific adsorption-mediated transcytosis can also contribute to the translocation of peptides, antibodies and lipoproteins across the BBB[4]. To minimise this risk, the healthy BBB itself imposes a highly efficient impediment for most of the clinically administered neuropharmaceuticals. On the other hand, the BBB dysfunction is highly implicated in auto-immune, neuropathological processes (Alzheimer and Parkinson's diseases), neuroinfections (meningitis and encephalitis), haemorrhagic and ischemic stroke and traumatic brain injury[5–7]. In this regard, the assessment of the BBB permeation for drug candidates at physiological and pathological conditions would be a primary concern for rational drug design and development through various *in silico, in vitro* and *in vivo* methods.

The goal of this review is to describe the state-of-the-art techniques and methods that have been used so far in pharmaceutical research to evaluate the BBB function and assess the ability of drug-like molecules to permeate the BBB.

We performed a literature search and study selection by seeking published biomedical research papers in PubMed. The criteria for search were as follows:

- article type: review, research article
- publication date: various
- species: mammals
- language: English
- key words: blood–brain barrier, *in silico*, *in vitro*, *in vivo* methods, drug-like compounds, rational drug design

We also used monographs dedicated to the BBB research and drug design strategies to bring readers the state-of the-art information in regard to describing issues.

Discussion

The authors have referenced some of their own studies in this review. These referenced studies have been conducted in accordance with the Declaration of Helsinki (1964) and the protocols of these studies have been approved by the relevant ethics committees related to the institution

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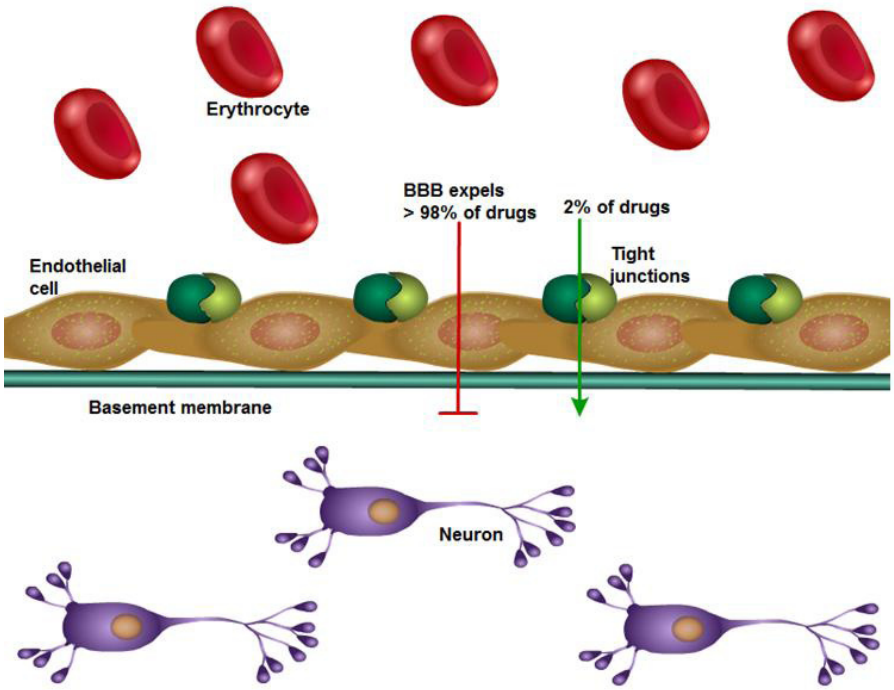

Figure 1: Schematic depiction of the blood–brain barrier permeability for different drug-like chemical substances.

in which they were performed. All human subjects, in these referenced studies, gave informed consent to participate in these studies.

In silico methods

The *in silico* prediction methods have acquired popularity in the last few decades in the BBB research because of their speed, flexibility, low cost and less time-consuming efforts in comparison to *in vitro* and *in vivo* approaches. Therefore, a new strategy has evolved based on the computational simulation and prediction of compound interaction with the BBB interface to expedite and improve rational drug design and discovery at its early stage.

To screen the virtual libraries that encompass up to hundreds of thousands and even millions of drug-like molecules, numerous procedures were devised based on their molecular descriptors and fingerprints. A standard high-throughput screening (HTS) is a method of choice to filter and determine the CNS-active drug/hit/lead-like compounds either by the descriptor- or by the molecular docking-based strategy (Figure 2).

As for the descriptor-based HTS, the great assessment in this direction was done by Lipinski and co-authors, characterised in a literature as the Rule of Five[8]. Despite the fact that the Rule of Five was widely adopted by both pharmaceutical industry and academia for its robustness (few false-negatives) and fast calculation speed, there were disadvantages of the method. Among those were lots of false-positive outcomes due to simple summation of molecular properties (molecular weight, sum of nitrogen and oxygen atoms, etc.) without considering the BBB transport mechanisms, such as multidrug P-glycoprotein (P-gp) transporter efflux and strong reliance on experimentally determined datasets.

Aside from the Lipinski's rule of thumb, the other methods were also elaborated to predict the ability of substances to permeate the BBB successfully and exert their pharmacological potential. Among them are various quantitative structure-activity relationship (QSAR) regression models based on the BBB partitioning values, such as logBB, taken from experimental data for various drug-like molecules[9]. The logBB parameter is defined as the logarithm value of steady-state brain to blood (plasma) concentration ratio for a drug of interest according to following equation:

$$\log BB = \log (c_{brain}/c_{blood})$$

In molecular descriptor-based analysis, the predicted logBB parameter was mainly derived from the notion of molecular polar surface area descriptor and octanol-water partition coefficient (logP) to assess compound hydrophobicity and H-bonding capacity (desolvation rate). These two last descriptors were vigorously discussed throughout the literature[10,11]. For instance, they were implemented continuously in the QSAR regression models through many mathematical formulas, such as Clark and Rishton equations[12,13]:

$$\log BB = 0.152 \log P - 0.0148 PSA + 0.139$$

$$\log BB = 0.155 \log P - 0.01 PSA + 0.164$$

On the other hand, the molecular docking-based methods have been successfully used to determine the P-glycoprotein substrates or inhibitors dealing with the phenomenon of active multidrug efflux by P-gp in the brain[14,15]. Despite its relative precision, this approach depends on the accurate crystallographic three-dimensional models of the protein structure and implements laborious ligand-receptor preparations and computationally slow genetic algorithms[16,17]. Therefore, this approach is particularly valuable when used in combination with previously described methods

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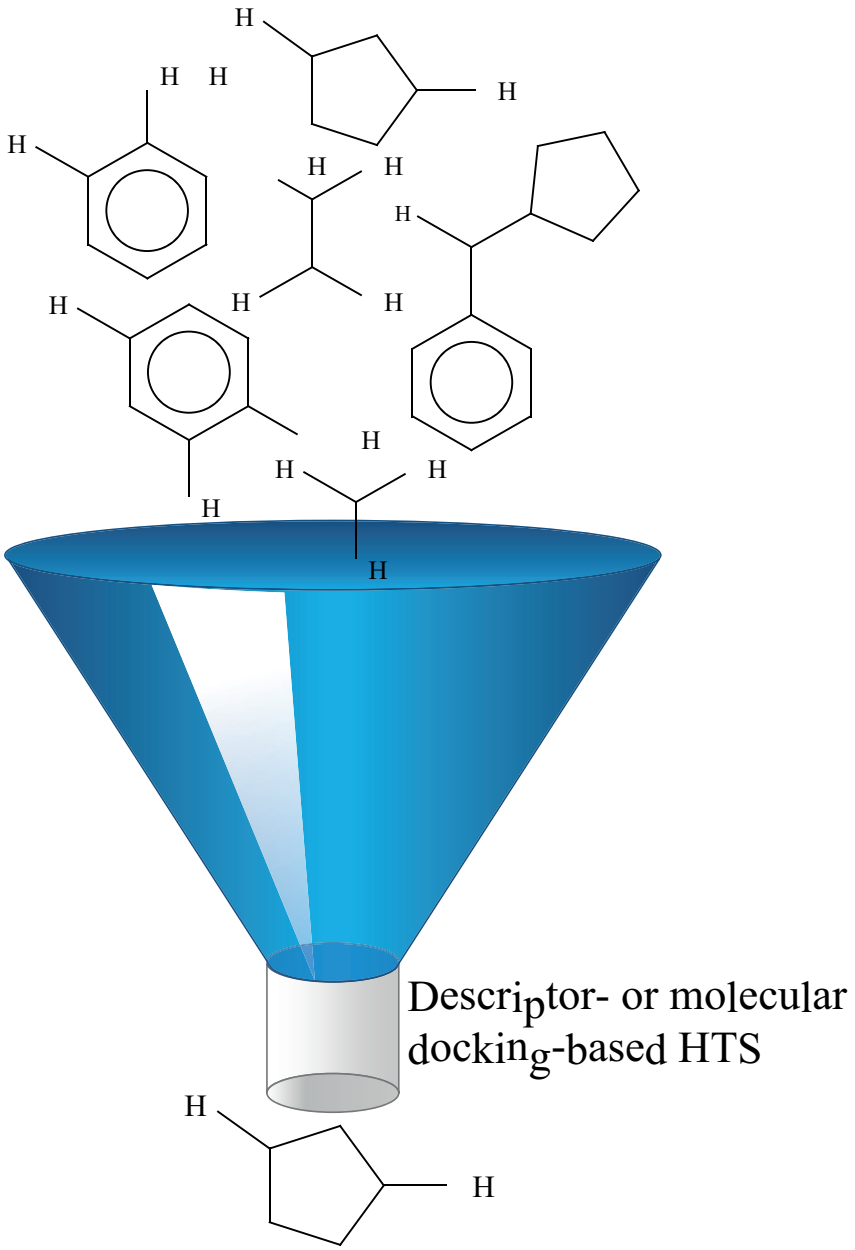

Figure 2: Schematic depiction of *in silico* methods to predict the BBB permeability of drug-like chemical compounds. The HTS methodology filters the virtual compound library through a descriptor- and/or molecular docking-based 'funnel' to generate the best results in accordance with Lipinski's Rule of Five, predicted logBB values and drug–P-gp interaction.

to exclude the role of active transport as a result of drug-P-gp interaction upon the BBB permeation of drug-like chemical compounds.

In vitro methods

In CNS research, BBB permeability properties of drug-like candidates are very important. Systems that can be used for HTS are favoured. Moreover, methods that allow for direct access to the brain endothelium with no interference from other brain structures are preferred. *In vitro* models allow for this which are the more common choice for such purpose. The translation of *in vivo* BBB permeability research to an *in vitro* setting calls for efficient *in vitro* methods of assessment.

A good *in vitro* BBB model should possess characteristics that mimic the BBB *in vivo*. A valid *in vitro* BBB model expresses TJ proteins among adherent endothelial cells and possesses negligible permeation to sucrose or electric current[18]. In addition, it is selectively permeable to molecules[19] and displays functional mechanisms of active extrusion[20] or active transport[21,22].

Cells of both cerebral and non-cerebral origin are used as *in vitro* models of the BBB. Isolation of brain capillaries and culture of brain capillary endothelial cells (BCECs) are both employed. However, due to the limitations of currently available immortalised BCEC lines since some of them have insufficient barrier properties, the cells of non-cerebral origin might be also used to build a barrier.

Isolated brain capillaries are used for BBB transport studies[23–25]. Freshly isolated capillaries directly reflect the situation at the luminal side of brain capillaries. In fact, they reflect the *in vivo* situation very well. However, they are not well suited for the BBB permeation studies since the luminal surface of the microvessels is difficult to access.

Primary BCECs also mimic the *in vivo* situation that makes them favourable in an *in vitro* model for BBB research. They provide a close phenotypic resemblance to *in vivo* BBB cells. However, it takes time to isolate, seed and incubate BCECs. Moreover, it is difficult to reproduce the same phenotypic and permeability properties for every experiment when using this cell type. Primary or low-passage porcine BCECs were the 'pioneer' cells used as an *in vitro* permeability model[26].

In order to overcome the problems concerning reproducibility of primary BCECs, immortalised

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BCECs are established for *in vitro* BBB permeation studies. For instance, the murine cerebrovascular endothelial cell line (cEND) was generated and its barrier properties were enhanced by glucocorticoid treatment[27–29].

Improvement of barrier properties has also been reported in porcine cerebral capillary endothelial cells[26], rat BCECs[30] and human dermal microvascular endothelial cell line (hDMEC/D3)[31]. Meanwhile, BCECs can also be co-cultured with astrocytes[32], C6 glioma cells[33] or pericytes[34] to improve barrier properties. These cells can be grown either with or without contact with BCECs in a Transwell culture system. Transwell models have been developed to study BBB permeation. Most permeability experiments employ this method. It can be a monodimensional system, wherein which only BCECs are grown on a microporous membrane or a two-dimensional system wherein the BCECs are co-cultured with other cells (Figure 3A–C).

However, all these systems lack the experimental replication of intraluminal blood cells together with bloodstream flow that imparts shear stress as it is occurring *in vivo*. The first *in vitro* BBB filter model was introduced in the 1980s using bovine brain endothelial cells[35]. The insert was composed of nylon mesh and polycarbonate tubing. A variety of chambers and inserts from different materials and pore sizes later became commercially available.

To compensate for the aforementioned lack in shear stress as affecting endothelial barrier function, dynamic BBB models were established[36]. In these models, hollow fibres that mimic capillaries and allow co-culture of other cell types were used (Figure 4). Bovine aortic endothelial cells co-cultured with glial cells were the first BBB model to adopt this method[37]. More recently, immortalised porcine brain endothelial cells co-cultured with glial cells[38] were used. The human cerebral microvascular endothelial cell line (hCMEC/D3) co-cultured with astrocytes grown in the lumen of hollow microporous fibres and exposed to a physiological pulsatile flow was also recently developed as a dynamic BBB model[39]. This method demonstrated that hCMEC/D3 cells cultured under pulsatile flow conditions have maintained *in vitro* physiological permeability barrier properties of the BBB *in situ* even in the absence of abluminal astrocytes. However, due to some technical demands of this approach, it may not be utilised as a high-throughput *in vitro* permeability screening system.

There are several *in vitro* models to choose for conducting BBB permeability studies, but there is no universal *in vitro* model that encompasses all the properties presented *in vivo*. Thus, it is advantageous if a combination of existing methods is used to come up with better experimental systems.

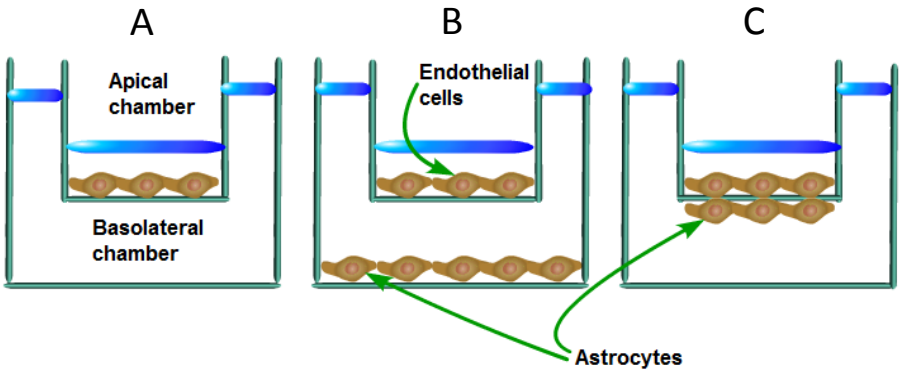

Figure 3: Schematic drawing of static *in vitro* Transwell models to study BBB permeation. 'A' represents the traditional endothelial monolayer as monodimensional system while 'B' and 'C' show the two-dimensional experimental setup with no or close contact cellular arrangements, respectively.

In vivo methods

While *in silico* and *in vitro* methods have many significant advantages to perform substantial screening of drug-like chemical compounds, the BBB permeation analysis should not rely solely on them. Therefore, *in vivo* techniques made it possible to correlate the data produced by previous methods, determine and confirm the final results and clarify the BBB molecular mechanisms considering the complexity of living organisms[40].

There are various *in vivo* methods (some of which are not in the scope of this review) that have been utilised to evaluate the BBB permeation mechanism, including the high-performance liquid chromatography (HPLC) analysis of brain homogenates, *in situ* brain perfusion and intracerebral microdialysis (Figure 5). Each of these invasive techniques is suitable to experimentally determine the logBB and/or logPS (logarithm of permeability–surface area product) values under appropriate physiological and pathological conditions.

The HPLC analysis of mouse or rat brain homogenates is a crude method of choice; it starts with a homogenate preparation by ultrasonication in Dulbecco's phosphate-buffered saline (PBS) or other matrices following high-speed centrifugation to produce a clear supernatant for further determination of drug concentration by HPLC. The major disadvantage

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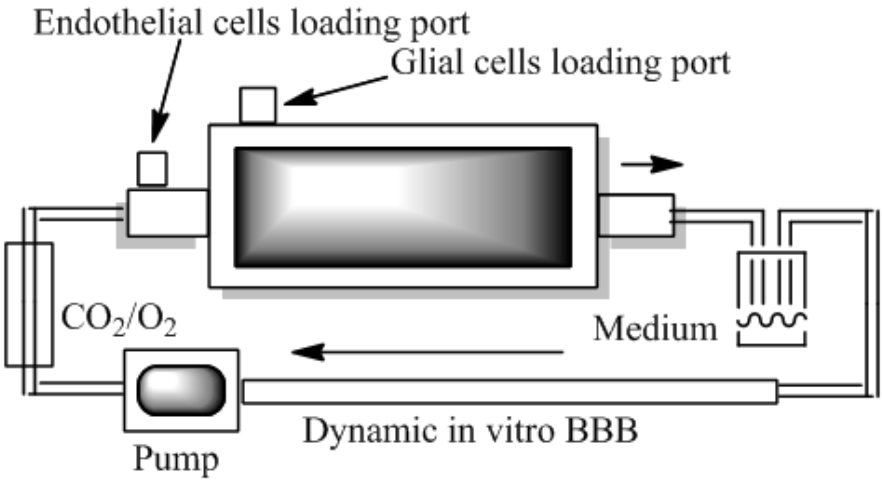

Figure 4: Schematic representation of a conventional dynamic *in vitro* BBB device ('bioreactor'). Endothelial cells are seeded intraluminaly in collagen 4 or fibronectin-coated hollow-fibre cylinder while astrocytes are seeded extraluminally.

of this approach is that the residual capillary blood in the brain might influence the results and, therefore, should be eliminated via brain reperfusion with PBS before surgery or *in situ* brain perfusion[41]. The other hurdles and difficulties in HPLC measurements include a high lipid and protein composition presented in the brain, which bind to a chemical entity (protein/lipid-bound drug fraction) and sediment along with it.

The *in situ* perfusion technique was first developed by Takasato et al.[42]. In this method, the right cerebral hemisphere of the rat is perfused *in situ* (in place), with the reference and test compounds retrograde via the external into the internal carotid artery in anaesthetised rats.

After perfusion, the animals are decapitated and the brain is analysed for reference and test compounds to quantify the logPS coefficient, which is a calculation method based on the rate of brain penetration for analysed chemical entity. The logPS parameter is calculated as follows:

$$\log \mathrm{PS} = \log\left[\frac{V_D - V_0}{t}\right]$$

where t is the duration of the perfusion period (min) and V_D or V_0 is the brain volume of distribution for the test and reference compound respectively and calculated as the brain/perfusate concentration at time t[43].

In situ brain perfusion does not alter the BBB integrity and can be used to accurately determine permeability coefficients for solutes ranging from 10^{-8} to 10^{-4} cm×s^{-1} [42]. The most frequently used animal for brain perfusions is the rat, although this method has also been successfully applied in dog, guinea pig and mice studies[44–46]. Although this method takes longer to perform compared to carotid artery single injection, it is more sensitive due to the prolonged duration. It also allows the estimation of PS product for those compounds that penetrate easily or very poorly. In the course of *in situ* brain perfusion, the compound being tested is not systemically exposed; therefore it avoids metabolism in the liver. In addition, many factors of the perfusate such as concentration and constituents can be controlled and varied[47]. A serious disadvantage of this method, however, is the large number of animals needed for complete kinetic analysis. In addition, radiolabelled or reference compounds are required for analysis.

Intracerebral microdialysis is a valuable tool in pharmaceutical research that is used to perform a direct sampling of cerebral interstitial fluid via establishing a dialysis catheter with semipermeable membrane into the brain[48]. Therefore, the molecule of interest from the brain will

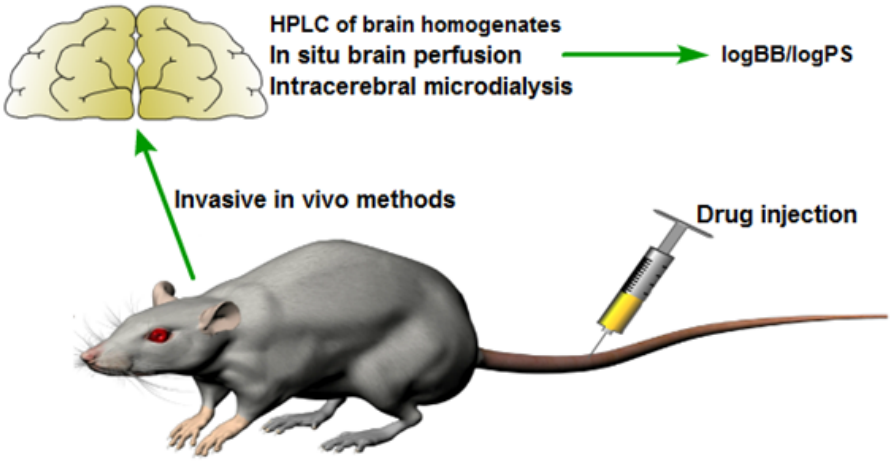

Figure 5: Schematic diagram of different invasive *in vivo methods*. Intravenous drug injection is followed by further analysis of drug concentration in the brain and blood (see text for details).

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traverse this membrane according to its concentration gradient (from high to low) that makes it possible to analyse within the collected fluid (microdialysate)[49,50]. This technique allows the possibility to monitor the drug concentration in the brain over time within the same animal and probe different brain areas as presumable drug targets. The potential drawbacks may include chronic BBB inflammation and disruption caused by this invasive procedure followed by increased BBB leakage and plasma protein extravasation[51]. Despite these shortcomings, this method of choice is still the only technique that provides information about the local concentration of unbound fraction of drug-like substances at any given time in freely moving animals.

Conclusion

Diverse *in silico, in vitro* and *in vivo* methods for the estimation of drug-like molecules transferred across the BBB have been devised for pharmaceutical research by industry and academia in the last few decades. Presently, all these methods have their intrinsic advantages and limitations emphasised in this current review. The more reliable *in vivo* methodologies are still not applicable for HTS of huge molecular databases generated by combinatorial chemistry manipulations. For that reason, additional *in silico* and *in vitro* techniques were widely adopted by bioscientists to expedite a compound selection process, fully assess the brain uptake for chemical entities and better understand the complex mechanisms underlying BBB transport. Therefore, the use of a combined screening process, including *in silico, in vitro* and *in vivo* methods, may achieve greater reliabilities in predicting and measuring the BBB permeation potential of promising drug candidates in humans.

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Abbreviations list

BBB, blood–brain barrier; BCEC, brain capillary endothelial cell; cEND, cerebrovascular endothelial cell line; CNS, central nervous system; HPLC, high-performance liquid chromatography; HTS, high-throughput screening; PBS, phosphate-buffered saline; QSAR, quantitative structure-activity relationship; TJ, tight junction.

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