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Blood Brain Barrier Permeation

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1. Introduction

The large surface area and the short diffusion distance from capillaries of the blood brain barrier (BBB) to the neurons facilitate the drugs and nutrients access to the brain. Penetration of chemicals to the BBB occurs using a combination of intra and intercellular passages. Tight junctions regulate the intracellular passage of molecules according to their physico- chemical properties (e.g. lipophilicity, ionisation and polarity), where inter cellular penetration is regulated by influx and efflux transporters, endocytosis and passive diffusion. Poor pharmacokinetic properties (absorption, distribution, metabolism and excretion) and toxicity are responsible for most of the failures in drug discovery projects. This problem is more evident for CNS drugs because of the restrict barrier function of blood brain barrier. The CNS drug discovery attracted more attentions since the diseases pattern has been changed during recent decades and aging disorders are one of the major health problems. Drug exposure is controlled by plasma pharmacokinetic properties of drug which are different from brain pharmacokinetic and can be studied using common pharmacokinetic studies, where BBB permeability depends on physicochemical properties of drug compound and physiologic function of the BBB (physical barrier, transport, metabolic, …) and need special study techniques. In this chapter, fundamentals of BBB, permeation mechanisms, penetration measurement methods and penetration prediction methods are discussed.

2. Fundamentals of BBB

2.1 Cellular properties of Blood Brain Barrier

BBB consisted of a monolayer of brain micro vascular endothelial cells (BMVEC) joined together by much tighter junctions than peripheral vessels and formed a cellular membrane which known as the main physical barrier of BBB (Abbott, 2005; Cardoso et.al., 2010). The main characteristics of this cellular membrane are, uniform thickness, no fenestrae, low pinocytotic activity, continues basement membrane and negative surface charge. In addition to the BMVECs, the neurovascular unit consisted of the capillary basement membrane, pericytes, astrocytes and microglia. The BMVECs are surrounded by a basement membrane which composed of structural proteins (collagen and elastin), specialized proteins (fibronectin and laminin) and proteoglycans. This structural specificity gives the basement membrane a cell establishment role. Pericytes are cellular constituents of microvessels

including capillaries and post capillary venules that covered about 22-32% of the capillaries and shared the same basement membrane. Pericytes are responsible for a wide variety of structural and non-structural tasks in BBB. In summary they synthesis some of structural and signalling proteins and they are involved in the BMVECs proliferation, migration and differentiation. More details and references about pericytes role in BBB can be found in the literature (Cardoso et al., 2010). Fine lamellae closely opposed to the outer surface of the capillary endothelium and respective basement membrane formed by astrocytes end feet. Like pericytes, astrocytes involve in various functional and structural properties of neurovascular unit.

Microglia is immunocompetent cells of the brain that continuously survey local micro environment with highly motile extensions and change the phenotype in response to the homeostatic disturbance of the CNS (Prinz & Mildner, 2011). The interactions of brain micro vascular endothelial cells with basement membrane, neighbouring glial cells (microglia and astrocytes), neurons and perivascular pericytes leads to specific brain micro vascular biology. Presence of matrix adhesion receptors and signalling proteins form an extensive and complex matrix which is essential for maintenance of the BBB (Cardoso et al., 2010). Figure 1 shows a schematic illustration of neurovascular unit and BBB cellular components.

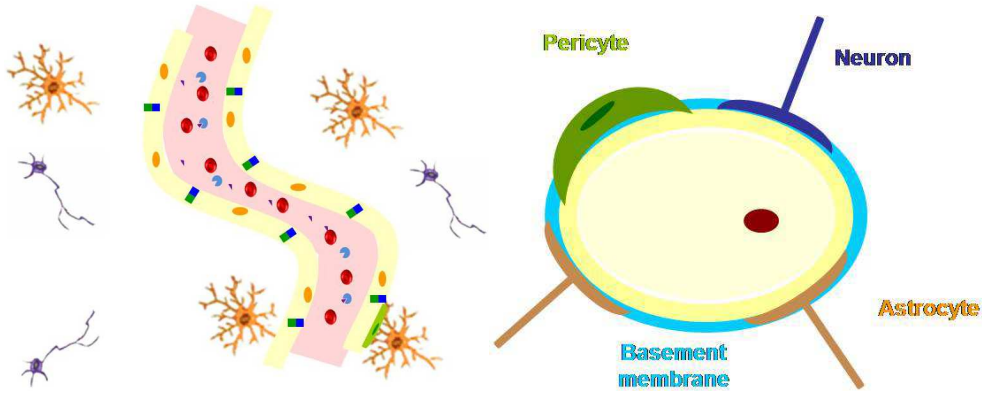

Fig. 1. Schematic illustration of the neurovascular unit and BBB cellular components adopted from (Cardoso et al., 2010).

2.2 Molecular properties of BBB

The BMVECs assembly are regulated by molecular constituents of tight junctions, adherence junctions and signalling pathways. Tight junctions are highly dynamic structures which are responsible for the barrier properties of BBB. Apical region of the endothelial cells sealed together by tight junctions and paracellular permeability of BMVECs are limited by them. Structurally tight junctions formed by interaction of integral transmembrane proteins with neighbouring plasma membrane. Among these proteins junction adhesion molecules, claudins and occludins (inter membrane) which bind to the cytoplasmic proteins (e.g. zonula occludens, cinguline, …) are well studied and their role in tight junctions and BBB have been evaluated (Figure 2). Beyond the main role in physical restriction of BBB, other functions such as control of gene expression, cell proliferation and differentiation have been

suggested for tight junctions. Below the tight junctions, actin filaments (including cadherins and catenins) linked together and form a belt of adherence junctions. In addition to the contribution in the barrier function some other events such as adhesion of BMVECs to each other, the contact inhibition during vascular growth, the initiation of cell polarity and the regulation of paracellular permeability have been suggested for adherence junctions. A dynamic interaction between tight junctions and adherence junctions through signalling pathways regulate the permeability of BBB. These signalling routes mainly involve protein kinases, members of mitogen – activated protein kinases, endothelial nitric oxide synthase and G-proteins. Dynamic interactions between these pathways control the opening and closing of the paracellular route for fluids, proteins and cells to move across the endothelial cells through two main types of signal transduction procedures (e.g. signals from cell interior to tight junctions to guide their assembly and regulate their permeability, signals transmitted from tight junctions to cell interior to modulate gene expression, proliferation and differentiation). The molecular mechanisms of these interactions can be found in the literature (Ballab et al., 2004; Abbott et al., 2006). In addition to the proteins with enzymatic activities, there are other specific proteins (drug efflux transporters, multi drug resistance proteins, organic anion transporting polypeptides) work as BBB transporters which are responsible for rapid efflux of xenobiotics from the CNS (Losscher & Potschka, 2005) and delivery of the essential nutrients and transmitters to the brain.

The combined effect of the special cellular and molecular properties of central nervous system result in the specific barrier functions of BBB which is important for preventing CNS from harmful xenobiotics. Because of these properties drug delivery to the CNS is among the most challenging drug development areas. In order to develop successful drug candidates for CNS disorders drug uptake mechanisms should be studied. In the next section, these mechanisms are briefly reviewed.

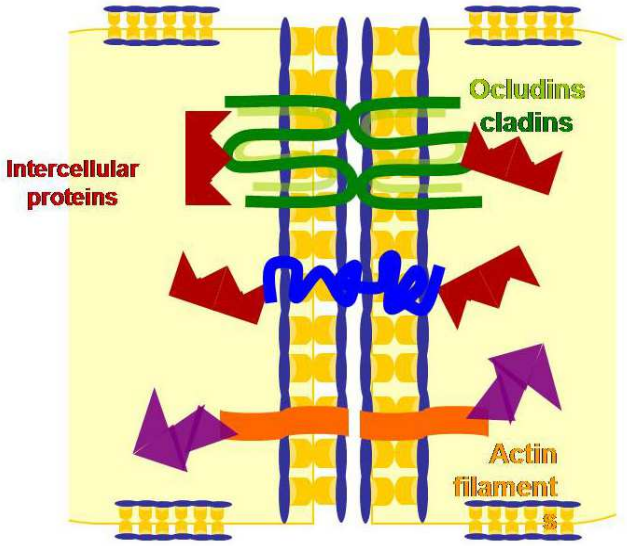

Fig. 2. Tight junctions and adherent junctions.

3. BBB permeation mechanisms

Like other cellular membranes in the body, permeation through BBB can occur by passive diffusion, endocytosis and active transport (Diagram 1). Combined effects of the mentioned mechanisms modulate the compound (e.g. Drugs) penetration to the brain.

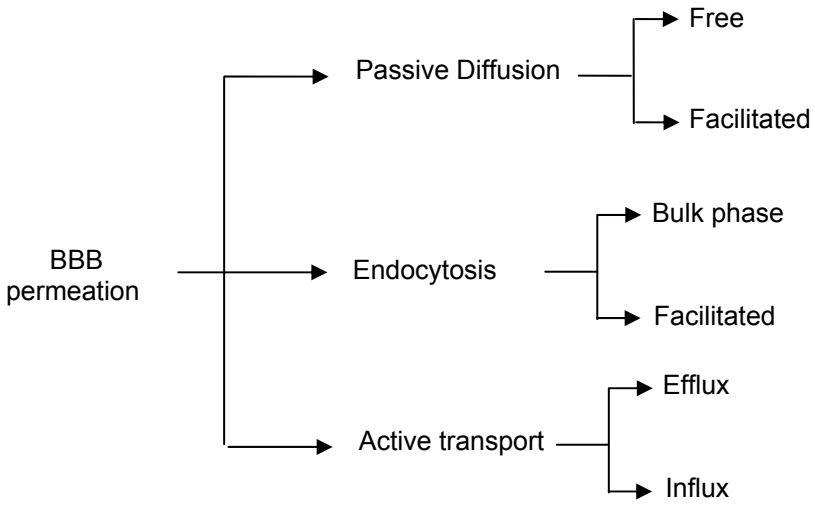

Diagram 1. Main permeation mechanisms in the brain.

3.1 Passive diffusion

A limited number of drugs and drug like compounds with high lipophilicity and low molecular size can penetrate to the brain mainly by passive diffusion. In order to overcome the surface tension difference between a compound and cellular membrane, physical work is needed and the smaller molecules will need less work. The uncharged forms of the weak acidic and basic compounds have higher permeability rate in comparison with charged molecules in physiologic pH of brain. The charged forms possess hydrophilic characteristics and hydrophilic drugs distribute within blood and cannot cross the endothelial cells and excreted from brain parenchyma. Therefore, the molecules with higher fraction of uncharged form in physiologic pH have higher permeability rate (Fischer et al., 1998).
Passive diffusion occurs via two mechanisms (Figure 3):

- Free diffusion in which some compounds move freely paracellularly (e.g. sucrose) between cells to a limited extent due to tight junctions or transcellularly (transcytosis) across the cells for lipophilic substances (e.g. ethanol) (Alam et al., 2010). These mechanisms are non-competitive, nonsaturable and occur in downhill concentration direction.
- Facilitated diffusion in which target compounds bind to a specific membrane protein and carry to the other side of the membrane through conformation change of the protein. This mechanism is a form of carrier mediated endocytosis which occurs from high to low concentration like free diffusion and contributes for transport of some amino acids, nucleosides, small peptides, mono-carboxylates and glutathione (Alam et al., 2010).

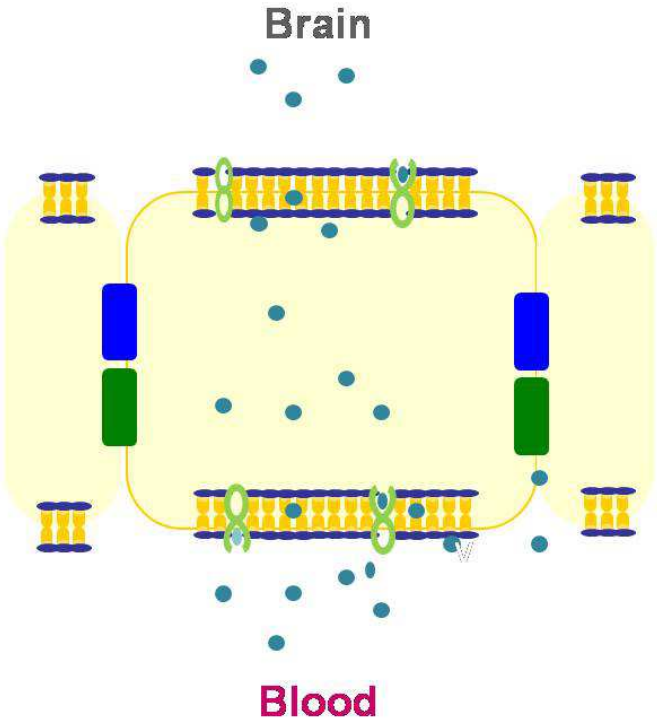

Fig. 3. Free and facilitated passive diffusion.

3.2 Endocytosis

In this method, substances (e.g. macromolecules) are engulfed by membrane and pass through the cell by vesicles and release in the other side (Kerns & Di, 2008). Endocytosis occurs via two main methods: bulk phase endocytosis (fluid phase or pinocytosis) and mediated or facilitated endocytosis (receptor and absorptive mediated). Fluid phase endocytosis is a nonsaturable, non-competitive and non-specific method for uptake of extra cellular fluids which is temperature and energy dependent.

Receptor mediated endocytosis facilitates the larger essential molecules uptake selectively using specific receptors present in luminal membrane. Hormones, growth factors, enzymes and plasma proteins are targets for specific receptors (Pardridge, 2007).

Absorptive mediated endocytosis is based on an electrostatic interaction between negatively charged plasma membrane luminal surfaces (glycocalyx which is a negatively charged proteoglycan or glycosaminoglycan) with cationic substances (e.g. peptides) and uptake it in a vesicle into the endothelial cell and release it on the other side (Figure 4) (Ueno, 2009).

This has lower affinity and higher capacity than receptor mediated endocytosis (Alam et al., 2010). Mechanism of vesicle formation (caveolin dependent, dynamin dependent and caveolin- dynamin independent) is not discussed in this chapter and more details could be found in the literature (Lajoie et al., 2010).

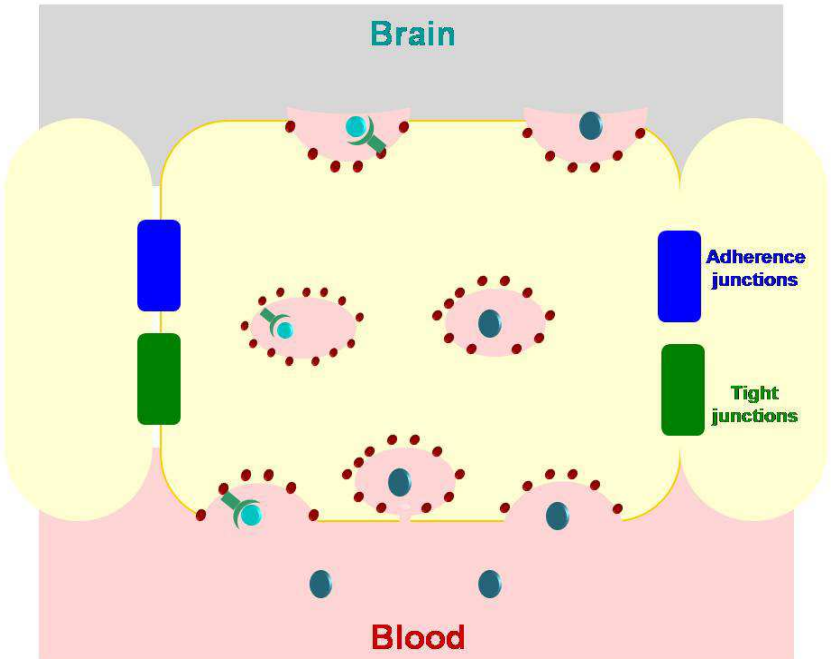

Fig. 4. Bulk phase and facilitated endocytosis.

3.3 Active transport

Hydrophilic drugs which cannot penetrate the brain through passive diffusion and lipophilic drugs which cannot penetrate the brain, in contrast of their suitable characteristics for BBB permeation are substrate for drug transporters of the BBB. Also some compounds are substrates for transporters and at the same time they are delivered by passive diffusion or endocytosis. Drug transporters are integral membrane proteins which is able to carry the drug usually against the concentration gradient into and out of the cell.

The overall exposure of xenobiotics to brain through these transporters depends on their location and expression level according to the normal and pathophysiologic conditions. Two types of drug transporters according to their driving forces (ATP dependent and ATP independent) are known. Active transporters broadly categorized as primary (ATP dependent), secondary or tertiary (ATP independent) (Murk et al., 2010).

There are two types of transporters:

1. Carrier mediated transporters which express on both the luminal and abluminal membranes and operates in both blood to brain and brain to blood directions.
2. Active efflux transporters which mediate extruding drugs and other compounds from brain (Alam et al., 2010). Although the main role of the drug transporters is carrying the drugs and other xenobiotics into and out of the brain but they are responsible for other cell processes such as inflammation, differentiation of immune cells, cell detoxification, lipid trafficking, hormone secretion and development of stem cells (Murk et al., 2010).

3.3.1 Influx transporters

Essential hydrophilic nutrients (e.g. glucose, amino acids, fatty acids, organic and inorganic ions) reach to brain through influx transporters and receptors. According to the structural similarity of the target drug to the biologic molecules; it can be delivered to the brain using appropriate transporter. Solute carrier family encodes most of the influx transporters which include facilitated, ion coupled and ion exchange transporters that do not need ATP (Eyal et al., 2009). These transporters are responsible for uptake of a broad range of substrates including glucose, amino acids, nucleosides, fatty acids, minerals and vitamins (Alam et al., 2010). The most well studied groups of these bidirectional transporters along with their properties and activities are summarized in Table 1.

3.3.2 Efflux transporters

Efflux occurs in BBB through both passive and active routes in order to detoxify the brain and prevent from drugs and xenobiotics exposures. There are several kinds of efflux transporters such as ATP binding cassette transporters (ABC), organic anion transport systems, amino acid transport systems and so on (Ueno, 2009). ABC transporters are primary active systems which are responsible for different efflux activities including P-glycoprotein (P-gp), multi-drug resistance proteins (MRPs), and breast cancer related protein (BCRP). P-gp (the most studied ABC transporter), located in luminal side of BBB, immediately pump most of the drugs and xenobiotics back to the blood and decrease the net penetration to the brain. A broad range of drugs, generally including un-conjugated and cationic substances (Table 1) are substrates for P-gp, where some of them are able to inhibit P-gp and lead to increased permeability of co-administered drugs. This fact can be used as a drug delivery strategy to the brain. Along with P-gp, MRPs and BCRP are responsible for main part of drug efflux in BBB and their effect are dependent to their localization and expression level in normal and pathologic conditions. Over expression of these transporters considered as one of the major reasons of pharmacoresistance of brain diseases and their inhibition, bypassing and regulating methods are important for CNS drug development (Loscher & Potschka, 2005).

3.4 Metabolism in BBB (Enzymatic barrier)

Existing enzymes in BBB can be regarded as second barrier after negative surface charge. These enzymes involve in disposition of drugs and xenobiotics before entering the endothelial cells of capillaries. Alkaline phosphatase, acid phosphatase, 5′-nucleotidase, adenosine tri-phosphatase and nucleoside di-phosphatase are among well studied enzymes distributed within BBB (Ueno, 2009).

4. BBB permeation measurement methods

The rate and the extent of drug transport to the brain are needed for drug discovery studies (both peripheral and CNS drugs) and different methods developed in order to study the pharmacokinetic profile of drug candidates. BBB permeability depends on physicochemical properties of drug compound and physiologic functions of the BBB (physical barrier, transport, metabolic pathways) and need special study techniques. These techniques include *in vivo*, *in vitro*, and *in silico* methods (Diagram 2) which are complement in most cases and researchers are able to define different aspects of drug passage to the brain using these methods.

Transporter name	Substrates	Sample drugs and nutrients	Influx/ Efflux
Organic anion transporting polypeptides	Anionic amphipathic molecules with molecular weight greater than 450 Daltons and a high degree of albumin binding	Fexofenadine, Digoxin, Methotrexate	Influx
Organic anion transporters	Anionic drugs and nucleotides	Benzylpenicillin, Valacyclovir, Zidovudine, Mercaptopurine, Methotrexate, Valproic acid	Influx
Organic cation transporters	Bidirectional transport of small hydrophilic positively charged compounds	Cimetidine , Desipramine, Metformin, Amantadine, Memantine, Cisplatine, Quinin	Influx / Efflux
System L.	Bidirectional transport of large neutral amino acids with branched or aromatic side chains	L-phenylalanine, L-tyrosine, L-tryptophan, L-lucine, Levodopa, α-Methyldopa, Baclofen, Melphalan, Gabapentin, Pregabalin	Influx / Efflux
Monocarboxylate transporters	HMG-CoA reductase inhibitors that contain a carboxylic acid moiety	Simvastatin, γ- Hydroxybutyrate	Influx
Nucleoside transporters	Purine and pyrimidine nucleosides	Adenosine	Influx
Hexose transporters	Hexose nucleosides	Glucose	Influx
Ion transporters	Bidirectional transport of small ions	Cl^-, Na^+, K^+, H^+, $HCO3^-$	Influx / Efflux
P-glycoproteins	A broad range of drugs and xenobiotics (normally un-conjugated, cationic substances)	Anti cancer drugs, corticoids	Efflux
Multi-drug resistance proteins	Drugs and xenobiotics (normally conjugated, anionic substances)	Anti cancer and anti HIV Drugs	Efflux
Breast cancer resistant proteins	Drugs and xenobiotics (overlap with P-glycoproteins and multi-drug resistance proteins)	Some anti cancer Drugs	Efflux

Table 1. Some of the well studied influx and efflux transporters of brain.

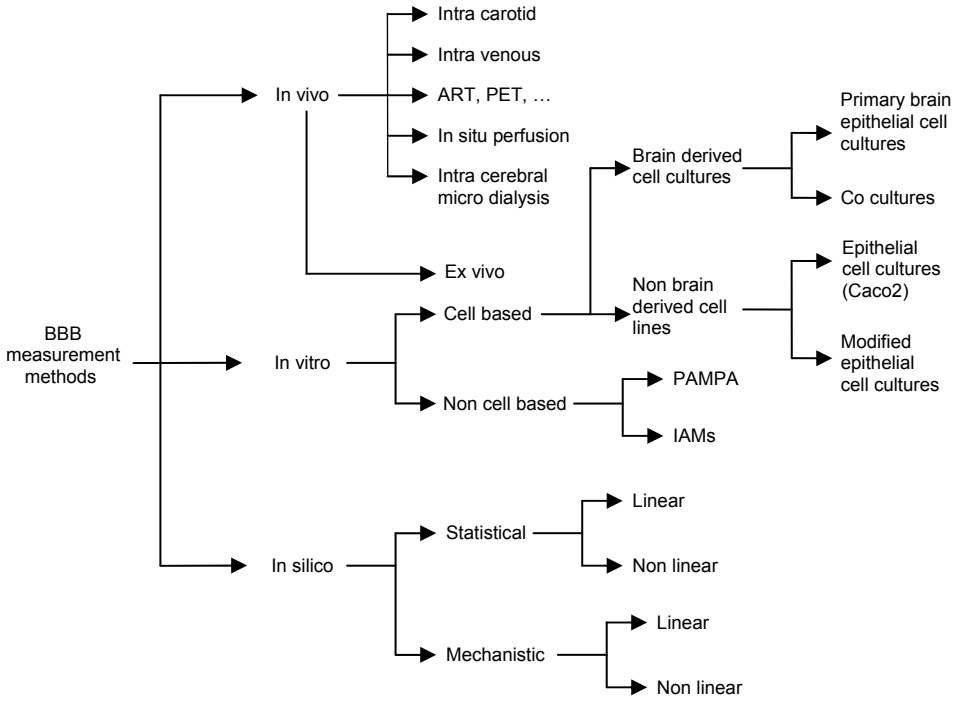

Diagram 2. Brain drug testing methods.

4.1 BBB permeation data

4.1.1 Bound and unbound drug concepts

The drug is available in blood in the free (unbound) and bounded (protein bounded, erythrocyte bounded, tissue bounded) forms. The unbound drug molecules equilibrate across the BBB and brain. The spaces that these equilibria occur are: blood, interstitial fluid, intercellular and intracellular fluids. Figure 5 shows these equilibria schematically. The speed of the equilibria to reach the steady state define the rate of drug distribution within brain, and the slowest one would be the rate limiting step. For poor CNS penetrantes, the BBB permeation or the diffusion of drug molecules within the brain tissue is the rate limiting step. Total brain concentration which allow us just to rank drug candidates according to their CNS total levels and general CNS penetrability can be measured using most of the *in vivo* methods, while there is just a few methods which are able to provide free fractions directly.

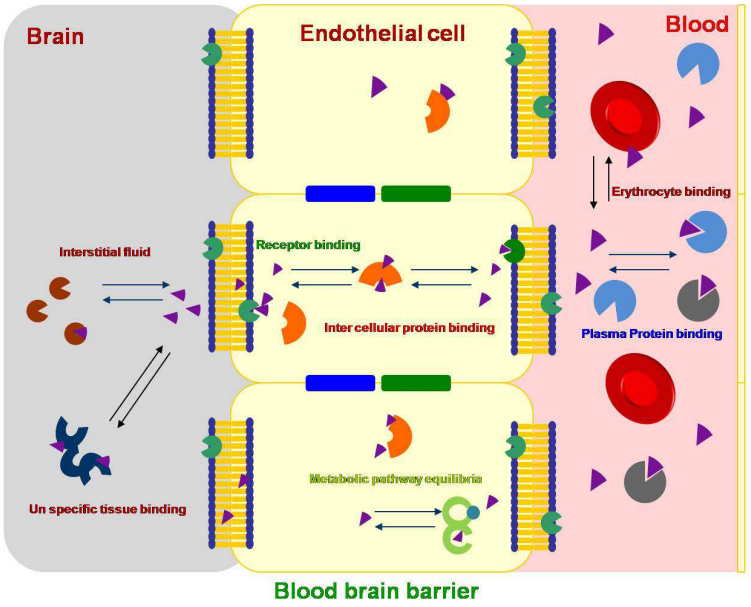

Fig. 5. Different equilibria in brain.

4.1.2 The importance of free drug measurement

The free drug is responsible for pharmacokinetic and pharmacodynamic properties of drugs and relation between dose and response is correct when free drug supplies in target tissue get into account. In this regard interstitial fluid and intra cellular fluid drug levels in brain are important data for drug discovery.

The traditional methods of brain homogenization destroy all compartments of brain (including brain tissue binding and plasma protein binding) and drug levels in specific compartments cannot be measured (Reichel, 2009). The plasma free fractions data cannot be used in CNS drug discovery studies, because of the different physiological properties, blood brain interstitial fluid free fractions. Some researchers used cerebrospinal drug levels (CSF sampling) as an estimate of the unbound drug levels in brain which is not so reliable because of lower tightness of cerebra-spinal blood barrier which leads to higher diffusion and overestimation of free drug concentration in brain (Read & Braggio, 2010). The microdialysis is the only *in vivo* method to provide such data directly, which is limited by its practicability.

4.1.3 The rate and extent of drug penetration to the brain

Neuropharmaceuticals should be able to permeate the BBB and enter the brain parenchyma in order to treat desired disorders whereas peripheral drugs should have limited entrance to the brain in order to decrease their neurological side effects. The drug entrance to the brain was evaluated and quantified using different methods, among them BUI, logBB, $K_{p,uu}$ etc, are well studied and frequently used to measure the rate and the extent of brain drug penetration (Jeffrey & Summerfield, 2010).

Brain uptake index (BUI%) is one of the earliest indicators of BBB permeability of compounds and is calculated by:

$$BUI\% = 100\frac{E}{E_{ref}} \tag{1}$$

where E denotes the first pass extraction and the E_{ref} referred to freely diffusible internal standard. This indicator provides information about the total concentration of the drug in the brain at early time point after administration (Lanevskij et al., 2010).
The logBB which describes the ratio between brain and blood (or plasma) concentrations and provide a measure of the extent of drug permeation is calculated using (Kerns & Di, 2008):

$$\log BB \; or \; K_p = \frac{AUC_{\;tot.brain}}{AUC_{\;tot.\,blood}} \tag{2}$$

The only information provided by K_p is passive lipid partitioning of the drug which is affected by metabolism, relative binding affinity to proteins and lipid content of brain and blood or plasma and it is not a net measure of BBB permeability (Abbott, 2004; Mehdipour & Hamidi, 2009). It is highly time dependent and in order to get an overall estimation, usually is measured under steady-state conditions.
Another approach based on unbound drug fraction, for quantifying the extent of brain penetration is recommended, which is calculated by:

$$K_{p,uu} = \frac{AUC_{\;\mathrm{u,\,brain}}}{AUC_{\;\mathrm{u,\,blood}}} \tag{3}$$

$K_{p,uu}$ affected by both passive diffusion and active influx/efflux and can give information about the permeation mechanism, beyond these, it is not affected by plasma protein and brain tissue binding which interfere in logBB values (Mehdipour & Hamidi, 2009). For drugs delivered by passive diffusion, this index will be close to unity while for efflux and influx substrates it will be less than and more than unity respectively (Hammarlund- Udenaes et al., 2008).
To assess the brain drug permeability rate, the unidirectional influx constant from blood to the brain (K_{in}) and the product of the BBB permeability surface area (PS) which is a measure of the unidirectional clearance from blood to brain have been developed. Both parameters expressed as ml/min/g of brain (Rooy et al., 2010). PS is able to reflect the BBB permeation step more accurately (Abbott, 2004) and is valuable parameter for follow up permeation ability of drug candidates in the pharmaceutical industry and although in pathologic conditions. PS gives an estimation of unbound drug in brain but it is affected by the possible association of the drug with active influx or efflux transporters (Hammarlund- Udenaes et al., 2008).
According to the measurement method K_{in} and PS can be calculated from Crone-Renkin equation:

$$K_{in} = F\left(1 - e^{\frac{-PS}{F}}\right) \tag{4}$$

where F could be considered as perfusion flow rate, or cerebral blood flow rate and PS is computed using:

$$PS = -F \times \ln\left(1 - \frac{K_{in}}{F}\right) \quad (5)$$

Methods for measuring efflux of the drugs out of the brain (brain efflux index (BEI)) have been developed which represent the elimination rate constant of the drugs in brain. Using these parameters, scientists can provide information about the mechanism of BBB permeation in which for passive diffusion the efflux and influx constants will be similar.
To measure all of these data, the remained drug in brain microvascular should be calculated and subtracted from total brain concentration.

4.2 *In vivo*

The resulted data from *in vivo* experiments are valuable and regarded as gold standard in CNS drug discoveries. This value comprises from the experiment which uses anesthetized or cautious animals which represent full physiologic condition for study and the obtained data reflect different aspects of BBB permeation. Demanding skilled scientists and equipped laboratories are the main disadvantage of these techniques.

4.2.1 Intra venous injection

Intra venous injection methods have been developed during primary CNS studies in order to assess the BBB permeability and brain distribution of the CNS drug candidates. The radio-labelled compounds are injected intravenously and blood samples are obtained in different time intervals and a single brain tissue can be obtained at the designated time point. The measured compound concentrations in plasma and brain plotted against the time and after calculating AUC values the logBB computed using equation 2. For each time interval three animals are needed and in order to get a plot using 7 data points, 21 animals are required which is the main limitation of the method (Rooy et al., 2010). The logBB are interesting for pharmaceutical companies, because they can be easily used to rank the goals and other pharmacokinetic parameters such as C_{max} and time length that the compound remains above *in vitro* determined effective concentration can be calculated. Recently these data are questioned about their ability to reflect the permeability properties of studied compounds mainly because: 1) The obtained concentrations are total, while the free fraction of the compounds are responsible for most of their pharmacokinetic properties and 2) It is a brain distribution value and the permeation rate of compounds cannot be obtained (Kerns & Di, 2008). The other parameters which can be calculated using the obtained data are rate parameters (i.e. K_{in} and PS).

4.2.2 Single carotid injection

Single intra carotid injection is one of the earliest BBB permeation study methods and can be done by injection of a given concentration of a labelled compound through common carotid artery of an animal along with a reference standard and experiment stopped after 5 - 15 seconds. Then the brain sampling is done and the brain uptake index (BUI%) can be calculated using the concentration of the compound and the reference standard (Pardridge, 2007). Because of the low sensitivity of the method (limited sampling time), this method has

been replaced by *in situ* brain perfusion which provide higher control on experimental condition (Kerns & Di, 2008).

4.2.3 *In situ* brain perfusion

The desired concentration of the studied drug was prepared using the perfusion fluid and the resulted solution is perfused directly to the brain through common artery of an anesthetized animal (commonly rat) for the suitable time and the brain sampling carry out on the predefined time intervals after stopping the perfusion (Amith & Allen, 2003). Similar to the intravenous injection method the remained intravascular perfusion fluid should be removed by brain flashing or calculated using an impermeable compound injection (Rooy et al., 2010). Direct perfusion enables scientists to study the BBB drug permeation in the absence of the first pass metabolism or drug elimination methods. Using this method, the mechanism of drug permeation can be studied using co-administered transporter inhibitors. But such as intravenous injection high resource demanding is a limitation for this method. The K_{in} and PS can be calculated using the obtained data from this method.

4.2.4 Quantitative auto radiography

Another method for CNS drug partitioning study is quantitative auto radiography which can be used for regional study of total drug exposure. Using this method, the amount of radio labelled compound is measured in desired regions (e.g. stroke affected areas, brain tumours) following oral, intravenous or subcutaneous administrations to animals. Similar to previous methods after blood sampling in various time intervals, the brain is taking out and after sectioning the frozen brain to suitable sections the radioactivity is measured. Intra vascular correction is needed here too. Obtaining the regional PS values is possible using this method and the resolution of obtained data is high because of the micrometer dimensioned studied sections (Bickel, 2005; Rooy et al., 2010).

4.2.5 Positron emission tomography

Positron emission tomography is a non-invasive method which is applicable in human. The suitable tracers are administered to the body and the emission is monitored using positron emission tomography scanners. The blood sampling is done in designed intervals and the brain and plasma distribution is measured using a curve fitting method. Similar to quantitative auto radiography the regional information about drug distribution is achievable using this method (Dash & Elmquist, 2003).

4.2.6 Intra cerebral microdialysis

Microdialysis is the only technique which is able to provide the concentration of CNS drug candidates in the interstitial fluid directly. A stereotaxic probe equipped with a semi permeable membrane implanted under anesthesia. The interior of the probe perfused with a physiological solution and samples are taken from freely moving animals and analyze using suitable separation techniques (commonly chromatographic systems) (Bickel, 2005; Alivajeh & Palmer, 2010). The studied compound can be administered orally, intravenously, subcutaneously or from other routes. This method is applicable for human and by implanting the probe in different regions of brain; specific data from different parts of brain (which have different properties) could be collected. The recovery of the probe is an important point in this method to get the absolute concentration data. Pharmacokinetic

parameters of CNS drug candidates including half-life, C_{max}, T_{max}, total exposure, volume of distribution, clearance, BBB influx and efflux rates for different brain regions and most importantly the $K_{p,uu}$ at steady state can be obtained and calculated using microdialysis driven data. These data can be used for pharmacodynamic studies and dosing regimens (Alivajeh & Palmer, 2010).
The methods reviewed in sections 4.2.1 to 4.2.6 give information about the overall exposure resulted from different passive or active influx and efflux systems.

4.2.7 Permeation mechanism study *in vivo*

During drug development the detailed information about the mechanism of permeation and possible efflux or metabolic instability are needed to design the structure of the desired drug and its delivery system. To get detailed information researchers have been used different methods such as: knockout or gene deficient animals for studying the effect of a specific transporter, special enzyme or transporter inhibitors (e.g. efflux inhibitors) or receptor antagonists to eliminate the desired transport effect from the study.
In order to study passive diffusion of drug candidates without interfering of other permeation mechanisms, a number of methods have been developed. For example, it is possible to use excess molar of unlabelled compound in order to saturate the transporters, enzymes or facilitated mechanisms. Also it is possible to use efflux transporters' inhibitors (e.g verapamil for P-gp). Beside these, by studying the Michaelis-Menten behaviour of drugs, it is possible to ensure that the permeation mechanism is passive diffusion (unsaturable) or not.

4.2.8 *Ex vivo*

E*x vivo* experiments are developed to study drug candidates more reliably out of the body in the simulated physiologic condition (pH, temperature, buffer, nutrients, oxygen) which have the advantage of being applicable in post mortem human samples obtained by autopsy. The resulted data from these experiments have been shown acceptable correlation with *in vivo* experiments. Although in this method impossible experiments and studies in living organism can be conducted, but the differences between the living organism and the slices obtained by autopsy according to the degradation of some proteins should be take into account (Cardoso et al., 2010).

4.3 *In vitro*

In order to do more rigorous investigations on the complex mechanisms occurred in endothelial cell membranes and in intracellular compartments (e.g. active and passive efflux and influx) in the BBB of a living organism, *in vitro* methods can be used. *In vitro* models of BBB should be simple, reproducible and mimic the *in vivo* conditions (both normal and pathologic). Most of the *in vitro* models of BBB are based on endothelial cells as the foundation of BBB and different animals are used to prepare cell cultures. The results should be interpret carefully because of the differentiations (the lower tightness of the developed cell lines, the phenotype modification and the absence of intercellular contact and in vivo signallings occur during the cell isolation). But it is a reliable method for high throughput screening experiments, in order to compare the penetration ability of a set of compounds (Cardoso et al., 2010). The main categories of *in vitro* models include

cell based and non cell based methods. Cell based models are simplification of *in vivo* system in which the brain and non brain derived cell cultures are used to study the permeation and transport of drug candidates. The brain derived cell cultures (primary endothelial cultures) show closest phenotype to the *in vivo* brain while their preparation and handling are more difficult than non-brain derived cell lines. Primary endothelial cultures prepared by isolating animal brain micro vessels and *seeding* in culture medium where the endothelial cells grow out and make suitable mono layers for experiments. In order to mimic the *in vivo* system more closely co-cultures included astrocytes have been developed which provide more physical and physiological features in comparison with primary cell cultures (Cardoso et al., 2010). Non brain derived models use the epithelial cell cultures (e.g. Caco 2) and modified epithelial cell cultures which are used for drug absorption studies in order to rank the permeability of CNS drug candidates. Non cell based *in vitro* models include the parallel artificial membrane permeability assay (PAMPA) and immobilized artificial membranes (IAMs) which used as HPLC columns and mimic the properties of biological membrane (Abbott, 2004). PAMPA models initially developed for study passive oral absorption and successfully applied in the pharmaceutical industry. Recently, it has been modified for using in BBB permeation studies and showed good correlation with *in vivo* findings (Mensch et al., 2010).

4.4 BBB permeation prediction methods (*in silico* methods)

In vivo, *ex vivo* and *in vitro* methods of assessing brain drug penetration leads to high quality data resemble most of the permeation mechanisms in BBB, but they are highly cost and time demanding and are not suitable for screening of large compound libraries. As soon as BBB studies have begun, attempts to predict the BBB permeation properties of drug candidates lead to primary structure activity relationships which later accepted as essential rules of CNS drug development. These structural features later used to develop quantitative relationships to predict the pharmacokinetic properties of CNS drugs. During years and improving the knowledge about the effect of different passive and active mechanisms of brain drug penetration, the prediction models improved and specific models to predict different aspects of BBB permeation have been developed. In order to develop a model first the prediction endpoint (dependent variable or experimental value) should be measured or obtained from the literature. The quality of these data is deterministic for developed model certainty. After selection of the data set, the inclusion of each point in data set should be evaluated and possible outliers should be determined. The next step is to split data set in training and test sets and measure or calculate the desired independent descriptors. The significant descriptors should be selected and the relationship between the dependent and independent variables should be developed using appropriate modelling method. While the model has been developed, its predictive ability along with other validation parameters should be calculated and the effect of selected descriptors on the experimental value should be defined. The details of each step are provided in following sections. Some commercial software have been developed to predict the brain drug penetration which can be used to get primary estimations about the CNS activity of a compound.

4.5 Prediction endpoints (Experimental data)

In order to get initial information about the BBB permeation of new drug entities, studying the existing information using different methods is more interesting than experimental

measurement. There are different (*in vivo* or *in vitro*) indicators which are able to evaluate the rate or extent of drug permeation to the BBB (see section 4.1.3). Among them logBB values have been used extensively for *in silico* methods in order to predict the extent of drug penetration to the brain and the related data sets can be found in the literature. Unbound drug fraction, logPS and BUI% have been used to develop the prediction methods, while some researchers used *in vitro* data (e.g. PAMPA derived P-gp binding affinity) for their studies (Dagenais et al., 2009). Beside these BBB+/- and CNS+/- data which have been extracted from logBB experiments and implications of brain disorders or targets about primary site of action of compounds respectively, were utilized for classification purposes (Klon, 2009). It seems that using the combined information derived from different indicators will be more useful than individual ones. The quality of selected data set should be considered according to the experimental method which used to obtain it (data set homogenesity). The homogenesity of logBB data sets have been questioned, but the studies showed that these combined data sets are applicable. Also the outliers should be determined using statistical methods or according to the experimental method. One of the most common statistical methods is to compute deviations of a single data point from mean dependent or independent variables or both of them and exclude highly deviated datum. In fact an applicability domain for each prediction method should be defined and the compounds out of this domain should be excluded from analyses. For experimental procedures it should be kept in mind that if special efflux inhibitors are used or not. In some methods, scientists are used unlabeled substrate to saturate the desired enzyme or transporter or receptor and the resulted data from these experiments should not be combined with others (Lavnevskij et al., 2010). The third point which should be kept in mind is that the number of the data points should be enough for developing statistical properties (e.g. regression coefficients) of the developed model and also for excluding a part of data as test set. If it is not possible the prediction capability of developed model cannot be evaluated and it will be applicable for the entire data set.

4.6 Descriptors

The structural features and physicochemical properties (Table 2) of the studied compounds should be extracted using the available experimental and computational methods (commercial software, fragment based methods, …). The most studied and evaluated descriptors to define the BBB permeation are those related with passive diffusion. Table 3 contains the details of most frequently used descriptors as well as their effects on BBB permeation. As can be seen from the table, the overall findings about the structural features (also known as the rule of five) of the CNS drug candidates are:

- High lipophilicity
- Low hydrogen binding
- Small molecular weight.

It should be noted that these rules should be used cautiously during drug design procedure. For example, although high lipophilicity increase the permeation rate but it causes the poor solubility, metabolic instability and higher membrane bounding which are not suitable properties for a drug candidate.

Descriptor	Topological descriptors Constitutional, Molecular properties, Quantum chemical, ACDLabs, free aqueous solubility energy
Software	Absolve, Dragon, Hyperchem, Volsurf, MOE, Cerius package

Table 2. Frequently used descriptors and software.

Property	The cutoff for BBB permeation
Molecular weight	< 400-500 Da
H bond donor	<3
H bond acceptor	<7
ClogP*	<7
logD7.4	1-3
Polar surface area	< 60-70 $A^{\circ 2}$
Rotatable bonds	<8
Flexibility	1.27
pKa	7.5-10.5
N+O	<6

* The studies showed that logPoct/water have poorer correlation with permeation data in comparision with ΔlogP or logD7.4. Recent studies showed that the ionization state of drug candidates in physiologic condition should be defined and the models should be developed accordingly (Lavenskij et al., 2009, 2010; Shayanfar et al., 2011).

Table 3. Descriptors used in rules of five methods and their cut off points (Di, 2008; Palmer, 2010)

4.7 Model development

After preparing a number of descriptors, the best descriptor or a combination of descriptors which are able to describe the desired dependent variable (prediction end point) should be selected. There are two approaches for descriptor selection:

4.7.1 Mechanistic approach

In this method, the studied property (e.g. BBB permeation) affecting parameters should be extracted from theoretical findings (several processes include in the overall result) and convert to mathematical representations. The provided descriptors depend on their effects (positive or negative, direct or inverse) on desired property should be correlated to the prediction end point and the resulted equation could be used for prediction purposes (Lavenskij et al., 2010).

4.7.2 Statistical approach

It is so important to exclude insignificant descriptors to prevent over fitting and biased results using a descriptor selection method. The number of descriptors depends on the modelling method. For simple multivariate regression methods, the number of descriptors depends on the number of data points, while for partial least square and principal component analyses methods it is not limited. In addition to the number of the descriptors

and their significances, the inter correlation between them should be checked and just one of the highly correlated descriptors should be kept in multiple linear regression methods, while this is not a problem for partial least square or principal component analyses. There are different methods for descriptor selection and more information can be found in the literature. It is better to keep the penetration mechanisms and approved relationships in mind in this step and avoids complete statistical methods.

4.8 Method development

As soon as the descriptors selected or provided in mechanistic approach, the model should be developed according to the purpose of the modelling. The *in silico* methods developed for following purposes in CNS drug studies:

4.8.1 Classification

It is important to know that if the desired compound is CNS active or not. To do this a border value should be defined for the scaled dependent variable. Different data sets have been used for these models:

- logBB data (BBB+/-),
- CNS active or inactive compounds (CNS+/-)
- P-gp substrate or non-substrate (Pgp+/-).

These models are applicable for screening studies (primary steps of CNS drug development) where the goal is to select the possible CNS active compounds from large compound libraries and in advanced steps of CNS drug studies where the possible reasons of efficacy failure are investigated. Different classification methods have been developed until now using different algorithms and descriptors. The review of these studies showed that the methods were more successful for CNS+ and BBB+ compounds than CNS- and BBB- ones. One reason for this approach is raised from efflux pumps which efflux some structurally suitable compounds from brain. Considering the efflux system substrates during method development will improve the prediction accuracy for these compounds. It should be noted that there is a difference between BBB+ and CNS+, since a drug could be penetrated into brain without measurable biological effect. However in some modelling studies these data were mixed up. It seems that in order to develop more accurate classifiers, some physiological properties of brain such as the extent of non-specific protein and tissue binding, the concentration of the target protein and specific receptors in the brain should be considered.

4.8.2 Permeability prediction (The rate and extent of penetration)

The logBB, $K_{p,uu}$ (for exposure extent studies) and logPS (for rate studies) have been frequently used to develop prediction models. The multiple linear regression and least square methods are among the most studied models providing simple and interpretable equations.

Detailed review of these equations could be found in the literature (Garg et al., 2008; Klon, 2009; Mehdipour & Hamidi, 2009; Shayanfar et al., 2011). The descriptors used for rules of five (Table 3) studies originally comprised from these equations and at least one of these descriptors or similar descriptors which provide relevant information can be found in these equations. In this regard, most of the time, medicinal chemists use the same descriptors to check the new data set or new methods. Lipophilicity descriptors, size and shape descriptors, ionization states of compounds, and polar surface area descriptors proved to

have effect on BBB permeation. The complexity of BBB permeation encouraged scientists to check non linear methods applicability in this field and some exponential linear equations and neural networks have been successfully developed. Although neural networks provided more accurate predictions in comparison with linear ones, their interpretation and reproducibility are in question and their usefulness for developing universal models which can be applicable for chemists have not been approved yet. In fact the best model for a chemist is a model which is able to answer him/her what is the possible modification for desired property improvement and the un-interpretable models are not able to answer this question. Because of this, using less accurate but well defined models are preferred to complicate but accurate ones.
The studies of unbound fraction of the drug in brain ($K_{p,uu}$) showed that the previously accepted trend of permeation (higher permeation for more lipophilic compounds) which was raised from log*BB* and log*PS* studies are not the same for unbound fraction, and lipophilicity have inverse relation with it. These findings showed that the absolute values for the effective descriptors are not suitable and a balanced range of descriptors should be defined for them (Lavenskij et al., 2010).

4.9 Validation

In order to check the sensitivity, specificity, prediction capability, reproducibility, error margins and chance correlations for the developed models, some validation statistics should be provided and using these parameters researchers will be able to make decision on selecting or rejecting a model in comparison with others. The details of these parameters and their usefulness for evaluating the model have been reviewed. For classification methods the lower failure in localization of compounds (both positive and negative) is better and for predictive models the higher correlation coefficients (both for training and test sets and cross validation sets), lower prediction errors (less than about 1 log unit deviation and relative mean squared errors less than 0.3)and lower correlation coefficients (e.g. <0.2) for Y randomized data sets are acceptable. These parameters are not absolute and it would be possible to accept a low quality model in the absence of the better one.

4.10 Prediction using commercial software

Using the developed models, some software has been developed in order to calculate the BBB permeation or P-gp binding affinity which can be used for estimation of compound permeation. These predictions are included in the most of the ADME prediction software which could be found on internet.

5. Conclusion

The importance of BBB for reaching CNS drugs to their targets and also undesired penetration of non CNS drugs to avoid their CNS side effects are briefly discussed. Short review of measurement methods of drug's penetration to CNS is presented along with a summary of computational aspects used for modelling purposes.
The molecular and cellular properties of BBB have been reviewed and the role of its compartments in the regulating of drugs and xenobiotics penetration to the brain has been discussed. Working as a regulatory interface BBB is able to work as a physical and physiological barrier which prevents peripheral drugs to penetrate the brain and reduce

their CNS side effects. This barrier activity causes some difficulties in CNS drug delivery and different measurement methods have been developed to study the rate and extent of drug delivery to the brain and the mechanism of delivery methods have studied using these methods. Beyond the experimental methods, prediction of these properties are studied in order to provide cheaper, simpler and more rapid methods for medicinal chemists who work in brain drug development field.

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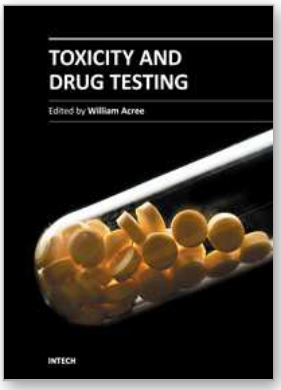

Toxicity and Drug Testing
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Modern drug design and testing involves experimental in vivo and in vitro measurement of the drug candidate's ADMET (adsorption, distribution, metabolism, elimination and toxicity) properties in the early stages of drug discovery. Only a small percentage of the proposed drug candidates receive government approval and reach the market place. Unfavorable pharmacokinetic properties, poor bioavailability and efficacy, low solubility, adverse side effects and toxicity concerns account for many of the drug failures encountered in the pharmaceutical industry. Authors from several countries have contributed chapters detailing regulatory policies, pharmaceutical concerns and clinical practices in their respective countries with the expectation that the open exchange of scientific results and ideas presented in this book will lead to improved pharmaceutical products.

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